Fish Physiol Biochem (2008) 34:19–24
DOI 10.1007/s10695-007-9141-x

Stability of RNA isolated from post-mortem tissues of Atlantic salmon (*Salmo salar* L.)

P. J. Seear · G. E. Sweeney

Received: 24 January 2007 / Accepted: 20 April 2007 / Published online: 12 June 2007

Abstract Studies of post-mortem interval on the stability of RNA from a number of various mammals have shown RNA to be stable for between 24 and 48 h following death. As yet there have been no studies looking at RNA stability in post-mortem tissues of poikilothermic fish. Brain, kidney, liver and muscle were collected from Atlantic salmon (*Salmo salar*) parr and samples of each tissue were placed into RNA*later*™ after 0–24 h post-mortem storage at room temperature. Electrophoretic analysis of the total RNA showed degradation of ribosomal RNA only in muscle from 8 h onwards. Probing of northern blots with β-actin showed that, in the brain, β-actin mRNA was stable for 24 h post-mortem but degradation of mRNA was observed after 8 h with the kidney and liver and after 4 h with the muscle. Expression of the weakly expressed thyroid hormone receptor β (TRβ) was detected by reverse transcriptase polymerase chain reaction (RT-PCR) in all tissues up to 24 h post-mortem although a reduction in PCR product was observed after 8 h with muscle and 24 h with kidney. Analysis with an Agilent 2100 Bioanalyzer showed that the RNA integrity number (RIN) of brain total RNA remained constant for 8 h post-mortem with only a small fall at 24 h post-mortem. The RINs of the remaining tissues indicated degradation at 8 h post-mortem with kidney and muscle and at 24 hours post-mortem with liver. Taken together these findings show that degradation of Atlantic salmon RNA is tissue dependent but stable for at least one hour post-mortem.

Keywords Brain · Degradation · Kidney · Liver · Muscle · Post-mortem interval · RNA stability

Abbreviations
RIN RNA integrity number
TRβ Thyroid hormone receptor β

Introduction

Accurate gene expression measurements in fish by techniques such as quantitative real-time PCR and microarray analysis depend on obtaining messenger RNA of a quality that is representative of that expressed in the cell immediately prior to death. Given that mRNA is degraded in the cell as part of normal RNA turnover (Wilusz et al. 2001), it is essential that post-mortem tissues are recovered and stored as quickly as possible in such a way as to limit

P. J. Seear (✉)
Biological Sciences Division, British Antarctic Survey, High Cross, Madingley Road, Cambridge CB3 0ET, UK
e-mail: paea@bas.ac.uk

G. E. Sweeney
Cardiff School of Biosciences, Cardiff University, Biomedical Sciences Building, Museum Avenue, Cardiff CF10 3US, UK
e-mail: SweeneyGE@cf.ac.uk

mRNA degradation. It is also desirable however, to recover a wide variety of tissues in order to maximize the information gained from each fish and this may mean that in the last tissues to be harvested the mRNA may have degraded more than in the first tissues.

RNA is highly unstable and is commonly assumed to degrade rapidly after death. However, studies that have looked at RNA stability in a number of post-mortem tissues of human (Johnson et al. 1986), cow (Fitzpatrick et al. 2002), pig (Malik et al. 2003), rabbit (Marchuk et al. 1998), and rat (Wetzel et al. 1994) have found that RNA is commonly stable for between 24 and 48 h post-mortem at room temperature (depending on the type of tissue). These studies have so far however, concentrated on mammals where the normal body temperature is 15–18°C higher than typical room temperature, presumably resulting in endogenous nucleases functioning at a suboptimal temperature. Consequently, we were interested in performing a RNA stability time course study on poikilothermic Atlantic salmon *Salmo salar* (L.) where at room temperature nucleases would be functioning closer to normal physiological temperature. In our current study we removed four tissues from an Atlantic salmon parr and incubated them for 0–24 h at room temperature prior to preservation in RNA*later*™ (Ambion). RNA integrity was analyzed using agarose gel electrophoresis, northern blotting, and reverse transcriptase PCR (RT-PCR). An Agilent 2100 Bioanalyzer was also used in order to determine a RNA integrity number (RIN) for each RNA sample. This user-independent RIN is calculated using a software algorithm (Schroeder et al. 2006) that takes into account the entire electrophoretic trace of the RNA as generated by the Agilent Bioanalyzer and has been shown to be more reproducible and reliable in assessing RNA quality than conventional methods (Imbeaud et al. 2005).

Methods

Tissue sampling

Atlantic salmon yearling parr were collected from Llynyfan Hatchery, Llanddeusant, Mid Wales in 2003 and 2004. For one week, parr were held in a 5 m diameter, 70 cm deep, circular tank with continuous aeration and water flow (approximately 1 l min^{-1}) in a controlled-temperature (11°C) room. Brain (2004 sampling only), liver, muscle and a sample of kidney were removed from a single fish euthanized by immersion in 125 mg l^{-1} tricaine methanesulfonate (MS-222, Sigma) and each tissue divided into five approximately equal pieces. One piece from each tissue was immediately placed into 1 ml of RNA*later*™ (Ambion) and incubated at 4°C overnight before storing at −20°C. The remaining tissue fragments were placed into 1.5 ml tubes and incubated at room temperature (19°C). At set time points (1, 4, 8 and 24 h post-mortem) 1 ml of RNA*later*™ was added to a sample of each of the tissues and placed at 4°C for overnight incubation before storing at −20°C.

Total RNA extraction

Each tissue sample (100–200 mg) was homogenized in 1 ml of Tri Reagent® (Sigma) per 100 mg of tissue using 5 ml borosilicate glass-on-glass homogenizers (Jencons-PLS). Total RNA was extracted following manufacturer's instructions except that 1.5 ml Phase Lock Gel® tubes (Eppendorf AG) were used to allow easy separation of the aqueous from the organic phases. Spectrophotometry was used to quantify total RNA concentration.

Agarose gel electrophoresis

A 1% agarose gel with 0.5 μg ml^{-1} ethidium bromide was used to fractionate 1 μg of brain total RNA and 3 μg of kidney, liver and muscle total RNA. Electrophoresis was performed in 1× TBE at 100 V for 45 min before visualizing under ultraviolet light using the GelDoc-It imaging system (Ultra-Violet Products).

Northern blot analysis

For the northern blot 3 μg of kidney, liver and muscle total RNA and 1 μg of brain total RNA were fractionated through a denaturing formaldehyde 1.5% agarose gel in 1× MOPS buffer. RNA was transferred to a nylon membrane with 20× SSC overnight and fixed using a Stratalinker® UV Crosslinker (Stratagene). The membranes were prehybridized at 65°C in 6× SSC, 0.1% SDS, 5× Denhardt's solution and

100 μg ml^{-1} tRNA for 2 h. The prehybridized blots were then hybridized overnight at 65°C with a ^{32}P-radiolabeled 1,800 bp DNA fragment of Atlantic salmon *β*-actin [GenBank: AAB65430.1]. The *β*-actin probe was prepared using the Megaprime™ DNA labeling system kit (Amersham Biosciences). The membranes were washed twice in 1× SSC, 0.1% SDS at room temperature and twice at 65°C for 20 min each. Following washing, the membranes were wrapped in Saran wrap and exposed to Kodak MXB film with intensifying screens at −80°C.

RT-PCR analysis

For RT-PCR, 8 μg of muscle, kidney and liver and 1 μg of brain total RNA were DNase treated using DNA-*free* (Ambion). Briefly, 0.5 μg of DNase-treated total RNA was reverse transcribed using SuperScript™ II reverse transcriptase (Invitrogen) and 100 ng of random primers in a 20 μl reaction. This cDNA was then used as a template for PCR using primers specific for 18S rRNA (forward: 5′-TC AAGAACGAAAGTCGGAGG-3′ and reverse: 5′-A AAGATAGCGTCCGACAC-3′) and thyroid hormone receptor *β* (TR*β*) (forward: 5′-GTGGACATAG AAGCCTTC-3′ and reverse: 5′-AAAGATAGCGT CCGACAC-3′). The forward and reverse TR*β* primers were designed to anneal to different exons in order that DNA contamination could be identified. PCR was performed using 1 μg of cDNA in a total volume of 20 μl that contained 2× Red Taq® ReadyMix™ with 1.5 mM $MgCl_2$ (Sigma) and 1 μM of forward and reverse primers. PCR conditions for 18S rRNA were 95°C for 1 min before 13 cycles of 95°C for 30 s, 56°C for 1 min and 72°C for 30 s, followed by a final extension step of 72°C for 1 min and for TR*β*, the conditions were 94°C for 5 min before 30 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 30 s, with 72°C for 5 min final extension. PCR products were visualized by electrophoresing 15 μl of product in a 2% agarose gel with 0.5 μg ml^{-1} ethidium bromide in 1× TBE buffer.

Agilent Bioanalyzer analysis

The Agilent 2100 Bioanalyzer with the RNA 6000 LabChip® kit (Agilent Technologies) was used to analyze 200–300 ng of RNA and provide a RNA integrity number (RIN) for each sample.

Results

Agarose gel electrophoresis

Electrophoresis showed little degradation of ribosomal RNA extracted from kidney, brain and liver although low-molecular-weight bands and smearing are visible after 4 h with liver and after 8 h with brain, suggesting limited degradation (Fig. 1). With muscle however, rRNA degradation is more obvious with the high-molecular-weight bands seen at 0 and 1 h post-mortem absent after 4 h and the 28S rRNA band becoming less intense from 8 h onwards. The agarose gel of the muscle total RNA also shows low-molecular-weight bands below the 18S rRNA band after 4 h that become relatively intense after 24 h.

Northern blot analysis

To assess the mRNA integrity, RNA was transferred from the denaturing agarose gels onto nylon membranes and hybridized with a 1,800 bp cDNA fragment of the Atlantic salmon *β*-actin gene. A single mRNA transcript of the expected size of 2 kb was detected in all samples (Fig. 2). The degradation rate of the *β*-actin mRNA was found to vary between tissues. With the brain autoradiograph the *β*-actin band intensity remained constant throughout the time

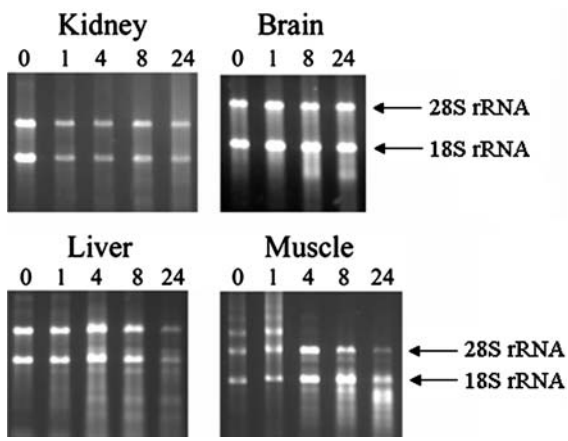

Fig. 1 Non-denaturing agarose gel (1%) of total RNA from post-mortem tissue stored at room temperature. Numbers above each lane correspond to the number of hours post-mortem. Gels shown are from the 2004 sampling, but are representative of 2003 tissue samples (except that brain RNA was not analyzed in the 2003 experiment). There was not sufficient RNA to run a sample of 4 h post-mortem brain

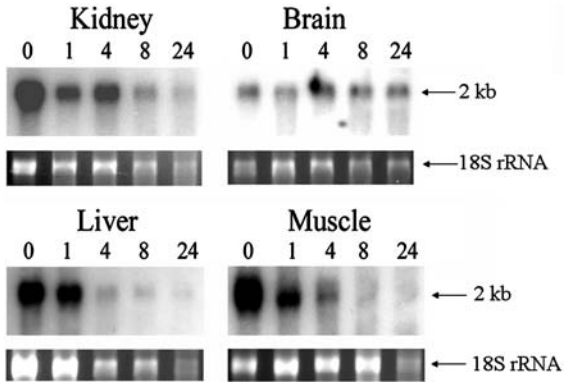

Fig. 2 Northern blot analysis for β-actin of post-mortem tissue incubated at room temperature for increasing periods. The 18S rRNA band of the corresponding denaturing formaldehyde agarose gel (1.5%) from which the northern was taken is shown below each blot. Numbers above each lane correspond to the number of hours post-mortem. The northern blots shown are from the sample taken in 2004, but are representative of samples from 2003 (except that brain RNA was not analyzed in the 2003 experiment)

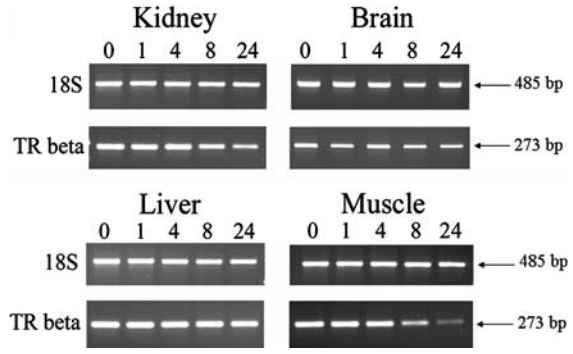

Fig. 3 RT-PCR analysis of post-mortem tissues stored at room temperature from 0 to 24 h. Numbers above each lane correspond to the number of hours post-mortem. RT-PCR was performed using 0.5 μg DNased total RNA from all tissue samples using primers to detect low-copy-number messages (TRβ) and primers that amplified the housekeeping 18S rRNA gene. Gels shown are from the 2004 sampling, but are representative of 2003 tissue samples (except that brain RNA was not analyzed in the 2003 experiment)

series indicating no mRNA degradation. The kidney autoradiograph shows a reduction in signal intensity at the 1 h time point, although no further reduction until 8 h post-mortem. With liver, although the reduction in signal intensity from 1 to 4 h post-mortem can be seen to be due more to unequal loading than mRNA degradation when the autoradiograph is compared to the agarose gel, degradation does appear to have occurred at 8 h post-mortem. The β-actin mRNA in the muscle was found to be the least stable, with a steady reduction in signal intensity up until 8 h post-mortem, at which time the mRNA appeared to be almost fully degraded.

RT-PCR analysis

The integrity of the mRNA was further assessed using RT-PCR to detect the presence of transcripts of the low-copy-number thyroid hormone receptor β (TRβ) gene. RT-PCR was also performed using primers specific to the 18S ribosomal RNA gene in order to normalize for variations in initial RNA sample concentration and reverse transcription efficiency. PCR conditions were optimized with respect to cycle number to ensure that the plateau phase—when the accumulation of product slows and stops—was not reached. Products of the expected length of 273 bp for TRβ were successfully amplified from all tissue samples (Fig. 3). With the brain and liver samples, there was no reduction in PCR product over time. With muscle and kidney however, there were reductions in PCR product at 8 and 24 h post-mortem respectively.

Agilent Bioanalyzer analysis

Table 1 shows the RNA integrity numbers of all the post-mortem tissue samples as measured and calculated by the Agilent 2100 Bioanalyzer. The range of the RIN is 1-10 with 1 representing a completely degraded RNA sample and 10 indicating intact RNA (Imbeaud et al. 2005). All tissue samples yield relatively intact RNA with an RIN of approximately 8 at 0 h post-mortem with varying rates of degradation after this time point. Brain RNA is the most stable with only very limited degradation occurring up to 8 h post-mortem, although there is a small drop in RIN at 24 h post-mortem. With kidney RNA, the RIN indicates limited degradation at 1 h post-mortem and again at 8 h post-mortem, but with no further degradation observed after this time point. Similar to kidney, there is some degradation with liver RNA at 1 h post-mortem but no further large drop in RIN until 24 h post-mortem. Muscle RNA remains stable up to 4 h post-mortem but degrades after 8 h and again after 24 h post-mortem to give the lowest RIN of all post-mortem tissues.

Table 1 RNA integrity numbers of post-mortem tissues stored at room temperature from 0 to 24 h (2004 samples only)

Tissue	Kidney	Brain	Liver	Muscle
0 h	8.5	8.0	8.5	7.8
1 h	7.1	7.0	6.9	8.3
4 h	6.9	7.9	6.5	7.6
8 h	5.3	7.2	6.3	5.7
24 h	5.3	6.3	4.8	3.9

Discussion

The stability of RNA in post-mortem tissues of Atlantic salmon was assessed by agarose gel electrophoresis of total RNA, probing northern blots with β-actin, detection of a low-copy-number gene (TRβ) by RT-PCR, and analysis with an Agilent 2100 Bioanalyzer. These four different analyses measured different aspects of the RNA and so varied in the assessment of RNA quality. Despite this variation, brain RNA appeared to be almost intact at all post-mortem time points according to all analyses with only a slight reduction in RIN at 24 h post-mortem. The other tissues varied in RNA stability with kidney and liver RNA showing a reduction in RIN after 1 h post-mortem. However, despite this degradation at 1 h post-mortem, the RIN of approximately seven is still relatively high considering that a RIN of 10 represents intact RNA. In their review on the effect of RNA integrity on real-time PCR performance, Fleige and Pfaffl (2006) recommend a RIN higher than five as good-quality total RNA. Taken together with the RINs of brain and muscle and the other analyses, it can be said that the RNA from all tissues was stable for up to 1 h post-mortem. This is remarkable given that unlike in mammals, the salmon RNases would be expected to function at near optimum at room temperature.

A number of previous studies have also found RNA in post-mortem brain tissue to be particularly stable over time, for example in human and rat (Johnson et al. 1986), and mouse (Trotter et al. 2002). In one study it was reported that rRNA and mRNA from rat brains were stable for up to seven days post-mortem (Inoue et al. 2002). Preece and Cairns (2003) showed that a post-mortem interval of 100 h only had a minimal statistically significant influence on mRNA levels in human post-mortem brain. In contrast liver RNA has been found to be relatively unstable (Marchuk et al. 1998) with one study on rats demonstrating degradation after only 24 h (Trotter et al. 2002). This variability in the stability of RNA with post-mortem interval is in agreement with the results presented here and is probably due to either a variation in the type or quantity of ribonucleases present in each type of tissue or the different rates of ribonuclease activation post-mortem. Fleige and Pfaffl (2006) found a large variability in the RIN of a number of bovine tissues that ranged from 4.56 with jenunum to 9.62 with corpus luteum. The authors reasoned that this variability may have been due to variations in the RNase activity, but also differences in tissue structure. The successful detection of the low-copy-number TRβ gene from all tissue samples suggests that RT-PCR would be sensitive enough to detect a wider range of messenger RNAs in these tissues up to 24 h post-mortem.

With only β-actin and TRβ investigated in this study it may be possible that other mRNA transcripts may be adversely affected by the length of the post-mortem interval, especially those known to have a short half-life in vivo (Tourrière et al. 2002). The use of microarray technology allows the investigation of a much wider range of mRNA transcripts and a study by Catts et al. (2005) used a cDNA microarray to investigate the effect of post-mortem interval on mouse brain RNA integrity. It was found that a subgroup of mRNA transcripts with AUUUA motifs in the 3′ UTR were more susceptible to degradation. The results from all four analyses in this study are consistent however, with the mammalian studies that all suggest that the cellular ribonucleases are not activated immediately post-mortem. In conclusion, these results demonstrate that RNA from brain, kidney, liver and muscle is of a high enough quality for use in northerns for up to at least 1 h post-mortem and up to 24 h post-mortem for use with RT-PCR. On a practical note this means that euthanized fish can be dissected slowly and carefully without fear of RNA degradation.

Acknowledgements The authors would like to thank Dr Sarah Rogers for supplying the Atlantic salmon β-actin clone and Dr Marco Campinho for his technical assistance. This work was supported by the Biotechnology and Biological Sciences Research Council under grant no. 72/EGA17676.

References

Catts VS, Catts SV, Fernandez HR, Taylor JM, Coulson EJ, Lutze-Mann LH (2005) A microarray study of post-mortem mRNA degradation in mouse brain tissue. Mol Brain Res 138:164–177

Fitzpatrick R, Casey OM, Morris D, Smith T, Powell R, Sreenan JM (2002) Post-mortem stability of RNA isolated from bovine reproductive tissues. Biochim Biophys Acta 1574:10–14

Fleige S, Pfaffl MW (2006) RNA integrity and the effect on the real-time qRT-PCR performance. Mol Aspects Med 27:126–129

Imbeaud S, Graudens E, Boulanger V, Barlet X, Zaborski P, Eveno E, Mueller O, Schroeder A, Auffray C (2005) Towards standardization of RNA quality assessment using user-independent classifiers of microcapillary electrophoresis traces. Nucleic Acids Res 33:e56

Inoue H, Kimara A, Tuji T (2002) Degradation profile of mRNA in a dead rat body: basic semi-quantification study. Forensic Sci Int 130:127–132

Johnson SA, Morgan DG, Finch CE (1986) Extensive post-mortem stability of RNA from rat and human brain. J Neurosci Res 16:267–280

Malik KJ, Chen CD, Olsen TW (2003) Stability of RNA from the retina and retinal pigment epithelium in a porcine model simulating human eye bank conditions. Invest Opthalmol Vis Sci 44:2730–2735

Marchuk L, Sciore P, Reno C, Frank CB, Hart DA (1998) Post-mortem stability of total RNA isolated from rabbit ligament, tendon and cartilage. Biochim Biophys Acta 1379:171–177

Preece P, Cairns NJ (2003) Quantifying mRNA in post-mortem brains: influence of gender, age at death, post-mortem interval, brain pH, agonal state and inter-lobe mRNA variance. Mol Brain Res 118:60–71

Schroeder A, Mueller O, Stocker S, Salowsky R, Leiber M, Gassmann M, Lightfoot S, Menzel W, Granzow M, Ragg T (2006) The RIN: an RNA integrity number for assigning integrity values to RNA measurements. BMC Mol Biol 7:3

Tourrière H, Chebli K, Tazi J (2002) mRNA degradation machines in eukaryotic cells. Biochimie 84:821–837

Trotter SA, Brill II LB, Bennett JP Jr (2002) Stability of gene expression in post-mortem brain revealed by cDNA gene array analysis. Brain Res 942:120–123

Wetzel DM, Bohn MC, Hamill RW (1994) Post-mortem stability of mRNA for glucocorticoid and mineralocorticoid receptor in rodent brain. Brain Res 649:117–121

Wilusz CJ, Wormington M, Peltz SW (2001) The cap-to-tail guide to mRNA turnover. Nature Rev Mol Cell Biol 2:237–246