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RESEARCH ARTICLE

Evaluating the role of NRF-1 in the regulation of the goldfish *COX4-1* gene in response to temperature

Ge Gao and Christopher D. Moyes*

ABSTRACT

Cold acclimation in fish typically increases muscle mitochondrial enzymes. In mammals, stressors that increase mitochondrial content are mediated though transcriptional regulators, including nuclear respiratory factor-1 (NRF-1). Focusing on the goldfish gene for cytochrome *c* oxidase (COX) subunit 4-1, we analysed the regulatory regions in various contexts to identify a mechanistic link between NRF-1 and cold-induced mitochondrial proliferation. Promoter analysis implicated two putative NRF-1 sites: one in the proximal promoter and a second in exon 1, which encodes the 5′ untranslated region (5′-UTR). Transfection into mouse myoblasts showed that deletion of a region that included the proximal NRF-1 site reduced promoter activity by 30%; however, mutagenesis of the specific sequence had no effect. Thermal sensitivity analyses performed in rainbow trout gonadal fibroblasts (RTG-2) showed no effect of temperature (4 vs 19°C) on reporter gene expression. Likewise, reporters injected into muscle of thermally acclimated goldfish (4 vs 26°C) showed no elevation in expression. There was no difference in thermal responses of *COX4-1* promoter reporters constructed from homologous regions of eurythermal goldfish and stenothermal zebrafish genes. NRF-1 chromatin immunoprecipitation of thermally acclimated goldfish muscle showed no temperature effect on NRF-1 binding to either the proximal promoter or 5′-UTR. It remains possible that the cold-induced upregulation of *COX4-1* expression is a result of NRF-1 binding to distal regulatory regions or through indirect effects on other transcription factors. However, the proximal promoter does not appear to play a role in mediating the thermal response of the *COX4-1* gene in fish.

KEY WORDS: Mitochondria, Oxidative phosphorylation, Goldfish, Thermal acclimation, Bioenergetics, Nuclear respiratory factor 1

INTRODUCTION

The ability to increase mitochondrial content in response to energy stress is an important capacity, enabling animal tissues to maintain energy balance when physiological or environmental conditions change. Mitochondrial biogenesis can be induced in mammals in response to exercise (Booth and Thomason, 1991), electrical stimulation (Williams et al., 1987), myogenesis (Moyes et al., 1997; Kraft et al., 2005) and cold stress (Cannon and Nedergaard, 2004; Ricquier and Bouillaud, 2000). Most of these challenges include periods of high energy demand. Paradoxically, fish induce mitochondrial biogenesis in response to cold water, when metabolic rates decline (Moerland, 1995; Somero, 2004; Egginton and Johnston, 1984; Egginton and Sidell, 1989; Bremer and Moyes, 2011). Mitochondrial proliferation in cold-acclimated fish may be a response to an energetic shortfall, representing a means to maintain energy homeostasis despite the debilitating thermodynamic effects of cold. Cold acclimation in goldfish is accompanied by increases in AMP/ATP and ADP/ATP, consistent with energetic stress (Bremer et al., 2016). The capacity for adaptive remodelling of muscle energetics in response to physiological and environmental challenges is common amongst vertebrates, yet there are differences in the genetic mechanisms that regulate mitochondria gene expression in fish versus mammals (Bremer et al., 2012). Thus, this paradigm is useful in exploring both the genetic mechanisms used by fish to respond to environmental challenges and the evolution of the control of bioenergetic gene expression in vertebrates.

Cytochrome *c* oxidase (COX), a complex mitochondrial enzyme composed of subunits encoded by both nuclear and mitochondrial genomes, is an intriguing model enzyme to study the ways that animals regulate genes to achieve an appropriate metabolic phenotype. COX activity provides an estimate of mitochondrial capacity, and changes in COX activity are typically assumed to reflect a change in mitochondrial content because of the relatively fixed stoichiometries between OXPHOS complex levels (Leary et al., 1998). However, the relationship between activity and content can be regulated by cardiolipin and other membrane lipids (Yamaoka et al., 1988, 1990; Sparagna et al., 2007), and there is a growing appreciation for post-translational regulation of COX enzyme kinetics (Arnold and Langer, 2002; Fontanesi et al., 2006, 2008). Understanding how COX levels change in response to physiological stressors requires an appreciation of the complexity associated with COX biosynthesis. New COX requires the incorporation of at least 10 nuclear-encoded gene products, and more if multiple paralogs for subunits are expressed. The complexity of COX synthesis is reflected in the heterogenous responses of the various nuclear-encoded COX genes. Under conditions that lead to a modest increase in COX activity, the mRNA for individual subunits may decline, remain unchanged, increase in parallel or increase many fold more than COX activity (Duggan et al., 2011). Furthermore, at least some of the increase in COX subunit mRNA can be attributed to decreases in mRNA degradation rather than simply increases in synthesis (Bremer and Moyes, 2014). The complex relationships between COX gene expression and COX activity epitomize the importance of considering both transcription and non-transcriptional mechanisms for adaptive remodelling of metabolism. Why any of the COX genes respond to temperature in the way they do is entirely unknown.

We studied fish *COX4-1* because it is a gene that typically increases its mRNA level upon cold acclimation and often to a greater extent than COX enzyme activity rises (Duggan et al., 2011; Bremer et al., 2012). In mammals, COX genes are regulated by

Department of Biology, Queen's University, Kingston, Canada K7L 3N6.

*Author for correspondence (chris.moyes@queensu.ca)

C.D.M., 0000-0003-3506-2837

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many factors, but a common theme is a role for the DNA-binding protein nuclear respiratory factor-1 (NRF-1) and its mediator, PPARγ coactivator-1α (PGC-1α; also known as PPARGC1A) (Evans and Scarpulla, 1989; Suske et al., 1988; Dhar et al., 2007; Booth and Baldwin, 1997; Yan et al., 1995; Kraft et al., 2005). In mammals, PGC-1α is important because of its ability to interact, directly or indirectly, with many DNA-binding proteins known to affect mitochondrial genes, including NRF-1 itself (Scarpulla, 2011). Although there may be differences in the PGC-1α axis in fish and mammals, owing to structural peculiarities in several regions of PGC-1α that bind NRF-1 (LeMoine et al., 2010; Bremer et al., 2016), it is likely that the suites of DNA-binding proteins that regulate oxidative genes in mammals perform similar functions in fish. In an analysis of the effects of thermal acclimation, we identified NRF-1 as a transcription factor that increases in parallel with the observed changes in *COX4-1* mRNA (Bremer et al., 2012). Of the many proteins known to interact with PGC-1α, only NRF-1 displayed increases in both mRNA and nuclear protein content in cold-acclimated fish. Since NRF-1 is known to regulate mammalian *COX4-1* (Dhar et al., 2007), these observations implicated NRF-1 as a critical transcription factor controlling cold-induced increases in COX genes in goldfish.

We began this study expecting to verify that the increase in *COX4-1* mRNA levels in cold acclimation was caused by NRF-1 regulation of the *COX4-1* proximal promoter. However, despite the presence of two putative NRF-1 elements in the *COX4-1* gene, we could find no evidence that these elements were involved in regulating the response of *COX4-1* to temperature, or that the capacity to increase *COX4-1* expression at cold temperature resides in the proximal promoter.

MATERIALS AND METHODS

RNA extraction, cDNA synthesis and DNA isolation

RNA was isolated from goldfish (*Carassius auratus* Linnaeus 1758) white muscle tissue using TRIzol (Invitrogen, Carlsbad, CA, USA) and RNA was reverse transcribed to cDNA using the QuantiTect Reverse Transcription Kit (Qiagen, Valencia, CA, USA). Genomic DNA was isolated from goldfish tissue using phenol/chloroform extraction (Sambrook and Russell, 2001).

Rapid amplification of cDNA ends (RACE) and rapid amplification of genomic DNA ends (RAGE)

RAGE and 5′-RACE were used to sequence the *COX4-1* gene. Both methods of sequencing required three rounds of PCR reactions (HotStar HiFidelity Polymerase kit and Long Range PCR Kit; Qiagen, Valencia, CA, USA) using three tailing primers and three *COX4-1* reverse primers (Table S1).

For 5′-RACE, cDNA was produced and amplified from RNA via reverse transcription using a reverse primer that annealed to the *COX4-1* in exon 1. The cDNA produced in this reaction was polyadenylated to create a poly-A^+ tail on the cDNA ends (Scotto-Lavino et al., 2006). This product was then amplified using Q-T17 in combination with COX4-1R1. A second round of PCR used Q-outer as forward primer and COX4-1R2. A third round of PCR uses Q-inner and COX4-1R3. The final product was gel purified, cloned and sequenced. A similar approach was used in RAGE, except the reverse transcription step was omitted and the first template was created using COX4-1R1 in combination with genomic DNA. This single strand PCR product was tailed and analysis continued as described for RACE. The sequences for the goldfish *COX4-1* gene are provided in Fig. S1.

Constructing plasmids with deletion mutant inserts

With the sequence information, we were able to design the cloning constructs. The plasmid vector chosen for this experiment was pGL2-basic, a firefly luciferase reporter vector (Promega, Madison WI, USA). Deletion mutant constructs were built to identify the critical regions of the promoter. Primers were designed to amplify various sizes of the promoter (Table S2), each with *Xho*I and *Mlu*I restriction enzyme sites to enable directional cloning into the reporter. The amplified products were then digested and cloned into the vector. To create the NRF-1 mutant, primers were constructed to alter the putative NRF-1 element (5′-GGGCAT-3′) to introduce three mutations (5′-GcGgAa-3′). Zebrafish (*Danio rerio* Hamilton 1822) primers (Table S2) were designed for amplifying the *COX4-1* promoter, based on the published sequence from Ensemble (ENSDARG00000032970). A promoter of 963 bp was amplified through PCR, as for the goldfish promoter, and cloned into pGL2-basic.

Mammalian cell culture

A mouse skeletal myoblast cell line (C2C12) was chosen to examine the *COX4-1* promoter. The cells were maintained in Dulbecco's Modified Eagle Medium (DMEM, Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS; Invitrogen) and 1% penicillin-streptomycin (Sigma-Aldrich, St. Louis, MO, USA). The cells were maintained at 37°C and 5% CO_2 and grown in 12-well plates with 1 ml cell culture medium in each well. The cells were obtained directly from ATCC and propagated in house.

Transfections occurred when cells were 40-50% confluent. Each well was transfected with a mixture of FuGENE6.0 (7.5 µl), a pGL2 basic plasmid construct (1 µg) and pRL-CMV (0.05 µg) encoding the *Renilla* luciferase. FuGENE was obtained from Roche Applied Science (Laval, Canada) and reporters were from Promega (Madison, WI, USA).

Luciferase activity assays utilized the Promega dual-luciferase reporter assay system and a Molecular Devices Lmax Luminometer. Transfected myoblasts were harvested 24 h after the transfection using 1× passive lysis buffer. For transfected myotubes, 24 h after the transfection the culture medium was replaced with serum reduced from 10% FBS to 2% horse serum (Sigma-Aldrich, St Louis, MO, USA) to induce myogenesis. The differentiated cells were harvested 24 h later using passive lysis buffer. Harvested cells were stored in 1.5 ml tubes at −80°C for 16 h before luciferase measurement. The firefly luciferase activities were expressed relative to *Renilla* luciferase activity, to correct for well-to-well variation in transfection efficiency.

Fish cell culture

A rainbow trout (*Oncorhynchus mykiss* Walbaum 1792) gonadal fibroblast cell line (RTG-2) was maintained in Leibovitz's L-15 cell culture medium (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% FBS and 1% penicillin-streptomycin. The cell line was grown at 19°C under 100% air. The cells were obtained from Niels Bols (University of Waterloo) and propagated in house.

Prior to the transfection, cells were grown on 12-well plates until they were approximately 70% confluent. The cells were then transfected with plasmid constructs and control pRL-CMV plasmids. The transfected cells were maintained at 19°C for 48 h. At this point, half of the plates remained at 19°C and the other half were transferred to 4°C. After 48 h, cells were harvested and analysed for luciferase activities as described above.

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Animal experiments

Goldfish of undetermined sex were purchased from a local pet store. The fish (5-7 cm fork length) were quarantined in a 140 litre aquarium with dechlorinated water at 20°C for 8 weeks in the animal care facility (BioScience Complex, Queen's University) before thermal acclimation. The fish were kept under a 12 h light:12 h dark photoperiod and fed daily with Laguna Goldfish and Koi Food. At the end of the quarantine period, half of the fish were transferred to an aquarium and temperature acclimation commenced, with water temperatures adjusted (1°C day^{-1}) to 26°C or 4°C. The fish were acclimated to their corresponding warm and cold temperatures for 6 weeks before injection experiments.

For the transfections, fish were anesthetized (0.4 g l^{-1} tricaine methanesulphonate and 0.8 g l^{-1} NaHCO3) and injected with 25 µl containing 25 µg reporter plasmid mixed with Trypan Blue (0.2%) and phosphate-buffered saline. Each fish was injected with three zebrafish plasmids and three goldfish plasmids in epaxial muscle using a Hamilton syringe. The injected fish were returned to the same tanks for 7 days before the white muscle tissue was harvested. Collected tissue was homogenized in 200 µl of 1× passive lysis buffer and immediately frozen at −80°C. Corrections were made for transfection efficiency using *Renilla* luciferase activities. All animal protocols were conducted following approval by the Queen's University Animal Care Committee, under the guidelines from the Canada Council on Animal Care.

Cytochrome *c* oxidase activity

COX enzyme activity was determined by measuring the oxidation of cytochrome *c*. Goldfish white muscle samples (~50 mg) were homogenized in 20 volumes of ice-cold extraction buffer (25 mmol l^{-1} K_2HPO_4, 1 mmol l^{-1} EDTA, 0.6 mmol l^{-1} lauryl maltoside, pH 7.4) using a Tenbroeck tissue grinder (Wheaton Industries, Millville, NJ, USA). Homogenates were added to 96-well plates (Corning, Corning, NY, USA) with assay buffer (25 mmol l^{-1} K_2HPO_4, 0.6 mmol l^{-1} lauryl maltoside, pH 7.4) and reduced cytochrome *c* (0.05 mmol l^{-1}). Enzyme activity was determined kinetically at 25°C and 550 nm using a spectrophotometer (Molecular Devices, Sunnyvale, CA, USA). Measurements were done in triplicate.

Chromatin immunoprecipitation (ChIP) assay

To examine the NRF-1 and *COX4-1* gene interaction, a ChIP assay was carried out using SimpleChIP Plus Enzymatic Chromatin IP Kit (Cell Signaling Technology, Danvers, MA, USA). The same white muscle tissue samples that were used to carry out COX activity and *COX4-1* mRNA analysis were used with 25 mg tissue for each ChIP. To crosslink DNA and protein, powdered tissue was combined with 37% formalin in phosphate-buffered saline at a final formaldehyde concentration of 1.5%. Samples were shaken for 20 min at room temperature. Glycine was added to stop the crosslinking, and the tissue was homogenized using a Tenbroeck tissue grinder. Micrococcal nuclease (0.1 µl) was added to digest the DNA into chromatin, with shaking at 37°C for 20 min. Each ChIP used 5 to 10 µg of digested, crosslinked chromatin. Digested chromatin was incubated with ChIP-grade Protein G magnetic beads and NRF-1 antibody (ab34682, Abcam, Cambridge, MA, USA) or rabbit IgG (Cell Signaling, Danvers, MA, USA) overnight with rotation. The DNA-protein complexes were reverse crosslinked and digested by incubating for 2 h at 65°C with 5 mol l^{-1} NaCl and 100 µg ml^{-1} proteinase K. DNA was then purified using a spin column, as per the manufacturer's instructions.

Real-time PCR

Primers were designed to specifically amplify the putative NRF-1 binding region on the gene promoter as well as on exon 1. The efficiency of the forward primer 5′-CAACGCTCTGCCCATCTA-TTT-3′ and reverse primer 5′-CAGACGATATAGAGGCCACA-GT-3′ amplifying putative NRF-1 site on exon1 and the forward primer 5′-CAAGATTCAAGATTAAGGGTTGGGCATACTA-3′ and reverse primer 5′-ATTTAGGTTATCCCCTCCCATCT-3′ amplifying putative NRF-1 site on proximal promoter was determined by real-time PCR with an appropriate dilution series of genomic DNA. Reactions contained 2 µl of template DNA from the ChIP, 2 µl of each forward and reverse primer (7.25 µmol l^{-1}), 12.5 µl GoTaq Master Mix (Promega, Madison, WI, USA) and 3.5 µl of double distilled H_2O. The analyses were carried out using an ABI 7500 Real-Time PCR System (Foster City, CA, USA). The protocol was as follows, 10 min at 95°C followed by 40 cycles of 15 s at 95°C, 15 s at 60°C, 34 s at 72°C. All samples were run in duplicate, comparing the DNA extracts from ChIP conducted with and without the NRF-1 antibody.

Statistical analysis

All data are presented as mean+s.d. The C2C12 and RTG-2 transfection experimental data are expressed relative to the full-length proximal promoter construct. The significance between the groups was assessed using ANOVA with *post hoc* Dunn's test. Each *N* value represents replicate measurements of an extract from a single well transfected with a unique plasmid dilution on a separate day or passage.

The *in vivo* transfections were analysed using two-tailed *t*-tests, taking into consideration that comparisons between zebrafish and goldfish were paired, while comparisons between acclimation states were unpaired. Three fish samples showed no detectable luciferase activity and were excluded from the analyses. The groups of fish were determined randomly, and no steps were taken to blind the investigator to the groups.

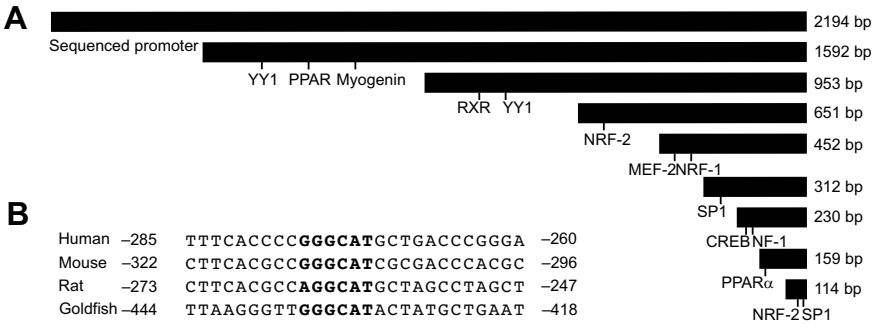

Fig. 1. Goldfish cytochrome *c* oxidase subunit 4 isoform 1 (*COX4-1*) promoter analysis. (A) Select putative binding sites are identified in relation to the deletion mutants used in transfection analyses. The size of the promoters is indicated to the right of each deletion mutant. Identification of transcription factor binding sites was done by using the TRANSFAC 6.0 database. (B) *COX4-1* promoters contain a conserved putative NRF-1 binding region in mammals (Dhar et al., 2007) and goldfish. The sequence corresponding to the core element shown in humans is highlighted.

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RESULTS

Characterization of the goldfish *COX4-1* gene proximal promoter

The sequence of the goldfish *COX4-1* 5′-UTR and proximal promoter was analysed using TRANSFAC 6.0, which identified putative transcription factor binding sites. We analysed 2194 bp of promoter nucleotide sequence (Fig. S1). The goldfish *COX4-1* gene has a short exon 1 with only 24 nucleotides, and the ATG start codon is located within the second exon. Like many nuclear genes encoding mitochondrial proteins, the goldfish *COX4-1* promoter lacks a TATA box.

Fig. 1A summarizes the putative elements for select transcription factors that are known to be expressed in muscle or are commonly involved in mitochondrial biogenesis. The identification of NRF-1 elements within the goldfish promoter was made challenging by the lack of a simple consensus sequence and a failure to detect any NRF-1 binding sites using TRANSFAC 6.0. However, mouse *Cox4i1* (the homologue to goldfish *COX4-1*) has been shown to bind NRF-1 in a region (5′-GGGCAT-3′) that shows high homology with the promoters for the rat and human orthologues. We searched the goldfish gene for a homologous sequence and found a putative element in the proximal promoter (Fig. 1B).

We used the reporter constructs of goldfish *COX4-1* to carry out transfection studies in mouse myoblasts (Fig. 2A) and differentiated myocytes (Fig. 2B) to further investigate and characterize the goldfish *COX4-1* proximal promoter. In each case, the deletions were compared to the 1592 bp promoter construct. In comparing myoblasts to myocytes, differentiation approximately doubled expression of the longest *COX4-1* reporter (Fig. 2C).

In proliferating myoblasts (Fig. 2A), the relative luciferase activity was not affected by deletions until the promoter was shortened to 159 bp. The activity of the 159 bp promoter was only about 30% that of the longer constructs, and not significantly different from the empty vector. Likewise, in differentiating myocytes (Fig. 2B), shortening the promoter length to 159 bp reduced luciferase activity to the point where it was not significantly different from the empty vector. However, in myocytes, another significant decrease in reporter activity was seen when the promoter was shortened from 452 to 312 bp. The critical deleted region includes the putative NRF-1 binding site, as well as sequences that are consensus elements for other critical transcription factors, including MEF-2. With an emphasis on a potential regulatory role for NRF-1, we mutated the putative NRF-1 binding region on the 1592 bp promoter construct and compared its luciferase activity to the native promoter. Luciferase activity did not differ between the wild-type and mutant promoters lacking the putative NRF-1 site (Fig. 2D).

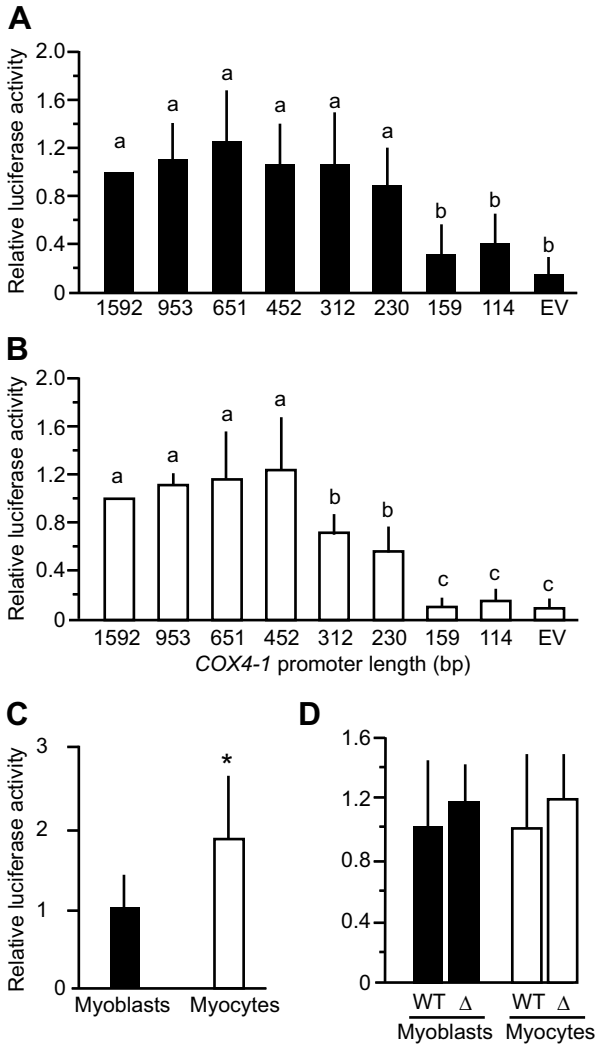

Fig. 2. Effects of goldfish *COX4-1* promoter deletion on promoter activity. C2C12 cells were transfected with the deletion mutant constructs or empty vector (EV). All data were corrected for transfection efficiency using the pRL-CMV. Luciferase activity (+s.d., n=5) is expressed relative to the activity of the longest construct. (A,B) Comparison of promoters of different lengths, expressed relative to the longest promoter in myoblasts (A) and myocytes (B). Differences between bars with different letters are significant (P<0.05). (C) Expression of the 1592 bp promoter in myoblasts and myocytes. *P<0.05, ANOVA with Dunn's test. (D) Effects of mutation of the putative NRF-1 site in the 1592 bp promoter in myoblasts and myocytes. For details of number of samples analysed, see Materials and Methods.

Fish cell culture

The main purpose of the cell culture experiments was to develop a system that could be used to identify a cold-responsive element, which we hypothesized to be the putative NRF-1 sites. This is challenging because the phenomenon of cold-induced activation of the *COX4-1* gene is seen in muscle, and the available cell lines are derived from other tissues, and not all of them remain viable in the cold. Rainbow trout gonad cells (RTG-2) show slower proliferation in the cold but cell survival was not affected.

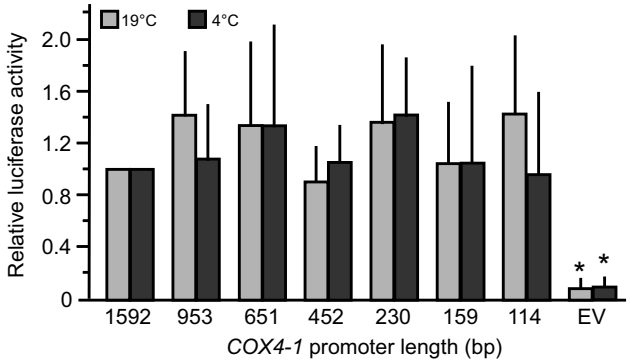

Fig. 3. Temperature effects on goldfish *COX4-1* promoter activity in RTG-2. Deletion mutant constructs or empty vector (EV) were transfected into RTG-2 cells then 48 h later, transferred to test temperatures (4 or 19°C) for 48 h. Data are expressed relative to the longest promoter tested and reported as mean (+s.d.) for six independent trials. For details of number of samples analysed, see Materials and Methods. *P<0.05, ANOVA with Dunn's test.

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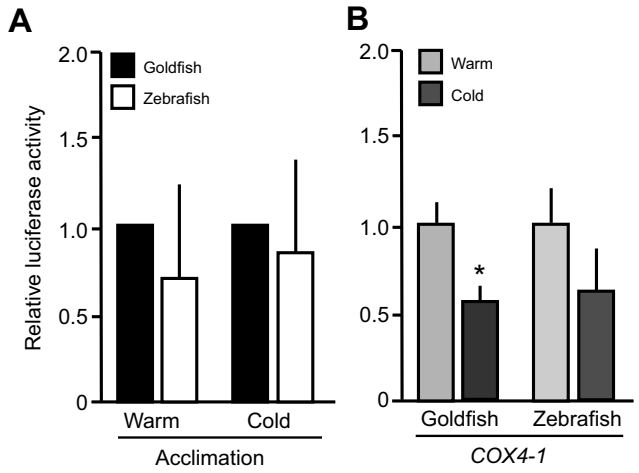

Fig. 4. Temperature effects on goldfish and zebrafish *COX4-1* promoter activity. Goldfish were injected with *COX4-1* reporter plasmids into white muscle, containing Trypan Blue for localization. Injections consisted of a luciferase plasmid driven by fish *COX4-1* proximal promoter with pRL-CMV (*Renilla*) as a transfection control. Each fish was injected three times on one side with reporter constructs. All data were corrected for transfection efficiency with *Renilla* and reported as mean+s.d. (A) Species effects were assessed where paired comparisons were possible with each zebrafish promoter triplicate compared with the goldfish promoter triplicate derived from the same goldfish, acclimated to either warm (n=12) or cold (n=8) water. (B) Acclimation effects were assessed within each species using unpaired comparisons (t-test), using absolute values of luciferase (corrected for *Renilla*) (n=8-13). Data are reported for each species, scaled to the data from warm-acclimated fish. *P<0.05, ANOVA with Dunn's test.

Progressive deletion of regions of the promoter had no significant effect on expression in RTG-2 cells at either 19°C or 4°C (Fig. 3). In each case, expression remained in excess of 20-fold greater than the empty vector. The shortest construct analysed, 114 bp, included a region that contained putative Sp1 sites.

To test whether the goldfish *COX4-1* promoter is responsive to temperature in RTG-2 cells, we compared the absolute luciferase activity of the longest promoter construct at the two temperatures. Since both reporters were compared against *Renilla*, we expected the luciferase activity to be higher with the *COX4-1* reporter incubated in the cold. However, there was no significant difference between temperature groups in luciferase activity, corrected using *Renilla* (data not shown). These results argued against the proximal promoter possessing a cold-responsive element, although this response might be tissue specific. In other words, the critical element might be in the proximal promoter, but RTG-2 cells might lack the transcription factor that responds to cold in muscle, or the transcription factor might not be cold responsive in this cell line. Furthermore, we did not determine if cold exposure resulted in an increase in nuclear-localized NRF1 protein. As a result, we took these same constructs and injected them into goldfish in an *in vivo* study.

Goldfish *in vivo* transfections

The longest promoter constructs from the goldfish and zebrafish *COX4-1* genes were injected into muscle of goldfish that had been acclimated to warm (26°C) or cold (4°C) temperatures (Fig. 4). The injections included pRL-CMV vector to eliminate thermal effects on general transcription and translation, and isolate the effects on the goldfish promoter itself. Luciferase activities were measured 1 week after the injection.

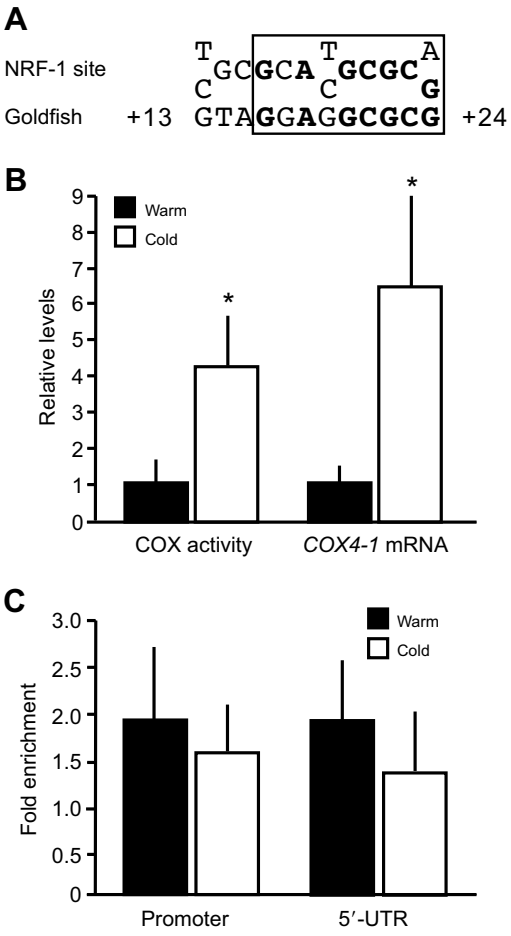

Fig. 5. COX activity and temperature effects on NRF-1 enrichment. (A) Comparison of the NRF-1 consensus element and a putative NRF-1 site on the goldfish *COX4-1* gene in the 5′-UTR. (B) The effects of acclimation on COX activity (n=5) and COX subunit mRNA (Bremer et al., 2012) are presented for the tissue samples used in ChIP analyses, expressed relative to the values in cold-acclimated fish. *P<0.05, ANOVA with Dunn's test. (C) ChIP analyses (n=5) are summarized for putative NRF-1 binding sites in the proximal promoter and 5′-untranslated region. All data are expressed relative to the control (ChIP lacking NRF-1 antibody) and presented as +s.d. There was no significant effect on temperature on either region of DNA.

Although we expected that the construct would have higher relative expression in the cold, consistent with what is seen with the native gene (Fig. 5B), we saw 50% lower luciferase activity in the cold-acclimated group (Fig. 4B). Given the utility of the model, we also used it as an opportunity to assess if promoters behaved differently in fish that had evolved different thermal sensitivities. Zebrafish cannot survive acclimation to 4°C, but acclimation to 9°C causes no increase in *COX4-1* mRNA (Duggan et al., 2011). We transfected goldfish with the *COX4-1* proximal promoter from both zebrafish and goldfish. The two promoters showed a similar pattern in relation to acclimation temperature (Fig. 4A).

Chromatin immunoprecipitation

The previous experiments examined the proximal promoter for an indication of cold responsiveness, however by focusing on the proximal promoter, we were unable to assess the potential role of a putative NRF-1 element in the first exon (Fig. 5A). We employed ChIP analyses to directly assess the *in vivo* NRF-1 binding to the putative elements on the promoter and 5′UTR. The fish tissue we used was from goldfish white muscle samples from a study where

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cold acclimation increased COX activity 5-fold and *COX4-1* mRNA levels almost 7-fold (Fig. 5B).

ChIP analysis involves fixing DNA-binding proteins to their elements, fragmenting DNA, immunoprecipitating the protein, and detecting the presence of the DNA fragment of interest in the immunoprecipitate using real-time PCR. Fig. 5 summarizes the results of ChIP analysis of NRF-1 binding to the putative elements in the promoter (Fig. 5C) and 5′-UTR (Fig. 5D). The ChIP containing the NRF-1 antibody produced more amplifiable template than the negative control in each extraction. However, cold acclimation did not increase NRF-1 binding to either of the putative elements. Thus, these analyses cast doubt on whether either putative element is functional, but regardless, suggest that changes in *COX4-1* mRNA with thermal acclimation are not being driven by changes in NRF-1 binding in the region of the proximal promoter.

DISCUSSION

The ability of animals to survive stress depends on the capacity to sense a perturbation and respond appropriately, either reversing the disruption, or remodelling in a way that makes cells and tissues better able to cope with the challenges. Temperature is an interesting stress because it can cause acute damage to proteins as well as more subtle metabolic effects, driven by enzyme kinetics or thermodynamics. In mammals, the response to cold is a hypermetabolic challenge as animals expend energy to thermoregulate, presumably resulting in a physiological stress comparable to aerobic exercise. In contrast, fish experience all of the complex effects of changes in temperature. In many species, thermal acclimation results in remodelling of tissues and metabolic pathways (Egginton and Sidell, 1989; Guderley, 1990; Hardewig et al., 1999; Bremer and Moyes, 2011; O'Brien, 2011; Duggan et al., 2011). Goldfish is a useful model to study stress in general because it can survive extreme metabolic challenges, including temperature. We have used it to study the transcriptional regulation of cold-induced mitochondrial biogenesis, including the transcriptional regulators that induce mitochondrial remodelling (LeMoine et al., 2008; Bremer et al., 2012) and coordination of genes encoding subunits of multimeric enzymes (Duggan et al., 2011).

This research extends these studies by focusing on the cold-induced increase in *COX4-1* mRNA, which is attributed primarily to increases in the rate of transcription (Bremer and Moyes, 2014). Mammalian studies on muscle mitochondrial biogenesis have variously implicated many transcriptional regulators, including NRF-1, NRF-2, retinoic acid X receptor α, estrogen-related receptor α, thyroid receptor α and PPARs (Scarpulla, 2011). In goldfish cold acclimation, many of these regulators increased their mRNA levels; however, only one – NRF-1 – increased its nuclear protein content in cold-acclimated fish muscle (Bremer et al., 2012). The *COX4-1* gene homologue in mammals (*Cox4i1*) is regulated by NRF-1 binding to a specific element in the proximal promoter (Dhar et al., 2007). Based upon the NRF-1 patterns seen in various fish during thermal acclimation (Bremer and Moyes, 2011) and the near parallel changes in levels of nuclear NRF-1 protein and *COX4-1* mRNA in goldfish (Bremer et al., 2012), we hypothesized that changes in the goldfish *COX4-1* gene were being driven by NRF-1 binding to the proximal promoter.

Is there an NRF-1 binding site on the goldfish *COX4-1* gene promoter?

NRF-1 is an important regulatory factor, activating mitochondrial genes during mitochondrial biogenesis in response to energetic stresses. It has a role in regulating suites of genes encoding mitochondrial proteins, including cytochrome *c*, Tfam, TFB1M, TFB2M, SURF1, VDAC and TOM20 (Gleyzer et al., 2005; Scarpulla, 2002; Kelly and Scarpulla, 2004; Evans and Scarpulla, 1989). Studies carried out in mouse suggest that NRF-1 regulates all of the genes encoding subunits of COX (Dhar et al., 2007). Assessing its role in genes in non-model organisms is not straightforward because NRF-1 elements and core sequences are highly variable (Evans and Scarpulla, 1990; Dhar et al., 2007), making it difficult to recognize NRF-1 sites.

We identified two regions of the goldfish *COX4-1* gene that bear many of the features of the typical and atypical NRF-1 elements characterized in mammalian genes. The mouse NRF-1 element in *Cox4i1* is considered atypical, differing from the NRF-1 consensus sequence (Dhar et al., 2007). When aligning the mouse *Cox4i1* NRF-1 element with the sequenced goldfish *COX4-1* promoter, we identified a putative NRF-1 site in the proximal promoter in a location similar to that seen in mammalian homologues (Fig. 1B). It shares the GC-rich palindrome motif that facilitates NRF-1 protein binding (Virbasius et al., 1993; Scarpulla, 2002). We also identified a putative NRF-1 binding site in the 5′-UTR (Fig. 5A). This sequence had features that were similar to the more typical NRF-1 consensus sequence derived from studies of diverse NRF-1 sites (Evans and Scarpulla, 1989; Suske et al., 1988; Chang and Clayton, 1985). To assess the ability of the gene to be regulated by NRF-1, we used a series of complementary *in vivo* and *in vitro* approaches.

Goldfish *COX4-1* promoter activity in mammalian cells

Mammalian C2C12 myoblasts are commonly used to assess reporter gene activity. They are readily transfected, and have the capacity to initiate the early stages of myogenesis upon serum starvation. This differentiation is accompanied by mitochondrial proliferation and a shift from glycolytic to oxidative metabolism (Moyes et al., 1997), increases in NRF-1 mRNA and an increase in NRF-1 reporter gene activity (Kraft et al., 2005), making them well suited to identify potential NRF-1 sites in heterologous promoters. Thus, we constructed a series of goldfish *COX4-1* promoter deletion mutants (Fig. 1A) to identify the regions that are critical for expression in proliferating myoblasts and differentiating myocytes.

In myoblasts, the analyses showed that sequential deletions of the distal regions had no effect on reporter activity until the promoter was reduced from 230 to 159 bp. Of note, deletion of the region between 452 and 312 bp, which possesses the putative NRF-1 site, was inconsequential in myoblasts (Fig. 2A). A lack of role for NRF-1 regulation of the proximal promoter in myoblasts was further supported by site-directed mutagenesis; mutation of the putative NRF-1 site in a longer promoter had no effect (Fig. 2D).

A slightly different story was seen in differentiating myocytes. The proximal promoter increased activity 2-fold in myogenesis (Fig. 2C), consistent with what is seen for the endogenous *Cox4i1* gene (Kraft et al., 2005). Sequential deletion of the proximal promoter had no effect until a 25% reduction in activity was seen when the promoter was shortened from 452 to 312 bp. Thus, in contrast to myoblasts, myocytes showed a reliance on a region that includes the putative NRF-1 site (Fig. 2B). However, site-directed mutagenesis of the putative NRF-1 site had no effect (Fig. 2D), arguing that the dependency on this region in differentiated muscle was due to other transcription factors. We did not further explore the features of the promoter that regulated expression in mouse myoblasts, but there were two critical regions, each of which possesses multiple transcription factor binding sites. The region from −452 to −312 contains a putative binding site for MEF-2,

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which is known to regulate COX genes (Wan and Moreadith, 1995; Lee et al., 2011). Deletion of the region from −230 to −159, which possesses sites for CREB and NF-1, also led to a decrease in expression of the goldfish *COX4-1* reporter in myocytes. Our main focus was on identifying the thermally responsive elements, focusing on NRF-1.

Is there a region on the *COX4-1* gene promoter needed for a temperature response?

While the mouse cell line was useful for exploring basic properties of the goldfish promoter, it is neither suitable nor appropriate for assessing the effects of temperature. To assess the effects of temperature, we employed a cold-tolerant rainbow trout cell line (RTG-2) that has been useful to study transfections of other metabolic genes (Rees et al., 2009). In assessing the effects of temperature, it is necessary to consider the possibility of direct effects of temperature on processes other than transcription of the gene of interest. Co-transfection of *Renilla* and luciferase constructs controlled for simple thermal effects on general transcription or translation.

Although the promoter was readily transfected and expressed at 4°C and 19°C, cold-temperature incubation did not cause an increase in the expression of the luciferase reporter relative to the *Renilla* control. Nonetheless, we used the deletion mutants to assess the relative importance of the various regions as we had performed in mouse muscle cells. However, these cells, derived from gonad, appeared to rely entirely on a different region for regulation of expression of the gene. There was no consequence of deleting most of the promoter and full expression was seen in constructs as short as 114 bp. This region possesses an Sp1 site, which is critical in many mitochondrial genes for regulating basal expression (Kraft et al., 2005). The lack of response to temperature, and the reliance on the most proximal elements meant that these studies shed no light on the role of the putative NRF-1 elements. In addition, we are unaware of studies that have assessed if gonad and/or fibroblasts undergo the same cold-induced mitochondrial proliferation seen in muscle.

With inconclusive results from RTG-2 cells, we continued the promoter analysis using *in vivo* injections of reporter genes directly into muscle of warm- and cold-acclimated goldfish. Cold acclimation of goldfish increases in *COX4-1* mRNA in white muscle (Duggan et al., 2011; Bremer et al., 2012), so we expected that *in vivo* transfection of a *COX4-1* proximal promoter construct into goldfish white muscle would lead to a corresponding increase in luciferase. Instead, *COX4-1* reporter activity was about 50% lower in cold-acclimated goldfish (Fig. 4B).

We used this opportunity to ask whether there were differences in the thermal sensitivity of the *COX4-1* promoters in fish that had evolved different thermal strategies. Both zebrafish and goldfish are cyprinids, but they differ in their thermal tolerance. Previous studies showed that zebrafish did not experience an increase in COX activity or *COX4-1* mRNA in the cold, with the caveat that it was not possible to cool zebrafish to the temperatures (<4°C) that trigger mitochondrial biogenesis in goldfish (Duggan et al., 2011). When homologous reporters were compared from zebrafish and goldfish *COX4-1* genes, they were affected in the same manner. Reporters from both species showed an approximate 50% reduction in activity in cold-acclimated goldfish, although the difference was significant only for the goldfish construct (Fig. 4B). Had we found evidence of evolutionary variation in the thermal responsiveness of fish *COX4-1* proximal promoters, we would have performed further studies with a broader range of phylogenetically appropriate species.

Does NRF-1 binding to *COX4-1* increase in cold-acclimated goldfish?

The *in vitro* and *in vivo* models that we used have benefits and limitations, but collectively, analyses of the goldfish *COX4-1* promoter failed to show evidence of cold-induced activation. Thus, in parallel with this transfection work, we explored the regulation of the *COX4-1* promoter *in vivo*. ChIP permits the identification of factors that are bound to specific regions of DNA *in vivo*. In addition to the putative element in the proximal promoter, this approach also allowed us to study the role of the putative element in the 5′-UTR, a region that was not evaluated in the promoter analyses.

The ChIP analysis showed that antibodies to NRF-1 enriched the precipitation of the putative NRF-1 elements in both proximal promoter and 5′-UTR, supporting a potential interaction between NRF-1 and the gene. However, the modest ChIP enrichment seen with warm fish muscle diminished when cold fish muscle was used (Fig. 5). Thus, if NRF-1 does indeed bind to these elements, the data suggest that the interaction is reduced in cold-acclimated fish. Therefore, consistent with the results from transfection studies, changes in *COX4-1* mRNA with thermal acclimation do not appear to be driven by NRF-1 binding to the proximal promoter.

Given the strong correlations between NRF-1 (mRNA and protein) and COX4-1 (mRNA and protein) in thermally acclimated goldfish (Bremer et al., 2012), the failure to show a direct mechanistic relationship was surprising. It may well be that the observed changes in NRF-1 exert effects outside the proximal promoter. It is also possible that in focusing on the state of thermally acclimated steady state, that we have missed the influence of regulatory factors and signal transduction pathways that are more prominent during the acclimation process itself. While we focused our efforts on assessing the link between differences in NRF-1 and COX4-1 levels in thermally acclimated fish, whether a different scenario plays a role during acclimation remains unknown. It is possible, for example, that the regulators that generate a change during acclimation differ from those that maintain a difference upon acclimation.

Broader implications

This study continues efforts to understand the regulatory basis of the metabolic reorganization that accompanies cold acclimation of fish. This paradigm is interesting because it touches on many important themes in comparative physiology and the evolution of environmental responsiveness.

When considering thermally responsive genes, it is unclear how much can be gained from background literature derived from homeothermic models, such as mammals. There will be some aspects of gene regulation that are analogous, and it may well be that fish metabolic genes respond to thermally induced metabolic perturbations rather than cold temperature per se. Based upon this assumption, we asked if NRF-1 regulation of the proximal promoter drives cold-induced increases in *COX4-1* expression. The lack of support for this hypothesis may have more to do with the regulation of the gene through distal interactions than a fundamentally different mechanism to sense and respond to cold versus more general energetic stresses. It may well be that a change in thermal environment in fish has complex effects, including elements of metabolic stress as well as kinetic and thermodynamic effects arising from temperature. The complex nature of a thermal stressor may be one reason why the various COX genes appear to be less coordinated in thermal acclimation (Duggan et al., 2011) than is expected in mammalian paradigms of mitochondrial biogenesis.

Because of its duration and predictability, seasonal temperature change is well suited to studying how animals use genetic mechanisms to modify the metabolic phenotype. Thus, the evolution of thermal responsiveness of individual genes provides insight into the mechanisms by which animals cope (or fail to cope) with thermal changes. Eurythermic animals are interesting models because they have all the same basal requirements of stenothermic animals yet must also have a capacity to modify their metabolism in response to conditions that would kill more stenothermic animals. The proximal promoter is the most important part of the control region in genes, and it stands to reason that basal regulation may be fundamentally similar in related animals. In this study, we found that the proximal promoter of *COX4-1* was unresponsive to temperature, and did not differ in thermal responses between eurythermic goldfish and stenothermic zebrafish when studied in the same cellular background. It seems likely that genetic control of lineage-specific traits, such as differences in thermal tolerance of closely related species, would reside outside the proximal promoter.

This study also contributes to a broader understanding of the evolution of transcriptional regulation in vertebrates. A great many transcriptional regulators are highly conserved in structure, but in the case of metabolic gene regulation, it appears likely that at least one transcription factor may play different roles in mammals and fish. PGC-1α, which is critical to integration of transcriptional cascades in mammalian metabolism, differs in structure in ways that suggest a distinction in function. Fish PGC-1α has mutations in its NRF-1 binding domain (LeMoine et al., 2010) that appear to leave it incapable of binding NRF-1 (Bremer et al., 2016). Likewise, PGC-1α also lacks a site that is essential for regulation by AMPK (Bremer et al., 2016), which is a critical energy transducer in eukaryotes. While this may cast doubt on the role of PGC-1α as a master regulator of mitochondrial biogenesis in fish, it may well be that NRF-1 plays a similar role in fish and mammals, acting through a different coactivator such as PGC-1β. Cold acclimation leads to an increase in nuclear content of NRF-1 protein (Bremer et al., 2012), but where NRF-1 binds and which genes it regulates in cold acclimation remain to be determine directly, however it appears likely that this control is exerted outside the proximal promoter of *COX4-1*.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
G.G. and C.D.M. contributed equally to the design, analyses and writing, with G.G. performing the bench work and C.D.M. providing the funding.

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