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HTF.WIR.1.15

ELSEVIER

Marine Environmental Research **39** (1995) 137–141
Elsevier Science Limited
Printed in Great Britain

0141-1136(94)00045-X

A Comparison of RT-PCR and Northern Blot Analysis in Quantifying Metallothionein mRNA Levels in Killifish Exposed to Waterborne Cadmium

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ABSTRACT

Reverse transcriptase PCR (RT-PCR) was evaluated as an alternative to Northern blot analysis in quantifying levels of metallothionein (MT) mRNA. Killifish were exposed to water-borne cadmium (Cd) at concentrations ranging from 0 ppb to 46 ppb and their levels of hepatic MT mRNA were quantitated by both Northern blot analysis and RT-PCR. Both methods provided comparable results in that low constitutive levels of MT mRNA were observed in fish exposed to clean water, dose-responsiveness was seen in fish exposed to increasing concentrations of Cd, and sensitivities of both techniques were similar in detecting induced levels of MT mRNA in Cd exposed fish. These results suggest that RT-PCR may provide a sensitive and quantitative method to evaluate gene expression in fish from small, non-invasively sampled tissues.

INTRODUCTION

Quantification of mRNA levels for inducible genes in aquatic organisms may be used to evaluate their exposure histories to toxicants, early biological effects, or understanding mechanisms underlying fluctuating levels of protein expression (Kreamer *et al.*, 1991; Haasch *et al.*, 1993). Evaluation of gene expression at the mRNA level offers the advantages of quantification of gene induction at specific gene loci and a lack of interference from post transctiptional processes which may impact on levels of protein expression or activity. However, conditions may often arise where it is difficutt or impossible to obtain target tissues in sufficient quantities to isolate enough total RNA to conduct traditional Northern blot analysis.

The development of PCR methodology has permitted both qualitative and quantitative analysis of DNA and RNA sequences from extremely small numbers of template molecules. In reverse transcriptase-PCR (RT-PCR), cDNA is quan-

titatively generated from total RNA. PCR, with gene-specific primers, is then used to produce sufficient quantities of the target cDNA to allow for its quantification. This process becomes specific, reproducible, and quantitative when external controls (mRNAs of known starting copy number) and internal standards (gene specific probes with a truncated sequence) are used to correct for variability in reverse transcription and PCR amplification.

Methallothionein (MT) genes can be induced in fish by treatment with certain heavy metals, such as cadmium (Cd), copper, mercury, and zinc (Zafarullah *et al.*, 1989) and MT protein induction may serve as a mechanism of cellular defense against metal toxicity. MT genes have been cloned from rainbow trout (Bonham *et al.*, 1987) and winter flounder and studies have indicated that MT gene expression is transcriptionally regulated in these species (Zafarullah *et al.*, 1988). As a result, it has been suggested that MT gene induction may serve as a sensitive and quantifiable marker of exposure and early biological effect of these heavy metals in aquatic organisms (Kille *et al.*, 1992).

This study is an evaluation of the use of RT-PCR to quantify levels of hepatic MT mRNA in killifish (*Fundulus heteroclitus*) which had been exposed to one of five water-borne Cd concentrations. Levels of MT mRNA detected by RT-PCR were compared to those determined by northern blot analysis on the same liver samples.

MATERIALS AND METHODS

Animals and treatments

Killifish were collected in Great South Bay, Long Island, New York, depurated, and exposed to non-filtered water containing 0, 2, 4, 12 or 46 ppb $CdCl_2$. Following an eight day exposure, fish were sacrificed and livers were snap frozen and stored at −70°C.

RNA isolation and Northern blot analysis

Total RNA was isolated using RNAzol reagent following the manufacturer's recommendations (Biotecx, Houston, TX) as described by Kreamer *et al.* (1991) and levels of MT mRNA were quantitated by Northern blot analysis (Founey *et al.*, 1988). Membranes (Nytran, Schleicher and Schuell) were hybridized at 65°C to ^{32}P radiolabeled, Cd induced winter flounder (WF) metallothionein cDNA probes (pGEMWFMTC4). Final wash conditions were 1 × SSPE/0.1% SDS at 65°C. Levels of MT mRNA were quantified from autoradiographs by densitometry.

Internal standard construction

To correct for variability in the efficiency of the reverse transcriptase (rTtH) reaction and PCR amplification, a MT mRNA internal standard was developed from the WF MT cDNA sequence by deletion of 6 bp (100–105) by site directed mutagenesis (Sambrook *et al.*, 1989). The deletion reaction contained, 2 mM dNTPs, 10 U vent polymerase, 1 × (PCR) buffer, 5 μM primers and 4 mM $MgCl_2$ which were added to 20 pmol of WF MTcDNA in a total volume of 50 μl and amplified at 94°C for 30 s, 55°C for 30 s, and 72°C for 45 s for 35 cycles.

Reverse transcription

A cocktail containing 12.5–50 μg template killifish RNA, 2.5 U rTth (Perkin-Elmer), 20 pmol oligo dT, 10 mM Tris pH 8.3, 50 mM KCl, 0.2 mM dNTPs, 1 mM $MnCl_2$, and 0–1 pmol truncated MT mRNA internal standard was incubated at room temperature for 10 min, 42°C for 10 min, and 72°C for 2.5 min followed by cooling (Sambrook *et al.*, 1989; Jesson-Eller *et al.*, 1993).

Primer design and PCR

Amplification primers were selected to produce 114 bp and 108 bp products for WF MT and truncated WF MT mRNA. Biotin was added to one of the primers to allow for the capture of PCR products with streptavidin beads. Hybridization probes were tagged with ruthenium (Ru) and designed to detect amplified MT (bp 95–115) and truncated MT (bp 95–118) products by luminescence. PCR conditions were 30 s at 94°C, 30 s at 55°C, and 45 s at 27°C for 30–40 cycles in a Perkin-Elmer 9600 PCR machine.

To determine the relationship between starting copy number of MT mRNA and truncated MT mRNA luminescence, the reverse transcriptase reaction was carried out with a series of dilutions for MT mRNA and truncated MT RNA followed by PCR. The reaction mixture was divided into 10 fractions; one was immediately placed on ice and used as the 0 cycle control, while the rest were amplified for 12–36 cycles.

Luminescence procedure

Luminescence was used to quantify MT PCR product by incubating the PCR products with Ru tagged hybridization probes at 94°C for 5 min followed by 5 min at the annealing temperature (T_α). Streptavidin beads were used to trap the PCR product through the biotin-linkage on one of the primers. Following termination of hybridization, streptavidin trapped PCR product was aspirated into a flow cell, and trapped on a working electrode. Through a series of reactions, ultimately resulting in luminescence by the Ru-tagged probe, PCR product number was determined (Jesson-Eller *et al.*, 1993).

Determination of initial MT mRNA copy number

Starting copy number of MT mRNA was determined by data from PCR cycles 9 through 30 (exponential portion of the curve) and the equation $y = b10^{xs}$ (where x = cycle number, s = slope of the curve, b = constant, and y = ECL output at a given cycle) ($r = 0.99$). When $x = 0$, a y value is generated that is related to starting copy number of MT mRNA.

RESULTS

Northern blot analysis on the complete set of samples revealed the dose-responsiveness of hepatic MT mRNA induction in killifish exposed to water-borne Cd (Fig. 1). Constitutively expressed low levels of MT mRNA were detected in fish which had been exposed to 0 ppb Cd. Approximately 8-fold induction of MT

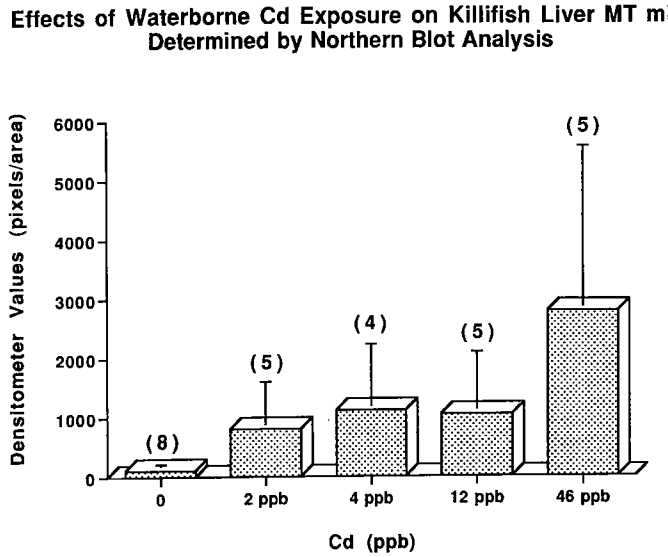

Fig. 1.

mRNA was observed in fish exposed to the lowest Cd concentration (2 ppb) and levels of MT mRNA induction increased to 26-fold in fish exposed to the highest concentration of Cd (46 ppb). An unexpectedly low level of MT mRNA was seen in killifish exposed to 12 ppb Cd. Northern blot analysis of MT mRNA levels in these samples provided a benchmark against which to evaluate the sensitivity and accuracy of RT-PCR.

RT-PCR was used in combination with ECL to quantitate levels of MT mRNA in a subset of these Cd exposed killifish. Specificity of the RT-PCR for killifish MT mRNA was established during optimization of PCR and hybridization conditions. Constitutive levels of MT mRNA levels were observed in the unexposed controls, but the MT mRNA copy number ($4 \times 10^3/100$ μg total RNA) was very low (Fig. 2). Five-fold induction of MT mRNA was detected at the lowest Cd concentration, 2 ppb. At the highest exposure concentration of Cd (46 ppb), copy number of MT mRNA was $102 \times 10^3/100$ μg total RNA or 25-fold induced over control values. Thus, the dose-responsiveness of MT mRNA induction in killifish to water-borne Cd exposure was also observed using RT-PCR.

RT-PCR and Northern blot analysis provided comparable results in quantitating MT mRNA levels in Cd exposed killifish. MT mRNA copy number determined by RT-PCR was dose-responsive and as sensitive as Northern blot analysis. The magnitude of MT mRNA induction detected by both methods at low (2 ppb) and high (46 ppb) Cd concentrations were similar. RT-PCR with ECL offers several advantages over Northern blot analysis including direct determination of mRNA copy number by non-radioactive means and the ability to analyze gene expression in small tissue samples. For environmental monitoring programs, RT-PCR will provide the ability to quantitate levels of gene expression in non-invasively secured target tissues and thus provide the potential to monitor the exposure history of individual fish over time.

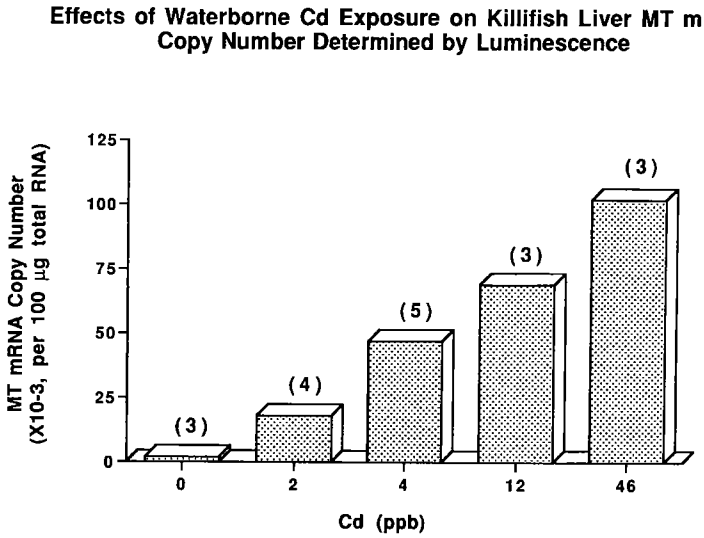

Fig. 2.

ACKNOWLEDGEMENTS

This study was supported by Perkin-Elmer Cetus, NIEHS Grant ES04895, and the Hudson River Foundation.

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