Upregulation of Telomerase Function During Tissue Regeneration

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Telomerase plays a primary role in the maintenance of telomeres in immortal, germ, and tumor cells in humans but is lacking in most somatic cells and tissues. However, many species, including fish and inbred mice, express telomerase in most cells and tissues. Little is known about the expression of telomerase in aquatic species, although the importance of telomerase for longevity has been suggested. We compared telomerase activity and telomere lengths among a broad range of tissues from aquatic species and found telomerase at significant levels in both long- and short-lived aquatic species, suggesting constitutive telomerase expression has an alternative function. Telomere lengths in these aquatic species were comparable to those observed in normal human tissues and cell strains. Given that a host of aquatic species with short life spans have telomerase and a tremendous capacity to regenerate, we tested the hypothesis that telomerase upregulation is important for tissue regeneration. During regeneration, telomerase activity was upregulated and telomere lengths are maintained with the shortest telomeres being elongated, indicating the importance for maintaining telomere length and integrity during tissue regeneration. Thus, the expression of telomerase in aquatic animals is likely not related to longevity but to their ability to regenerate injured tissue. Exp Biol Med 233:958–967, 2008

Key words: telomere; telomerase; stem cells; tissue regeneration; chaperones

Introduction

Telomeres are specialized protein-DNA structures at the ends of eukaryotic chromosomes that serve multiple functions, including stabilizing chromosome alignment during cell division and buffering incomplete DNA replication. Most human somatic tissues lack a mechanism to maintain telomeres, causing telomere erosion due to the inability of conventional DNA polymerases to fully replicate linear chromosomes (1–4). The enzyme telomerase is capable of maintaining telomere lengths in a variety of cell types in many different eukaryotic species, from yeast and ciliates to fish and mice (5). Telomerase is comprised of two essential core components: an RNA subunit (TR, telomerase RNA) that serves as the template for telomere addition, and a polymerase protein component (TERT, telomerase reverse transcriptase) that adds telomeric tracts to the ends of the chromosomes (5). Many additional associated proteins have been shown to functionally bind to telomerase, including the RNA binding proteins hnRNP A and C1 (6) and the Hsp90 chaperone complex (7). Exogenous expression of hTERT in normal human cells compensates for telomere loss, which results in the prevention of cellular senescence and extension of cellular life span (8), suggesting that telomere erosion is a

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Table 1. Telomerase, Telomeres, and Life Span of Aquatic Species

Species	Common name	Telomerase[a]	Telomeres	Life span (years)
Cucumaria frondosa	Sea cucumber	+	TTAGGG, 3–9 kb	7
Asterias vulgaris	Sea star	++	TTAGGG	7–8
Strongylocentrotus droebachiensis	Sea urchin	+	TTAGGG, 3–9 kb	24
Homarus americanus	Lobster	+++	TTAGG	50+
Carcinus maenus	Green sea crab	++	TTAGG	5–6
Squalus acanthias	Dogfish shark	+++	10–15 kb	35–40, max 70
Raja erinacea	Little skate	+++	10–15 kb	8
Danio rerio	Zebrafish	+	2–10 kb	3–4
Scomber scombrus	Mackerel	++	n.d.	17
Anguilla rostrata	American eel	++	10–15 kb	45–55
Fundulus heteroclitus	Killifish	+++	2–10 kb	4–5
Oryzias latipes	Japanese medaka	++++	3–12 kb	5
Oncorhynchus mykiss	Rainbow trout	++++	n.d.	6–8
Pleuronectes americanus	Flounder	+++	n.d.	11–12

[a] Quantitatively estimated as the ratio of telomerase-specific amplification to the internal control, where '++++' is 100% activity compared to Rainbow trout cell lines (14), '+++' is 40–80%, '++' is 10–40%, and '+' is less than 10%. n.d., not determined.

fundamental mechanism that contributes to the onset of cellular senescence.

Contrary to humans, inbred strains of mice have exceptionally long telomeres and active telomerase in nearly all tissues. Telomerase knockout mice, in which the TR component was homologously deleted, show varied phenotypes, and after 6 to 7 generations, telomeres shorten to the point where the mice eventually become sterile, presumably due to the diminished proliferative capacity of germ cells (9, 10). Because of the complexity of the mouse system in terms of telomere dynamics and telomerase function, recent efforts have focused on establishing alternative vertebrate models for the study of telomeres and telomerase, which include a variety of aquatic animals such as zebrafish and Japanese medaka (11, 12).

Previous studies relating to telomerase in fish utilized the widely accepted laboratory models of rainbow trout (13, 14), channel catfish (15, 16), and pufferfish (17). Recent evidence indicates a few other aquatic vertebrate species ubiquitously express telomerase, including American eel and zebrafish (11, 12). Most of these studies describe telomerase in aquatic animals without linking its expression to cell function. One group, however, has suggested that longevity in lobsters is due to high expression of telomerase activity (18). Here, we report several aquatic animals, both vertebrate and invertebrate species, express significant levels of telomerase activity in virtually all tissues sampled; but we show that some of these species have telomeres that resemble human telomere lengths, which differs significantly from inbred mice. We further demonstrate that telomerase activity is upregulated and telomeres maintained during tissue regeneration in three species of fish, suggesting that telomerase may be important for tissue renewal and regeneration after injury rather than for overall organism longevity.

Materials and Methods

Animals. Adult medaka (*Oryzias latipes*, CAB strain) and mummichog (*Fundulus heteroclitus*) were obtained from stocks maintained at the Aquatic Biotechnology and Environmental Laboratory, University of Georgia. Zebrafish (*Danio rerio)* were obtained from stocks maintained at Mount Desert Island Biological Laboratories (MDIBL). Invertebrates were obtained from the bottom of Frenchman's Bay off the coast of Mount Desert Island, Maine. All tissues were obtained from freshly euthanized animals (see Table 1 for a list of the aquatic animals analyzed) as part of another IACUC approved project/protocol for MDIBL investigators and not specifically for assessment of telomeres and telomerase activity.

Telomerase Activity Assay. Functional telomerase activity was assessed using the TRAP-eze® Telomerase Detection Kit (Chemicon, Temecula, CA), according to the manufacturer's instructions as described previously (19, 20, 21). The TRAP (telomeric repeat amplification protocol) assay is based on the use of a telomerase substrate (TS) primer, which is elongated by telomerase, followed by PCR amplification. Briefly, telomerase activity is extracted from tissue samples using a low speed drill for pulverization in NP40 lysis buffer (22), followed by centrifugation at 13,000 $\times$ g for 20 minutes. 0.1–2μg of total cellular protein was incubated with the TRAP cocktail for 30 minutes at room temperature. The PCR reaction was subjected to a hot start at 94°C for 3 minutes, followed by amplification at 94°C for 30 seconds and 60°C for 30 seconds repeated 27 times. TRAP products were electrophoresed on 10% PAGE and exposed to phosphorimager screens for 2 hours to overnight (Molecular Dynamics, Sunnyvale, CA). A 36-bp internal control (IC) serves to normalize sample-to-sample variation and quantification of relative telomerase activity levels. Telomerase activity was quantified as the ratio of the signal intensity of the telomerase ladder to the internal control.

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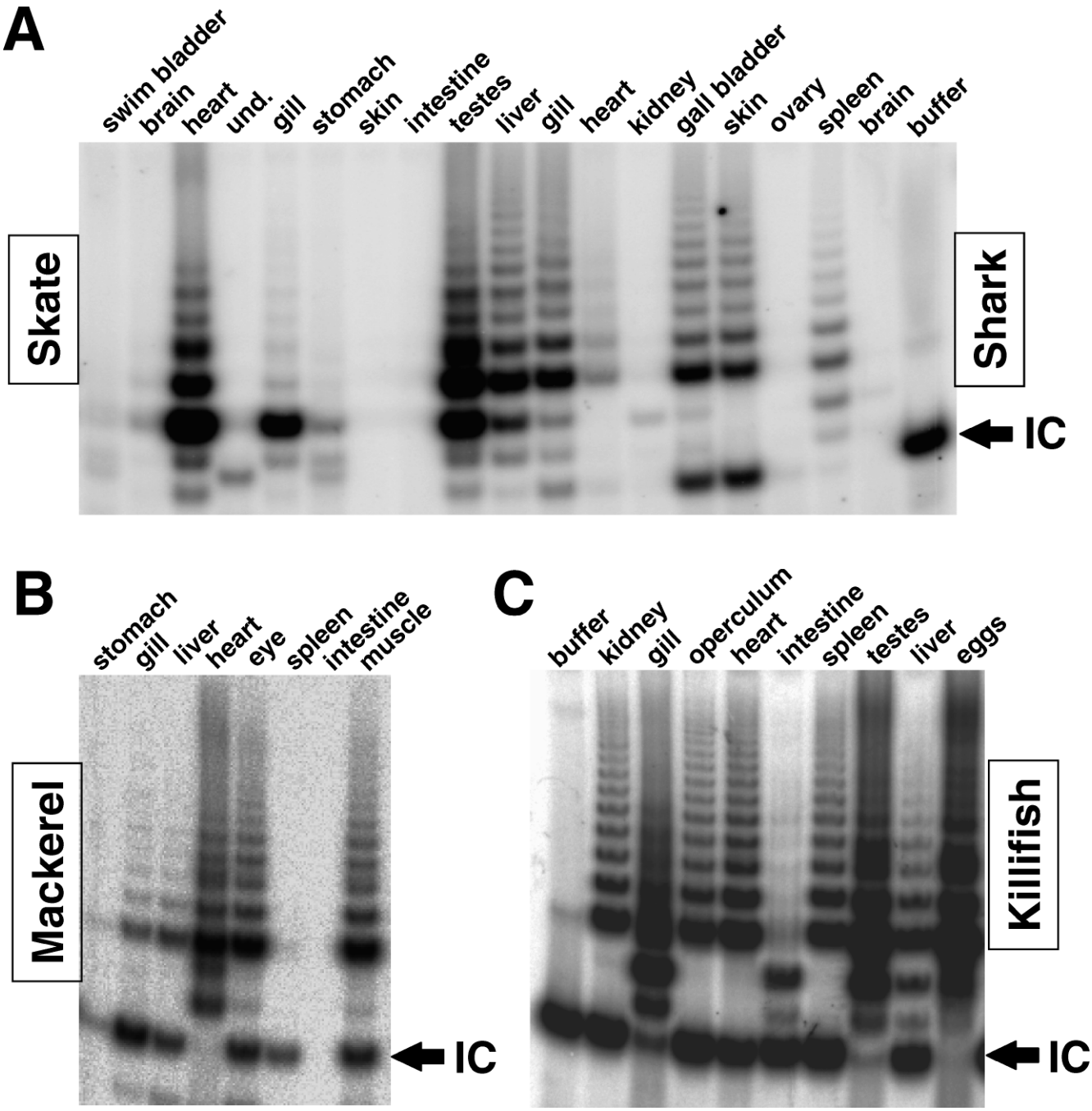

Figure 1. Detection of telomerase activity in tissues isolated from fish. Tissues were harvested from (A) little skate (*Raja erinacea*) and dogfish shark (*Squalus acanthias*), (B) mackerel (*Scomber scombrus*), (C) killifish (*Fundulus heteroclitus*) and tested for telomerase activity. Note the characteristic 6-bp laddering effect beginning at ~50bp, indicative of telomerase activity. Lysis buffer (buffer) serves as a negative control, and 'IC' denotes the 36-bp internal control that serves to quantitatively normalize sample-to-sample variation. Each species was tested for telomerase in 2–3 separate animals. Samples that lack a telomerase ladder and the IC are classified as PCR inhibited and were retested after a second extraction, as before (Gollahon and Holt, 2000; data not shown).

Identification of Telomere Sequence. To determine telomere sequence in the selected invertebrate species, non-radioactive TRAP assays (31 cycles) were performed using only the TS primer and the dedicated reverse primer (RP) (23) without inclusion of the internal control primers. PCR amplified extended products were directly cloned into pCRrTOPO vector using the TOPO TA Cloning Kit (Invitrogen, Carlsbad, CA), transformed into bacteria, and colonies selected. Only recombinant colonies will survive as PCR fragments are cloned into a lethal gene in the plasmid, and if not disrupted, colony survival is negligible. Recombinant colonies were miniprepped (Qiagen, Valencia, CA), sequenced, and screened for the presence of the TS and RP sequences and telomere-type repeat sequences.

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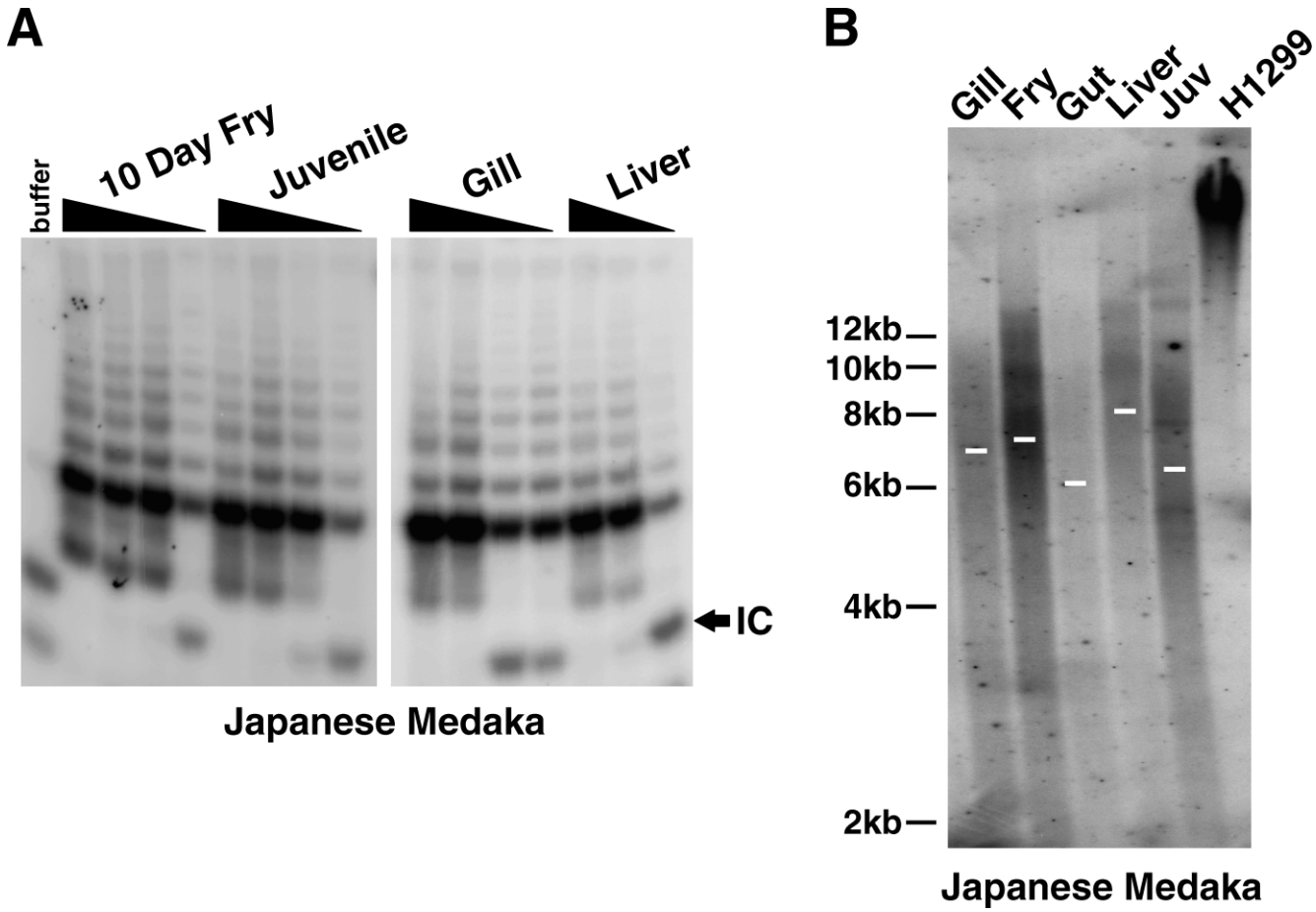

Figure 2. High levels of telomerase activity in Japanese medaka tissues with short telomere lengths. (A) Clarified lysates from medaka tissues in decreasing amounts of total protein (3 μg, 1 μg, 0.3 μg, 0.1 μg) were assayed for telomerase activity using the TRAP assay and electrophoresed on 10% PAGE. Gels were fixed and exposed to phosphorimager screens for 16 hours. 'IC' stands for the 36-bp internal control. Note that the 3-μg sample was not tested for the liver tissue in this representative gel. (B) Telomere lengths were assessed using the telomere amount and length assay (TALA) (Gan et al., 2001). Genomic DNA was isolated, digested, and hybridized with a ^{32}P-labeled $(TTAGGG)_4$ probe, followed by electrophoresis and exposure to a phosphorimager screen. The white bars indicate the median telomere length for each sample. H1299, a human non-small cell lung adenocarcinoma cell line, was included as a positive control.

Telomere Measurements. Telomere amount and length assay (TALA) was modified slightly from Gan et al. (24). Ten to twenty-five micrograms of isolated genomic DNA (Qiagen) was digested using six common 4-base restriction enzymes: Alu I, Msp I, Rsa I, Cfo I, Hae III, Hinf I (Invitrogen) and then hybridized to a radiolabeled telomere specific probe $(TTAGGG)_4$ for 2 hours at 55°C. Samples were cooled to 4°C, loaded onto a 0.8% agarose gel, and electrophoresed at 60 volts for 18 hours. Gels were dried and exposed to phosphorimager screens overnight. Telomere lengths were quantitated using ImageQuaNT Software (Molecular Dynamics) using an established protocol as previously described (25).

Tissue Regeneration Protocol. Fin tissues from 9 adult medaka, 4 adult fundulus, and 6 adult zebrafish were excised and flash frozen. The fin tissue was removed within approximately 2 mm of the base of the caudal peduncle. The fish were allowed to recover and housed in pairs in 10-gallon tanks at room temperature (22–25°C) with a light: dark cycle of 12:12 hours for the duration of the experiment. A second excision of fin tissues was repeated after 15 days for medaka and fundulus and after 7 days for zebrafish to obtain primarily regenerated tissue. Fins appeared to be nearly regenerated in all species. Fin clips were homogenized and lysates prepared as above and tested for telomerase activity using the TRAP assay.

Western Analysis. TRAP protein lysates (20 μg of total protein) were used for Western analysis for the chaperone proteins, hsp90 and its co-chaperone, p23, as well as β-actin (Sigma) for loading control as before (26).

Results

Detection of Telomerase in Aquatic Animals. Sporadic reports have shown telomerase activity in a few aquatic species, including fish and lobster (11–18), but there is little evidence available to explain why these species express the telomerase enzyme. We surveyed numerous

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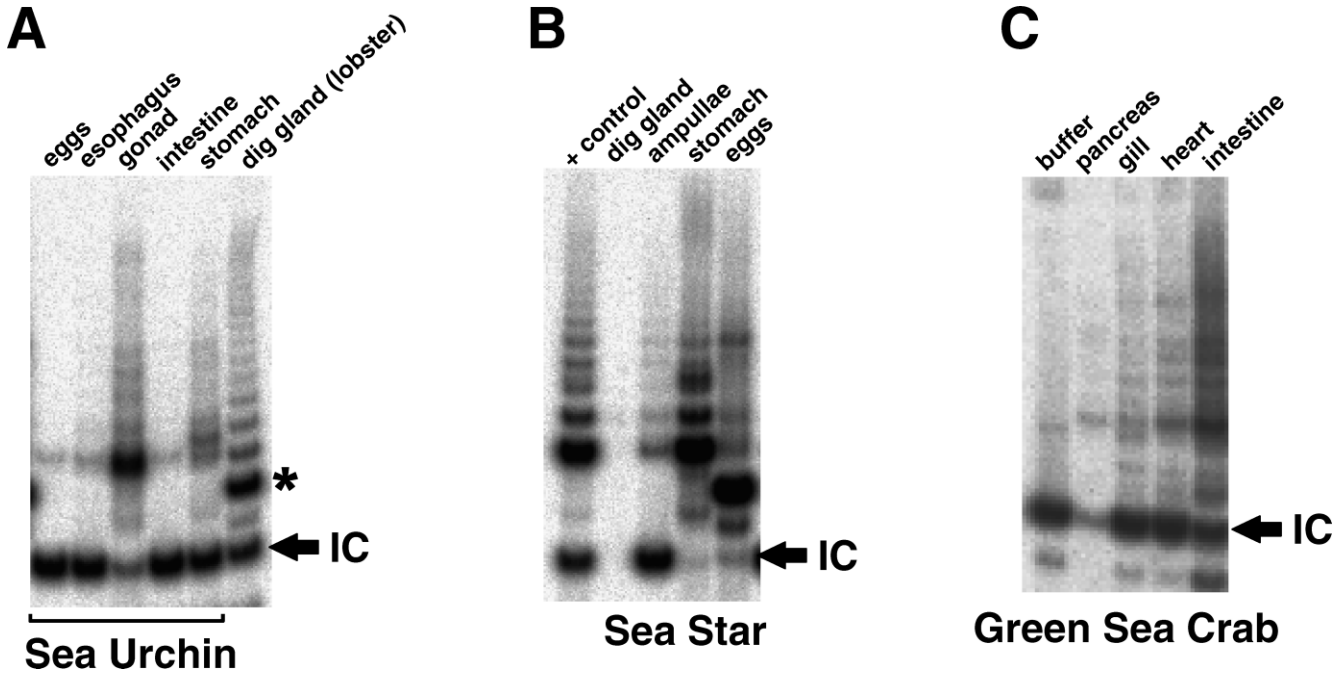

Figure 3. Unique telomerase laddering in select aquatic invertebrates. Tissues from the indicated species were harvested and proteins extracted (McChesney et al., 2005). Lysates were subjected to the TRAP assay and representative gels are shown for (A) sea urchin (*Strongylocentrotus droebachiensis*) and American lobster (*Homarus americanus*), (B) sea star (a.k.a. starfish, *Asterias vulgaris*), and (C) green sea crab (*Carcinus maenus*). Lysis buffer (buffer) serves as the negative control and IC as the 36-bp PCR control as before. Note the unusual banding pattern for green sea crab (C), which lacks a true telomerase laddering effect, and for lobster (last lane of (A), indicated by an *), where the telomerase ladder starts at ~44bp rather than 50bp. Each species was tested for telomerase in 2–3 separate animals.

aquatic animals to determine if expression of telomerase was common to both fish and aquatic invertebrate species. We detected significant levels of telomerase activity in nearly every tissue sampled (Figure 1 and Table 1).

Many of the tissue samples appeared to have some PCR inhibition as evidenced by the lack of both a telomerase ladder and the 36-bp internal control band, which prompted us to re-extract the previously extracted telomerase pellets (27). After the second round of extraction for many of the shark and skate samples (Figure 1A), we found low but detectable levels of telomerase activity in the remaining tissues (data not shown). Marine mammals were also tested (dolphin and sea lion) for telomerase activity and were found to lack the functional enzyme (data not shown). Taken together, our data suggest that expression of telomerase in fish, but not marine mammals, is a ubiquitous characteristic.

Further analyses of tissues isolated from Japanese medaka showed that medaka tissues display high levels of telomerase activity (Figure 2A). However, even with robust telomerase, the telomeres of medaka are relatively short, between 3kb and 12kb with an average of about 7kb (Figure 2B). Overall, most of the species tested had telomere lengths in the range of 2kb to 15kb, more similar to normal cells from humans and quite divergent from inbred strains of mice with 30–100kb telomere lengths (9, 10).

Tissues from a number of invertebrate aquatic species, including sea cucumber, sea star, sea urchin, green sea crab, and lobster, exhibited high telomerase activity in the vast majority of tissues as well (Figure 3 and Table 1). Green sea crab reproducibly showed an unusual telomerase-banding pattern (Figure 3C compared with Figure 2A), suggesting that its repeat sequence is not the vertebrate consensus, TTAGGG. There is a precedent for a non-hexameric repeat in aquatic species as the lobster telomere was shown to be the pentameric TTAGG sequence (18). To determine the telomere sequence in the invertebrate species, we cloned and sequenced telomerase (TRAP) products. We found that sea star, sea cucumber, and sea urchin all contain the hexameric telomere sequence, TTAGGG, found in vertebrates (Table 1), consistent with what has been found for telomeres in clams, mussels, and 2 marine worms (28, 29). We also confirmed that lobster is pentameric and showed that the green sea crab also has the TTAGG pentamer as its telomere sequence (Table 1), which at least partially explains the uncharacteristic telomerase laddering effects observed (Figure 3C).

Upregulation of Telomerase in Response to Tissue Regeneration. Given that tissues from most species examined have telomerase activity, we asked the question: What is the function of widespread telomerase activity in aquatic animals? As shown in Table 1, high levels of activity were present in fish that may live up to 4–5 years (medaka and killifish) (30–32), invertebrates such as sea star, sea cucumber, and green sea crab, as well as vertebrates like little skate and rainbow trout, that live an

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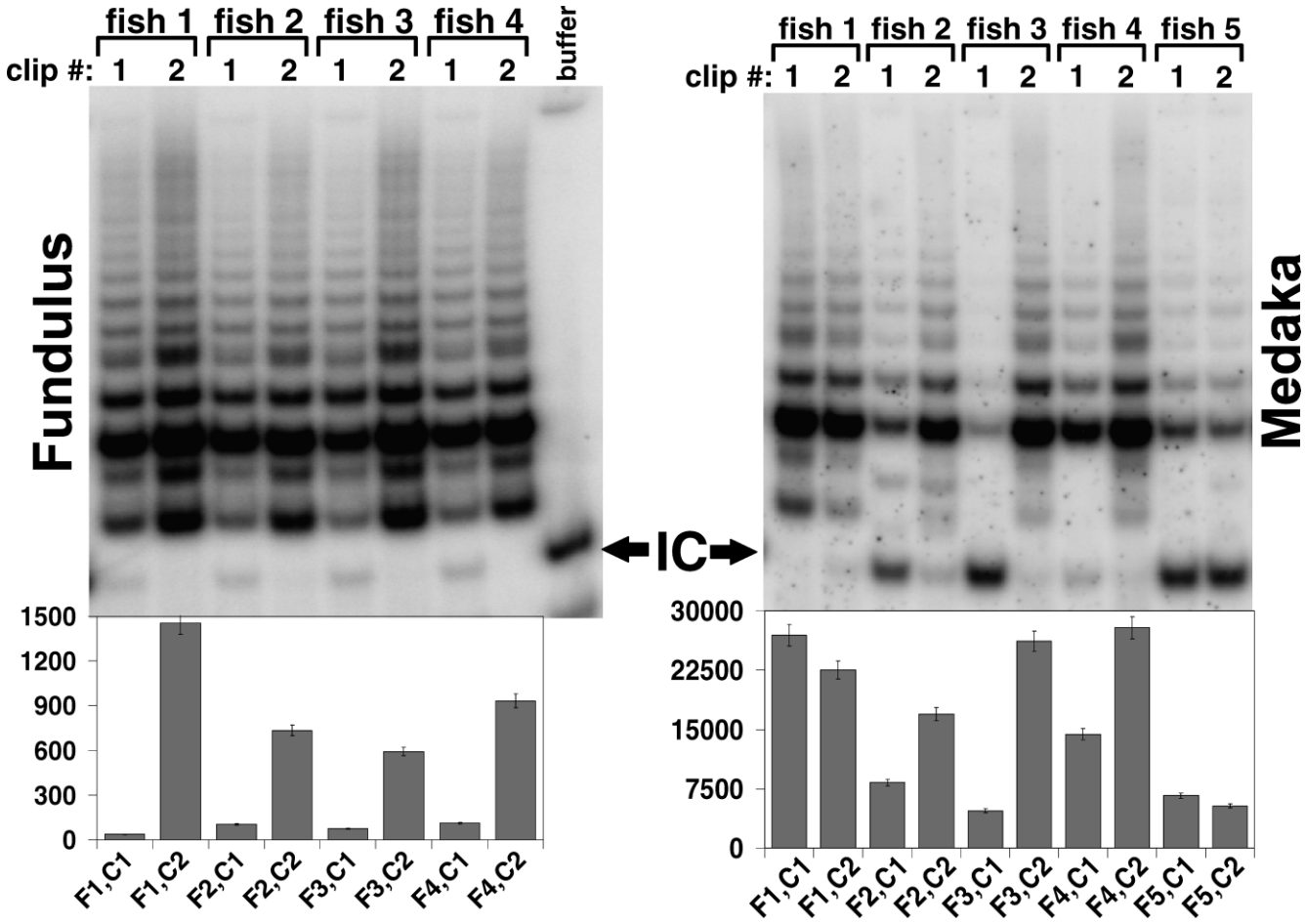

Figure 4. Upregulation of telomerase activity during tissue regeneration. Caudal fins were clipped at day 0 and reclipped after 7–15 days of regeneration. Individual fish were tested for telomerase activity using the TRAP assay. Shown is a representative telomerase gel for both killifish (*Fundulus heteroclitus*) and Japanese medaka (*Oryzias latipes*) with the characteristic 6-bp ladder indicative of telomerase activity and the 36-bp internal control (IC) used for quantitation. One tenth of one microgram of total cellular protein was used, and a reproducible increase in telomerase activity is observed for each of the killifish clips (clip 2 represents the regenerating tissues) when compared with the original clip (clip 1), while 3 out of 5 medaka fish showed an increase. Note that for medaka, fish #1 and fish #5 show no change in telomerase activity at the concentration of protein tested.

average of 5–8 years (33–37), and much longer lived species (38–41), such as lobster that are believed to live >50 years (42). Clearly, there is no positive relationship between telomerase activity levels and longevity. Because most of the aquatic animals tested have a tremendous capacity for tissue regeneration, we hypothesized that telomerase expression and its telomere maintenance function were directly related to the ability of these animals to regenerate injured tissue.

To test whether telomerase may be involved in tissue regeneration, we used the tailfin model (43, 44) for 3 species of fish: marine killifish (*Fundulus heteroclitus*), Japanese medaka (*Oryzias latipes*), and zebrafish (*Danio rerio*). The caudal fin tissues were removed ~2 mm from the base of the caudal peduncle, allowed to regenerate for either 7 (zebrafish) or 15 (medaka and killifish) days, and then assayed for telomerase activity at day 0 (initial clipping, clip #1) and during regeneration (reclipping, clip #2). Telomerase levels were elevated during tissue regeneration for each of the 3 species of fish (Figure 4 for killifish and medaka; data not shown for zebrafish). We assessed telomere lengths in the killifish fins (since these fin specimens were larger than those of medaka and zebrafish) to determine if there was a functional consequence for the telomerase increase. We found that, at a minimum, telomere lengths were maintained in 3 of the 4 fish tested (numbers below the lanes for calculated average telomere lengths; DNA extraction was below the level of sensitivity for fish #2), while the shortest telomeres for fish #1, fish #3, and fish #4 were elongated, both visually and quantitatively during regeneration. While the longest detectable telomeres were consistent between the initial clip and the regenerated one, the lower length of telomere detection shifts upward during the regeneration, as indicated by the white lines on the TALA blot (Figure 5).

To determine the potential mechanism of the telomerase increase during regeneration, we assessed the levels of molecular chaperones that are known to regulate telomerase

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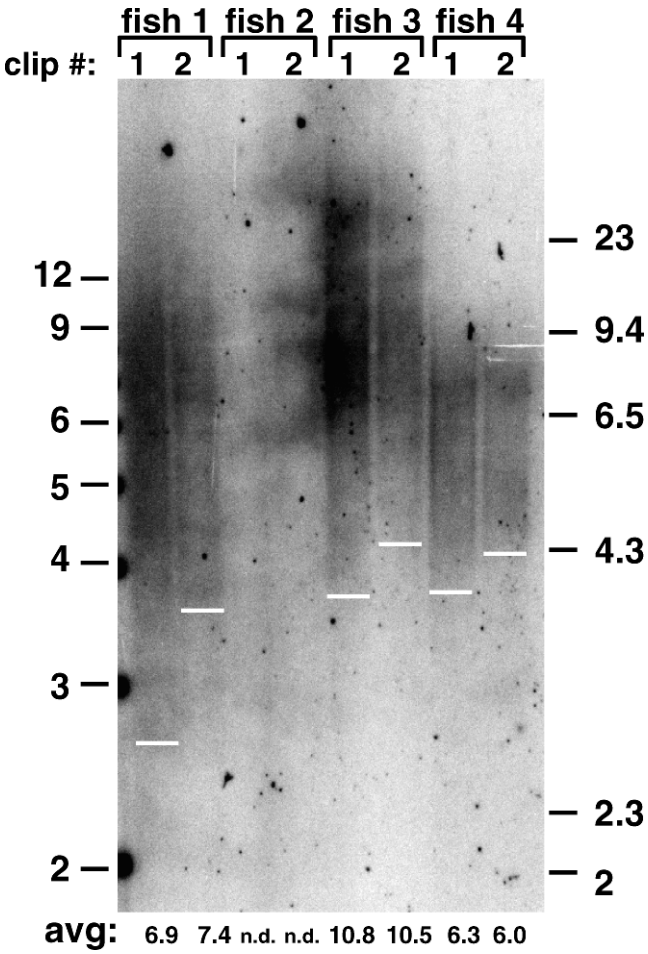

Figure 5. Maintenance of the shortest telomeres during regeneration. Fundulus (killifish) were clipped as described in Figure 4 and allowed to regenerate for 15 days. Because killifish fins are much larger than medaka or zebrafish, protein for telomerase activity and DNA for telomere length measurements could be extracted by dividing tissues from the same fish. Telomere lengths were analyzed, and averages were calculated as before (20, 25). White bars indicate the lower limit of telomere detection, showing that while overall telomere lengths are unchanged, the lengths of the shortest telomeres are increased. Unfortunately, genomic DNA yield extracted from fish #2 was below the sensitivity of the assay (n.d., not detected).

assembly and function (7, 45, 46). We have previously shown that during tumor progression, telomerase is upregulated via an Hsp90 chaperone-mediated mechanism (47). Figure 6 shows that the fundulus tail regeneration model has elevated Hsp90 in each tail clip tested and that the p23 antibody (JJ3 clone) did not react with fundulus p23, suggesting that the increase in telomerase function is at least partially due to an increase in chaperone-mediated assembly. Overall, our data are consistent with the possibility that telomerase upregulation is important after tissue injury in order to maintain telomeres, as well as elongating the subset of the shortest telomeres, during the rapid cellular proliferation associated with the regeneration process.

Discussion

Telomerase has become an important target in cancer therapeutics (5), and its role in the prevention of aging is well documented (8). Understanding the regulation of telomerase and the importance of telomere biogenesis in a variety of cellular processes will be critical for defining new strategies for either inhibiting the enzyme in cancer cells or activating it in aging cells. Here, we introduce several aquatic species as potentially useful and cost-effective alternatives to inbred strains of mice as animal models for the study of telomeres and telomerase, especially related to aging, cancer, and tissue regeneration.

We show that telomerase is expressed in nearly all tissues of both vertebrate and invertebrate aquatic species sampled; many of the tissues were from presumably fully differentiated organs (e.g., heart and brain). Japanese medaka tissues showed extremely high levels of telomerase activity compared with human tumor-derived or immortalized cell lines, but a more rigorous quantitative assessment needs to be accomplished prior to making this overall conclusion. Even with high telomerase, the fish tissues have telomere lengths that resemble those found in normal human cells strains and tissues, if not shorter, which will be of critical importance as we attempt to establish fish as a biologically relevant alternative telomere model for studying aging and cancer. We anticipate that this system will complement results obtained in the laboratory mouse, especially the telomerase knockout strain of mice (9, 10).

Unusual telomerase laddering effects were observed in some invertebrate animals; therefore, telomerase-extended products were sequenced to determine their sequence. We found that 3 of 5 invertebrate species had the vertebrate hexameric sequence, TTAGGG, while 2 other species have a pentameric sequence, TTAGG, consistent with previous reports for other aquatic invertebrates (18, 28, 29). Interestingly, the telomerase RNA template subunit was capable of recognizing the substrate primer in the telomerase reaction, even though the RNA template is responsible for synthesizing TTAGG repeats rather than TTAGGG sequences. These results further support the ability of telomerase, even from diverse species, to recognize a non-telomeric sequence for synthesis of telomeric tracts (21), an activity that may be important for the alternate functions of the telomerase enzyme.

The high level of telomerase activity in lobster tissue has been reported as directly related to longevity (18). However, in our survey of aquatic invertebrate and vertebrate animals, telomerase activity levels appear to be highly independent of life span. We propose that telomerase in aquatic organisms may be important for tissue regeneration based on the following observations: 1) telomerase is present in both short- and long-lived species, 2) these species have relatively short (or shorter) telomeres, which would need to be maintained during the increased cell proliferation associated with tissue renewal, 3) aquatic

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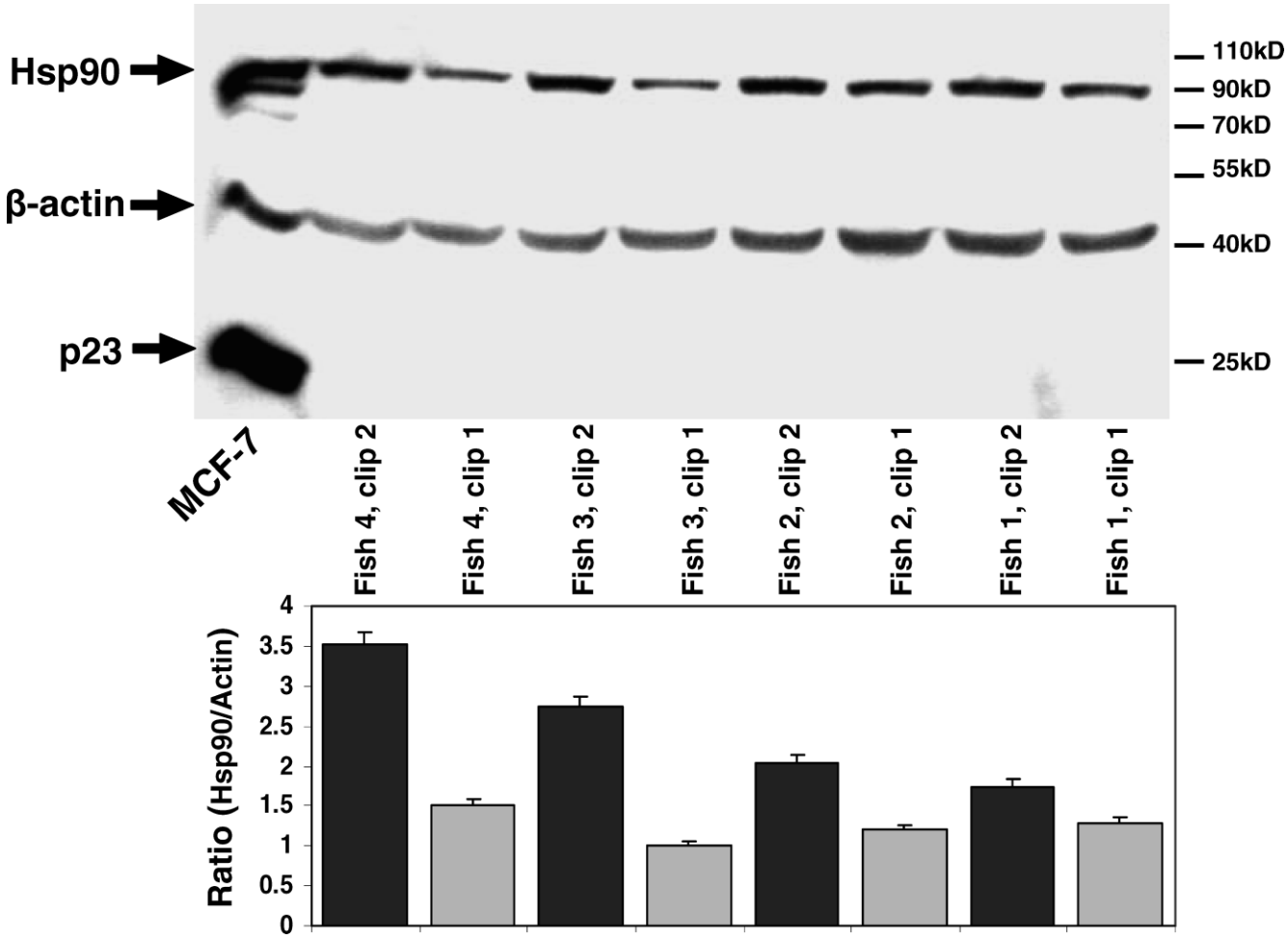

Figure 6. Elevated Hsp90 levels during regeneration. Fundulus tissue extractions were performed as described in Figure 4, and 20 μg of total cellular protein was used for SDS-PAGE (10%). Nitrocellulose membranes were probed with Hsp90 and p23 chaperone antibodies as previously described (Compton et al., 2006), and β-actin served as a quantitative control for protein loading. MCF-7 breast tumor cell extracts (15 μg) were used as a positive control for chaperone Western. Note that the p23 antibody failed to detect p23 in fundulus but that the Hsp90 and β-actin antibodies react with fundulus proteins.

mammals, which are typically not capable of regeneration, have no detectable telomerase activity, and 4) regeneration is a process that is shared by virtually all species of aquatic animals excluding mammals.

Fish and many aquatic invertebrates provide excellent models for studying the basic mechanisms of tissue injury and repair, including regeneration. One of our underlying hypotheses is that fish may be an outstanding source for stem cells, given the high levels of telomerase in most organs and the ability of fish to regenerate a variety of tissues. We show telomerase is upregulated during tissue regeneration in 3 different fish species, suggesting an integral role of telomerase in processes other than aging and cancer. Importantly, this increase in telomerase activity is correlated with maintaining or elongating telomeres. These data are consistent with the idea that telomerase preferentially elongates the shortest or critically short telomeres, as was shown using the telomerase knockout mouse (48). In an attempt to understand the mechanism of telomerase upregulation, we observed an increase in the Hsp90 chaperone during regeneration, suggesting that telomerase is at least partially regulated by elevated assembly of the holoenzyme complex, a concept that has been shown during human cancer progression model previously (47). Clearly, further investigation into the role of telomerase in cellular proliferation in aquatic animals is required, and the cloning of telomerase components and subsequent knockdown or knockout experiments will provide additional details on telomerase's mechanistic role in the response of fish cells to toxicological stimuli, carcinogenesis, regeneration, and organismal aging.

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