Compact intergenic regions of the pufferfish genome facilitate isolation of gene promoters: characterization of *Fugu* 3′-phosphoadenosine 5′-phosphosulfate synthase 2 (*fPapss2*) gene promoter function in transgenic *Xenopus*

Tara Stapleton[a], Artee Luchman[a], Jillian Johnston[a], Leon Browder[a], Sydney Brenner[b], Byrappa Venkatesh[b], Frank R. Jirik[a,*]

[a] *Department of Biochemistry and Molecular Biology, University of Calgary, 3330 Hospital Drive N.W., Calgary, AB, Canada T2N 4N1*
[b] *Institute of Molecular and Cell Biology, 30 Medical Drive, Singapore 117609*

Received 28 October 2003; revised 14 November 2003; accepted 14 November 2003

First published online 27 November 2003

Edited by Ned Mantei

Abstract **The highly compact nature of the pufferfish (*Fugu rubripes*) genome renders it a useful tool not only for annotating coding regions within vertebrate genomes, but also for the identification of sequences important to gene regulation. Indeed, owing to this compaction it will be feasible in many instances to initiate analyses using entire intergenic regions when mapping gene promoters; a strategy that is very rarely feasible with the expanded genomes of other species. Stemming from our interest in studying promoters expressed in chondrocytes, we selected for study the intergenic region upstream of *Fugu* 3′-phosphoadenosine 5′-phosphosulfate synthase 2, *fPapss2*, a gene required for the normal development of cartilage extracellular matrix. Functional characterization of the entire *fPapss2* 5′ intergenic region was carried out by monitoring expression of the enhanced green fluorescent protein (EGFP) gene reporter in the developing cartilage of transgenic *Xenopus laevis*. By evaluating a series of 5′ intergenic region deletions we defined a minimal *fPapss2* sequence of ∼300 bp that was essential for EGFP expression in tadpole cartilage. This functional analysis of an entire *Fugu* intergenic region, combined with the efficiency of *Xenopus* transgenesis, serves as a model for the rapid characterization of evolutionarily-conserved regulatory regions of other pufferfish genes.**

Key words: 3′-Phosphoadenosine 5′-phosphosulfate synthase 2; Enhanced green fluorescent protein; Gene promoter; Transgenic; Intergenic region; *Xenopus laevis*; *Fugu rubripes*

1. Introduction

By virtue of its approximately 380 Mb size, the genome of the pufferfish, *Fugu rubripes*, is amongst the most compact of vertebrate genomes. Despite its compact nature, however, the *Fugu* genome encodes a gene repertoire similar in scope to that of other vertebrates, including humans [1,2]. Thus, the overall gene density in the *Fugu* (one gene/12 kb) is almost an order of magnitude higher than that of mammalian genomes (one gene/100 kb). The compaction of the *Fugu* genome, due for the most part to the evolutionary depletion of repetitive elements and other non-essential DNA sequences that are found in the vast majority of vertebrates, has occurred primarily at the expense of intronic sequences and intergenic regions. As an example of the latter, the intergenic segments upstream of the *fPten*, *fMinpp1*, and *Fugu* 3′-phosphoadenosine 5′-phosphosulfate synthase 2 (*fPapss2*) genes are <2.5 kb in length [3]. As a result of these attributes, the *Fugu* genome has been proposed as a reference point to aid not only in the discovery of novel genes and the elucidation of the exon–intron structure, but also in the identification of regulatory elements that control gene expression [1].

The compact promoter regions of *Fugu* have been shown capable of faithfully recapitulating the complex expression patterns of mammalian genes, both in mammalian cell lines and transgenic rodents [4–10], indicating that key *cis* elements required for mediating expression can be conserved between fishes and mammals. Comparative analyses of *Fugu* and human/mouse promoter regions are useful in identifying conserved non-coding sequences. Such conserved sequences represent strong candidates for potential regulatory elements [4,6,8,11–14], and can be easily validated through transgenic or cell culture systems. Since the intergenic regions are often remarkably short in *Fugu*, the entire intergenic region can often be easily amplified by polymerase chain reaction (PCR) for use in expression studies. The recent completion of the draft sequences of mammalian and pufferfish [2] genomes has provided an unprecedented opportunity to study the regulation of vertebrate genes by the comparative genomics approach.

Owing to an interest isolating gene promoters that are highly expressed in chondrocytes, we initiated an analysis of the 5′ region of the *fPapss2* gene. The enzyme encoded by this gene, PAPSS2, is required for the sulfation of many molecular species, including large extracellular matrix macromolecules such as the proteoglycans of cartilage [15]. For this reason, *Papss2* has a critical role in normal vertebrate cartilage and skeletal development [15,16]. Indeed, attesting to its importance, mutations of *Papss2* orthologs are responsible for murine brachymorphism (*bm*) as well as a human disorder, spondyloepimetaphysial dysplasia, first described in a Pakistani kindred [15,17–20]. Transcripts derived from the human *PAPSS2* gene, also known as *HSK2* [20], while abundant in

*Corresponding author. Fax: (1)-403-210 8127.
E-mail address: jirik@ucalgary.ca (F.R. Jirik).

0014-5793/03/$30.00
doi:10.1016/S0014-5793(03)01353-X

cartilage, are also present at varying levels in a variety of other tissues. These included adult liver, lung, placenta, ovary, peripheral blood lymphocytes, adrenal gland, skin, and heart [16,21]. In a study to characterize *Fugu Pten*, the *fPapss2* ortholog was identified [3], making possible a search for functional promoter activity within the 5′ intergenic region of this gene.

This study, therefore, focused on evaluating the *fPapss2* 5′ intergenic region for its ability to direct expression of the enhanced green fluorescent protein (EGFP) reporter gene to the developing cartilage of transgenic *Xenopus laevis* tadpoles. *X. laevis*, a well-established model system for developmental studies, represents a powerful tool for analyses of in vivo gene regulation using transgenes to direct the expression of fluorescent proteins. The latter are readily visualized within tissues of interest owing to the transparent nature of *Xenopus* tadpoles.

2. Materials and methods

2.1. Generation of transgenic X. laevis

Xenopus adults were maintained in accordance with University of Calgary guidelines for animal care. Sperm nuclei were prepared according to the method of Amaya and Kroll [22], with the modifications of Sparrow et al. [23]; that is, protease inhibitors were not used, and digitonin (ICN Biomedicals) was used in place of lysolecithin to release sperm nuclei. Sperm were then diluted to 1×10^5 nuclei/μl with sperm storage buffer [22], and aliquots snap-frozen in liquid nitrogen prior to storage at −80°C. For microinjection, nuclei were thawed and prepared by mixing 2.5 μl (2.5×10^5 nuclei) with equimolar amounts of each plasmid construct as described [22]. Following 10 min incubation at room temperature, the nuclei–plasmid mixture was diluted to 500 μl with sperm dilution buffer [22] with gentle mixing. Injection needles were 'back-loaded' with the reaction mixture in preparation for microinjection, and then nuclear transplantation was performed according to the method of Sparrow et al. [23].

2.2. Transgene construct production

The complete 5′ intergenic region of *fPapss2* was amplified by PCR using a *Fugu* cosmid, #141O7 (Greg Elgar's cosmid library, HGMP-RC, UK), as the template [3] with the forward (gctgcttttaaccagcctg), and reverse (tctctcgactcgaaggcg) primers, which also introduced *Bgl*II and *Hin*dIII sites, respectively. The 1725 bp amplified product was then cloned into the *Bgl*II and *Hin*dII sites of the pEGFP-1 vector (Clontech). *fPapss2* promoter constructs having progressive 5′ deletions were generated using the following forward primers, which also introduced a *Hin*dIII restriction site: 1218 bp, gagagggtgctccaatgtggaac; 788 bp, gggcacaggacattagatacg; 436 bp, cgtctttgaacctctggccg; 349 bp, cttgcttcaaatcctccgcagcag; 224 bp, gaaccattggctgttggaaag; 81 bp, ggcctcagtcgagcagaactttcgg. The same reverse primer, 5′-ggaggtctggctattggatggg, consisting of sequences −32 to −53 bp upstream of the translation initiation codon and which also introduced a *Kpn*I site, was used in all the amplifications. Individual PCR products were cloned into the pEGFP-1 vector following digestion of this plasmid with *Hin*dIII and *Kpn*I. Plasmids containing the 1.2, 0.79 and 0.44 kb 5′ *fPapss2* sequences in pEGFP-1 were linearized by digestion with *Stu*I prior to microinjection. For those containing the 1.73, 0.35, 0.22, and 0.08 kb 5′ fragments the plasmids were cut with *Afl*II and *Stu*I digestion and then agarose gel electrophoresis was used to remove a plasmid fragment containing the SV40 promoter, neomycin resistance cassette. The enhancer elements within this promoter have the potential for altering expression of promoter fragments as we found in the case of the 0.22 and 0.08 kb fragments, where variable EGFP expression was seen in the transgenic tadpoles (data not shown). A promoter-less pEFGP-1 vector was used as a negative control. The *Xenopus* cardiac actin gene promoter, designated XCAR, that generates DsRed expression in developing tadpole muscle, was generously provided by E. Amaya (University of Cambridge).

2.3. Imaging of EGFP in live tadpoles

Tadpoles were screened for transgene expression using a Zeiss M2-BIO microscope fitted with a GFP filter set. Transgenic tadpoles were anesthetized in benzocaine, and images of fluorescent tadpoles were captured and processed using Openlab 3.1.5 (Improvision).

3. Results and discussion

Transgenic tadpoles were examined for EGFP expression directed by the entire 5′ intergenic region of the *fPapss2* gene, a 1.73 kb sequence that extends from the polyadenylation signal of the upstream *fMinpp1* gene to a site ∼30 bp upstream of the translation initiation codon of *fPapss2* [3]. Examination of the resultant founders revealed strong EGFP signals within the cartilage of transgenic tadpoles, with the highest levels of EGFP first being present in the developing chondrocranium of early stage tadpoles (Fig. 1A), and subsequently, within the various cartilaginous tissues of the developing limbs of metamorphosing tadpoles (see below). To further isolate the promoter region of *fPapss2* required for expression of EGFP in the chondrocranium, and hence to localize the region(s) containing key regulatory elements for tadpole chondrocyte expression, progressive 5′ deletions of the 1.73 kb intergenic fragment were obtained by PCR (Fig. 2) and subcloned into the pEGFP-1 plasmid. Loss of EGFP expression was seen between the 0.35 and the 0.22 kb segments (Fig. 2). However, since the expression pattern right up to the stage 58 tadpole was identical to that of transgenic tadpoles generated using the 1.73 kb segment, we concluded that all the *cis*-acting elements required for chondrocyte expression up to this developmental stage were intact

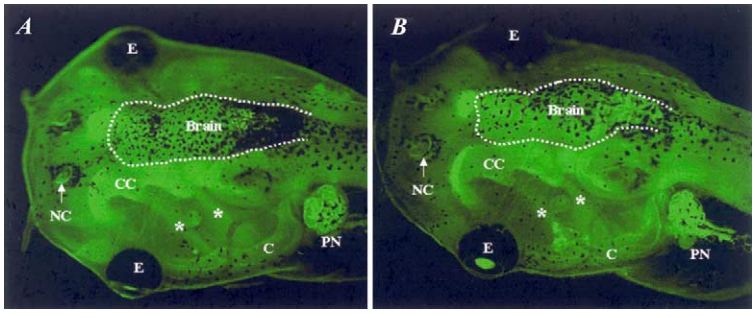

Fig. 1. The *fPapss2* 1.73 kb 5′ intergenic region (A) and a 0.35 kb 5′ segment (B) are both able to direct expression of EGFP (green signal) to the developing cartilage of transgenic tadpoles. The two images are cephalic halves of representative stage 51 animals demonstrating strong EGFP expression in the chondrocranium (CC), gill arch cartilage (C) and nasal cartilage (NC). The position of the developing brain is indicated by a white dotted line. Both specimens have been rotated on their longitudinal axis to expose the left half of the chondrocranium directly underlying the brain. The signals in the lens of the left eye (E) and pronephros (PN), also seen in control tadpoles, are due to autofluorescence.

within the 0.35 kb fragment (Fig. 1B). Likely due to integration in the vicinity of an endogenous enhancer, tadpoles generated from the 0.22 and 0.08 kb fragments occasionally demonstrated low levels of EGFP expression (data not shown), however, the vast majority of embryos microinjected with either of these two transgenes failed to generate detectable EGFP signals.

Demonstrating that the *fPapss2* promoter was most highly expressed in tadpole cartilage, and also that EGFP signal intensity was not simply a function of the cell density of any given tissue, a vector encoding the red fluorescent protein, DsRed, under the control of a *Xenopus* muscle-specific gene promoter, XCAR [24], was co-injected with the *fPapss2* EGFP plasmid. As seen in the representative double transgenic tadpoles shown in Figs. 3 and 4, the *fPapss2* promoter-directed expression of EGFP primarily to cartilaginous tissue, possibly with some overlap with XCAR expression at specific sites, such as the tail and oral musculature of stage 55 tadpoles (Figs. 3A–C and 4). While the *fPapss2* promoter did not lead to detectable EGFP signals in developing abdominal wall muscle fibers (Fig. 3A–C), EGFP signals in the tail region may have been due coexpression of EGFP and DsRed in muscle cells. However, it is possible that the EGFP expression in tails was due to the presence of chondrocytes. DsRed-expressing muscle bundles (which also expressed low levels of EGFP) in the stage 55 hindlimb were clearly separable from EGFP-positive cartilaginous rudiments (Fig. 3F).

fPapss2 (1.73 kb) segment-directed EGFP expression was seen in the cartilage right up to the stage 58, and was clearly evident in the developing interphalangeal joints of the hindfoot (Fig. 5A). Older animals, as they neared metamorphosis, tended to show reduced EGFP signals, owing in part to diminished skin transparency and the increased presence of melanin-containing cells. Interestingly, EGFP signals appeared in the region of the bony sheath that forms in the mid-portions of femur and tibia in stage 58 tadpoles (Fig. 5), suggesting that *fPapss2* promoter might be directing EGFP expression to osteoblasts, perhaps during the process of endochondral ossification when much new extracellular matrix is being laid down. EGFP signals were also present in other tissues of the hindlimb, including skin and developing muscle bundles (Fig. 5B). Adult human muscle was previously reported as being negative for *PAPSS2* expression [21], thus the fact we saw it in the tadpoles may be indicative of a unique feature of the *Fugu* gene promoter fragments we tested. However, it is also possible that *PAPSS2* expression is developmentally regulated, and that this gene is expressed in the human embryonic muscle. Despite evidence of PAPSS2 expression in adult mammalian liver and heart [16,21] we did not see EGFP in these sites in tadpoles. Perhaps adult frogs would show expression at these sites. It is also possible that EGFP was expressed in tadpole heart and liver, but at reduced levels. EGFP is a relatively insensitive reporter of gene activity, particularly as compared with reporters detected by virtue of their enzymatic activity, such as β-galactosidase. Finally, it is conceivable that the fish promoter is not capable of fully recapitulating the expression pattern of mammalian *PAPSS2* gene. Further investigation will be required to clarify these various questions.

Both the 1.73 and 0.35 kb intergenic 5′ segments resulted in

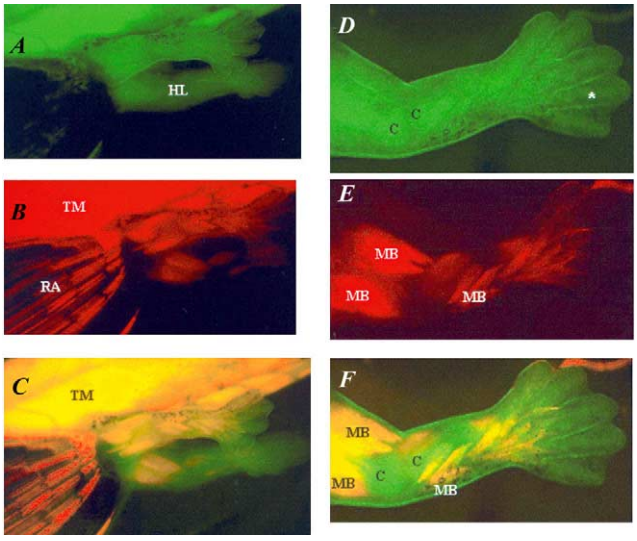

Fig. 3. Coexpression of EGFP, and DsRed under the control of *fPapss2* (1.73 kb) and XCAR promoters, respectively, following co-microinjection of the two transgenes. A: *fPapss2*-directed EGFP expression within hindlimb (HL) cartilages and proximal tail chondrocytes and/or musculature (TM) of a representative stage 55 transgenic tadpole. B: XCAR promoter-directed DsRed expression is present within tail musculature (TM), rectus abdominus muscle fibers (RA), and leg muscles of the same tadpole. C: Overlay of red and green fluorescence in the above regions, with yellow signifying coexpression of both reporters in the same tissue. D: EGFP expression in the cartilaginous femoral and tibial bone precursors (C) of the developing hindlimb, as well as in the primordial digits (asterisk) of a representative stage 55 transgenic tadpole. E: DsRed expression reveals XCAR promoter activity clearly defining the major muscle bundles (MB) of the developing hindlimb in the same specimen. F: Overlay of red and green fluorescence showing EGFP- and DsRed-expressing structures in relation to one another.

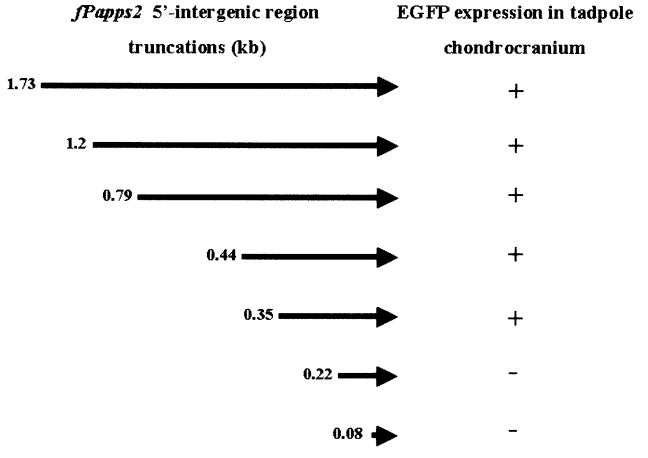

Fig. 2. Successive deletions of the *fPapss2* 5′ intergenic region were evaluated for their ability to direct expression of EGFP in transgenic tadpoles. Sequence coordinates were from the *Fugu Pten* locus sequence (GenBank accession number AF325922) [3]: complete intergenic region (from region of *fMinpp1* polyadenylation signal to start of *fPapss2*) 1.73 kb, bp 46 272–47 998; 1.22 kb, bp 46 302–47 520; 0.79 kb, bp 46 302–47 090; 0.44 kb, bp 46 302–46 738; 0.35 kb, bp 46 302–46 651; 0.22 kb, bp 46 302–46 526; 0.08 kb, bp 46 302–46 383. Chart shows 5′ *fPapss2* segments that were capable of directing EGFP expression to developing cartilage in transgenic tadpoles. Results were derived from examination of the EGFP expression patterns in >25 founders produced for each of the 1.2, 0.79, 0.44 kb 5′ *fPapss2* sequences, and >50 founders for each of the 1.73, 0.35, 0.22, and 0.08 kb 5′ *fPapss2* sequences.

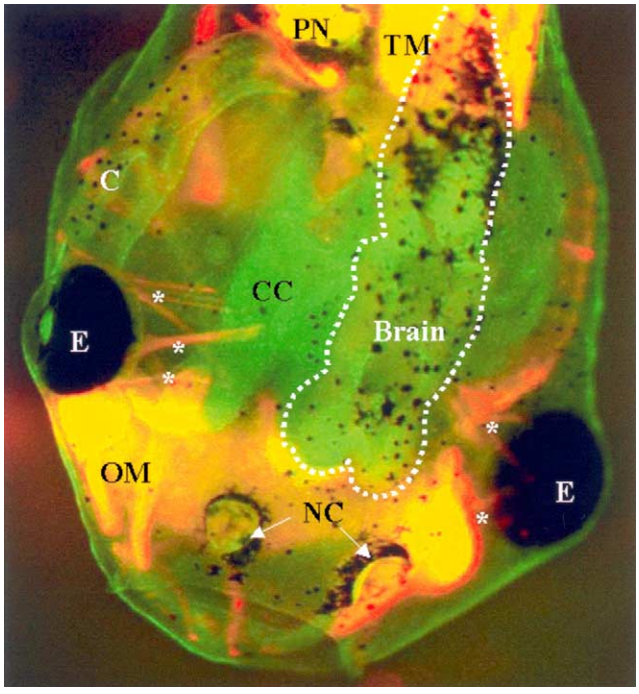

Fig. 4. The *Fugu Papss2* 0.35 kb 5′ intergenic region directs expression of EGFP primarily to cartilage (green signal). XCAR-directed DsRed expression is seen in muscle. The image is of the cephalic half of representative stage 51 transgenic tadpole, demonstrating DsRed signals in all muscle tissues, including the delicate extraocular muscles (indicated by asterisks), as well as the oral (OM) and tail musculature (TM). EGFP (green) expression can be seen primarily in chondrocyte-rich sites, including the chondrocranium (CC), gill arch cartilages (C), and nasal cartilages (NC). Expression is also seen in the cartilaginous components and/or muscles of the oral (OM) and tail (TM) musculature where there is overlap of EGFP and DsRed signals (yellow). The position of the brain is indicated by a white dotted line. The specimen was rotated on its longitudinal axis to expose the EGFP-rich chondrocranium that directly underlies the brain. Autofluorescence signals are evident in the lens of the eye (E) and the pronephros (PN).

a high frequency of founders, with approximately 80% of developing embryos proving 'positive' for transgene expression (data not shown). Furthermore, all resulting tadpoles generated with both 5′ fragments exhibited comparable EGFP signal intensities and tissue-specific expression patterns, suggesting relative independence of the *fPapss2* regulatory elements from so-called chromatin 'position effects' or variegated patterns of expression that can be seen in transgenic animals [25]. It should be noted that in this study we confined our analyses to the 5′ intergenic region of the *fPapss2* gene, and we have therefore not excluded the possibility that regulatory elements, such as enhancers, silencers, and locus control regions, capable of regulating *fPapss2* gene expression exist outside the confines of the region that we evaluated in transgenic tadpoles.

In order to identify putative binding sites for known transcription factors within this region, we analyzed the *fPapss2* 0.35 kb promoter region using AliBaba2.1. This program identified six potential Sp1 binding sites, in addition to one of each of the following (amongst others): YY1, AP-1, C/EBPalpha and C/EBPdelta. Interestingly, the proximal 2 kb of the human *PAPSS2* (*HSK2*) promoter contains nine potential Sp1 and one AP-1 binding sites [20], suggesting that these transcription factors may play a role in the regulation of *Papss2* genes in vertebrates. Interestingly, a comprehensive mutational analysis of the human PAPSS2 promoter revealed the importance of two transcriptional start site proximal Sp1 sites to promoter activity (using the luciferase reporter) in transiently transfected HeLa and SW13 cells [26]. Given that Sp1 and AP-1 are ubiquitously expressed, it is unlikely that they determine the relatively cartilage-specific expression pattern of the *fPapss2* promoter in transgenic tadpoles, although combinatorial interactions of these transcription factors with some novel tissue- and/or stage-specific transcription factor(s) in chondrocytes cannot be excluded.

To attempt to identify conserved potentially novel *cis*-acting elements that might mediate cartilage-specific expression, we compared 0.35 kb *fPapss2* promoter with the proximal 2 kb of the human *PAPSS2* promoter using the program Pipmaker (http://bio.cse.psu.edu/pipmaker). This analysis did not reveal any conserved elements that were shared between the *Fugu* and human promoters. It should be noted that many *cis*-acting elements consist of very short sequences (6–10 bp) with varying degrees of degeneracy across species [27]. It is, therefore, likely that the programs we employed may have failed to identify novel functional *cis* elements within the *Fugu* and human *Papss2* promoters. Further characterization of the compact *fPapss2* through nuclear extract binding assays and deletions/mutations of putative transcription factor binding sites, followed by expression studies in transgenic *Xenopus*

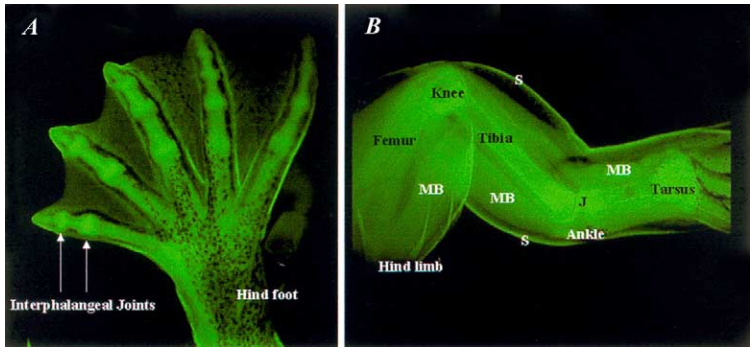

Fig. 5. Expression of EGFP (using the 1.73 kb *fPapss2* segment) within cartilage, and muscle bundles (MB) of a maturing hindlimb obtained from a representative stage 58 transgenic tadpole. A: Hindfoot, clearly showing the cartilaginous precursors of the digits, including an increased level of EGFP expression corresponding to the positions of the developing interphalangeal joints. B: In the stage 58 tadpole the midregions of the femur and tibia are encased by a developing bony sheath (also EGFP positive), whereas the ends and joints (J) of these structures are cartilaginous with increased EGFP signals. EGFP expression is also seen in skin (S) and large muscle bundles (MB) of the hindlimb.

tadpoles, should help in identifying these elements and tracing their evolution from fishes to mammals.

Applying the relatively high-throughput method of amphibian transgenesis to study the pufferfish genome has the potential to facilitate not only the rapid isolation of functional promoter regions (and enhancers), but also the discovery of novel and compact tissue-specific gene promoters for use in mammalian transgenic systems. In this respect, it is clear that *Fugu* gene promoters can be activated and regulated in a tissue-specific manner in transgenic rodents ([6,9,10], reviewed in [1]). *Fugu* gene promoter compaction not only makes the promoters relatively easy to work with using conventional cloning techniques, but also implies that these promoters may be able to function independently despite their close proximity to one another. Thus, it may be technically feasible to generate individual transgene constructs in which two or more gene products are placed under the control of different *Fugu* promoters. This would allow, for example, in vivo studies of gene–gene interaction across different tissues, or between cell types within a given tissue, via the use of a single transgene. Such an approach would obviate the need for the time-consuming breeding strategies that are typically necessary when combinations of different transgenes are contemplated.

Lastly, this study provides an example of how the pufferfish genome allows one to initiate a promoter analysis study using the entire intergenic region as the departure point, an approach that is rarely feasible with the genomes of other organisms. As 5′ regulatory elements may be widely dispersed in typical genomes, the compact nature of *Fugu* intergenic regions may well prove critical when evaluating new transgene promoters for their ability to obey the developmental cues responsible for regulating tissue- and differentiation stage-specific gene function.

Acknowledgements: This work was supported by grants from the National Centres of Excellence – Canadian Arthritis Network and an Establishment Grant from the Alberta Heritage Foundation for Medical Research (AHFMR) (to F.R.J.); and a grant from the Natural Sciences and Engineering Council of Canada (to L.B.). A.L. held a Fellowship Award from the AHFMR, and F.R.J. was the recipient of AHFMR Medical Scientist, and Canada Research Chair Awards.

References

[1] Venkatesh, B., Gilligan, P. and Brenner, S. (2000) FEBS Lett. 476, 3–7.
[2] Aparicio, S., Chapman, J., Stupka, E., Putnam, N., Chia, J.M., Dehal, P., Christoffels, A., Rash, S., Hoon, S., Smit, A., Gelpke, M.D., Roach, J., Oh, T., Ho, I.Y., Wong, M., Detter, C., Verhoef, F., Predki, P., Tay, A., Lucas, S., Richardson, P., Smith, S.F., Clark, M.S., Edwards, Y.J., Doggett, N., Zharkikh, A., Tavtigian, S.V., Pruss, D., Barnstead, M., Evans, C., Baden, H., Powell, J., Glusman, G., Rowen, L., Hood, L., Tan, Y.H., Elgar, G., Hawkins, T., Venkatesh, B., Rokhsar, D. and Brenner, S. (2002) Science 297, 1301–1310.
[3] Yu, W.P., Pallen, C.J., Tay, A., Jirik, F.R., Brenner, S., Tan, Y.H. and Venkatesh, B. (2001) Oncogene 20, 5554–5561.
[4] Aparicio, S., Morrison, A., Gould, A., Gilthorpe, J., Chaudhuri, C., Rigby, P., Krumlauf, R. and Brenner, S. (1995) Proc. Natl. Acad. Sci. USA 92, 1684–1688.
[5] Kimura, C., Takeda, N., Suzuki, M., Oshimura, M., Aizawa, S. and Matsuo, I. (1997) Development 124, 3929–3941.
[6] Venkatesh, B., Si-Hoe, S.L., Murphy, D. and Brenner, S. (1997) Proc. Natl. Acad. Sci. USA 94, 12462–12466.
[7] Rowitch, D.H., Echelard, Y., Danielian, P.S., Gellner, K., Brenner, S. and McMahon, A.P. (1998) Development 125, 2735–2746.
[8] Kammandel, B., Chowdhury, K., Stoykova, A., Aparicio, S., Brenner, S. and Gruss, P. (1999) Dev. Biol. 205, 79–97.
[9] Brenner, S., Venkatesh, B., Yap, W.H., Tay, A., Chou, C.F., Ponniah, S., Yue, W. and Tan, Y.H. (2002) Proc. Natl. Acad. Sci. USA 99, 2936–2941.
[10] Camacho-Hubner, A., Rossier, A. and Beermann, F. (2000) Genesis 28, 99–105.
[11] Bagheri-Fam, S., Ferraz, C., Demaille, J., Scherer, G. and Pfeifer, D. (2001) Genomics 78, 73–82.
[12] Gottgens, B., Barton, L.M., Gilbert, J.G., Bench, A.J., Sanchez, M.J., Bahn, S., Mistry, S., Grafham, D., McMurray, A., Vaudin, M., Amaya, E., Bentley, D.R., Green, A.R. and Sinclair, A.M. (2000) Nat. Biotechnol. 18, 181–186.
[13] Montpetit, A. and Sinnett, D. (2001) Oncogene 20, 3437–3442.
[14] Gilligan, P., Brenner, S. and Venkatesh, B. (2002) Gene 294, 35–44.
[15] Schwartz, N.B. and Domowicz, M. (2002) Glycobiology 12, 57R–68R.
[16] Kurima, K., Warman, M.L., Krishnan, S., Domowicz, M., Krueger Jr., R.C., Deyrup, A. and Schwartz, N.B. (1998) Proc. Natl. Acad. Sci. USA 95, 8681–8685.
[17] Li, H., Deyrup, A., Mensch Jr., J.R., Domowicz, M., Konstantinidis, A.K. and Schwartz, N.B. (1995) J. Biol. Chem. 270, 29453–29459.
[18] Ahmad, M., Haque, M.F., Ahmad, W., Abbas, H., Haque, S., Krakow, D., Rimoin, D.L., Lachman, R.S. and Cohn, D.H. (1998) Am. J. Med. Genet. 78, 468–473.
[19] ul Haque, M.F., King, L.M., Krakow, D., Cantor, R.M., Rusiniak, M.E., Swank, R.T., Superti-Furga, A., Haque, S., Abbas, H., Ahmad, W., Ahmad, M. and Cohn, D.H. (1998) Nat. Genet. 20, 157–162.
[20] Kurima, K., Singh, B. and Schwartz, N.B. (1999) J. Biol. Chem. 274, 33306–33312.
[21] Xu, Z.H., Otterness, D.M., Freimuth, R.R., Carlini, E.J., Wood, T.C., Mitchell, S., Moon, E., Kim, U.J., Xu, J.P., Siciliano, M.J. and Weinshilboum, R.M. (2000) Biochem. Biophys. Res. Commun. 268, 437–444.
[22] Amaya, E. and Kroll, K.L. (1999) Methods Mol. Biol. 97, 393–414.
[23] Sparrow, D.B., Latinkic, B. and Mohun, T.J. (2000) Nucleic Acids Res. 28, E12.
[24] Gurdon, J.B., Mohun, T.J., Brennan, S. and Casico, S. (1985) J. Embryol. Exp. Morphol. 89, 125–136.
[25] Martin, D.I. and Whitelaw, E. (1996) Bioessays 18, 919–923.
[26] Shimizu, C., Fuda, H., Lee, Y.C. and Strott, C.A. (2002) Biochem. J. 363 (Pt. 2), 263–271.
[27] Dermitzakis, E.T. and Clark, A.G. (2002) Mol. Biol. Evol. 19, 1114–1121.