Analysis of 148 kb of Genomic DNA Around the *wnt1* Locus of *Fugu rubripes*

Klaus Gellner^{1,2} and Sydney Brenner

Molecular Sciences Institute, Berkeley, California 94704 USA

The analysis of the sequence of ~150 kb of a genomic region corresponding to the *wnt1* gene of the Japanese pufferfish *Fugu rubripes* confirms the compact structure of the genome. Fifteen genes were found in this region, and 26.6% of the analyzed sequence is coding sequence. With an average intergenic distance of <5 kb, this gene density is comparable to that of *Caenorhabditis elegans*. The compactness of this region corresponds to the reduction of the overall size of the genome, consistent with the conclusion that the gene number in *Fugu* and human genomes is approximately the same. Eight of the genes have been mapped in the human genome and all of them are found in the chromosomal band 12q13, indicating a high degree of synteny in both species, *Fugu* and human. Comparative sequence analysis allows us to identify potential regulatory elements for *wnt1* and *ARF3*, which are common to fish and mammals.

[The sequence data described in this paper have been submitted to GenBank under accession no. AF056116.]

With 400 Mb, the pufferfish *Fugu rubripes* (*Fugu*) has one of the smallest genomes of all vertebrates, but the number of genes is similar to that of mammals (Brenner et al. 1993; for review, see Angrist 1998). The size difference is attributable to a marked reduction in intron size and intergenic distances, as well as to the paucity of dispersed repetitive sequences. Sequencing of its compact genome has several uses. The high gene density facilitates gene discovery (Trower et al. 1996) and because there is some conservation of gene order between fish and man (Elgar et al. 1996), it can be used for the analysis of linkage in the more complex mammalian genomes. Information on the *Fugu* genome, however, is still sparse. Linkage data for genomic fragments >100 kb are available only for the *Hox* gene clusters (Aparicio et al. 1997). We therefore decided to analyze a larger genomic fragment for its gene content and synteny to human. We have chosen the region around *wnt1*, because human *wnt1* maps to chromosome 12q13, where a large amount of mapping information is available.

In addition, the expression of *wnt1* is highly regulated and very well studied in vertebrates (Echelard et al. 1994). Regulatory elements involved in these processes, however, are difficult to identify. An efficient approach is the comparative sequence analysis of non-coding regions with the compact *Fugu* genome that has been successfully used previously (Marshall et al. 1994; Aparicio et al. 1995; Poepperl et al. 1995). In this study we have extended this approach to the *wnt1* region.

The 150 kb of sequence described in this paper contains 15 genes and, overall, 26.6% of the sequence is coding sequence, confirming the high gene density

in the *Fugu* genome. For eight genes, the human counterparts have been mapped, and all are assigned to human chromosomal position 12q13. This supports the idea that the *Fugu* genome can in some instances serve as a good model system for human in genome research. We also could identify putative regulatory elements for the *wnt1* and *ARF3* genes. One of these elements has already been used in functional assays (Rowitch et al. 1998), which confirmed the importance of the element for the regulation of *wnt1* expression.

RESULTS AND DISCUSSION

In an initial screen with a specific *Fugu* probe, we isolated a cosmid containing *wnt1*. By two steps of cosmid walking from each end of the initially isolated cosmid 138e3, we ended up with five overlapping cosmids (Fig. 1), which were then fully sequenced. The entire *Fugu* genomic sequence of 148640 bp (GenBank accession no. AF056116) has been analyzed with a combination of different methods. All subclones were submitted to "gap-blast" homology searches (Altschul et al. 1997) to reveal any specific homologies with sequences in the databases. Additionally, after aligning all sequences to obtain one contig, the whole sequence was analyzed with the gene prediction program XGRAIL (Xu et al. 1994) to find potential exons. For some genes, a second gene prediction program, FGENES, was used (Solovyev et al. 1995). Exon-intron boundaries were also analyzed by NETGENE (Brunak et al. 1991).

The combination of these approaches led to the discovery of 15 genes within the 148-kb fragment (Fig. 1). These can be divided into three groups. Genes of the first group are highly homologous to their mammalian counterparts (Table 1A), genes of the second group have some homology to known genes and are

¹Present address: Universität Jena, Institut für Spezielle Zoologie und Evolutionsbiologie, 07743 Jena, Germany.

²Corresponding author.

E-MAIL kgellner@pan.zoo.uni-jena.de; FAX 493641 949162.

Figure 1 Schematic representation of the genomic organization of the *Fugu wnt1* locus and adjacent genes. Cosmids from the Elgar *Fugu* library that were fully sequenced are shown as dumbbells. Also cosmids 28L23, 34D20, and 133H20 cover this region and were sample sequenced (data not shown). Arrows indicate the direction of transcription of the identified genes. Gene names are shown below.

most likely new members of the same family (Table 1B), whereas genes of group three do not show any striking homology to any known mammalian genes (Table 1C).

Group I: Highly Homologous Genes

The nine genes of the first group code for activin A receptor type IB (*ACVR1B*), ALL-1 related protein (*ALR*), *wnt1*, *wnt10b*, ADP-ribosylation factor 3 (*ARF3*), the receptor tyrosine kinase *erbB3*, proliferation associated protein 1 (*PAS1*), ribosomal protein *L41*, and LDL receptor related protein (*LRP*). All deduced protein sequences show 45% or more identity to their human counterparts. For the majority of the exons of the nine genes, prediction of coding sequences was consistent with both XGRAIL prediction and blast analysis. The first exons of some genes (e.g., the secreted protein *wnt10b* and part of the intracellular domain of *erbB3*), however, are not conserved and therefore are difficult to predict from the genomic sequence. In these cases, additional information was obtained using the program NETGENE (Brunak et al. 1991).

Eight of the nine genes have been mapped to a definite chromosomal position in the human genome, and all are assigned to 12q13 (Arheden et al. 1988; Kraus et al. 1989; Forus and Myklebost 1992; Hirai et al. 1996; Lamartine et al. 1997; Prasad et al. 1997; Roijer et al. 1998). In addition, murine *wnt1* and *wnt10b* (previously also called *wnt12*) are tightly linked within 2.3 cM on murine chromosome 15 (Adamson et al. 1994). The linkage between the two *wnt* genes may be an evolutionary conserved pattern, because also the *Drosophila wnt1* homolog, *wingless*, and *Dwnt4*, are closely linked (Graba et al. 1995). Furthermore, human *wnt3* and *wnt15* are found in the chromosome band 17q21 within a distance of 125 kb (Bergstein et al. 1997). It would be most logical to assume that the linkage of

two *wnt* genes occurred once in evolution, and that in the evolution of the vertebrates this pattern may have duplicated on several chromosomes (Ruddle et al. 1994).

For *L41*, the precise location has not been determined in human or mouse. The *L41* gene encodes a ribosomal protein that is only 25 amino acids long and is interrupted by two introns, with the middle exon only 23 bp in length. Because in other eukaryotes there are several unclustered copies of genes for ribosomal proteins (Woolford et al. 1979; D'Eustachio et al. 1981), some of which are pseudogenes, we cannot state if the *Fugu* gene is normally expressed.

Conserved synteny between *Fugu* and human was found previously for the *c-fos* region (Trower et al. 1996) and the PDGF-receptor region (How et al. 1996). It may therefore be possible in many cases to use the *Fugu* genome for positional cloning of genes important in human diseases, an efficient process because of the smaller size of introns and intergenic regions in *Fugu*. The sequence information of the *Fugu* gene can then be used to isolate the human gene of interest. We do not have sufficient data as yet to define the average length of conserved synteny between *Fugu* and human. In addition to the genes discussed in this paper, however, *Fugu* homologs have been isolated for both the HoxC cluster and the genes for adenylate cyclase VI and L-type calcium channel polypeptide, which also map to human chromosome 12q13 or 12q12–q13 (Elgar et al. 1996; Aparicio et al. 1997). Further genome walking experiments could extend the present region of synteny.

Group 2: *Fugu* Genes with Some Homology to Known Vertebrate Genes

Of the 15 genes defined within the 148 kb contig, 2 show some homology to human genes but are classi-

Table 1. Identified *F. rubripes* Genes on a Stretch of 148 kb of Genomic DNA*A: Genes with vertebrate homologs*

Fugu and human name	Length of Fugu protein (amino acids)	Percent identity	Accession no.	Assigned to chromosome
<i>ACVR1B</i>	506	83 human 88 zebrafish	P36896 X94119	12q13
<i>ALR</i>	4825	45 human	2358285	12q13
<i>wnt1</i>	370	76 human 92 zebrafish	P04628 P24257	12q13
<i>wnt10b</i>	390	60 human 60 mouse	1932789 P48614	12q13
<i>ARF3</i>	181	100 human	P16587	12q13
<i>erbB3</i>	1328	51 human	P21860	12q13
<i>PAS1</i>	392	79 human 79 mouse	1216526 P50580	12q13
<i>L41</i>	25	100 human	P28751	N.A.
<i>LRP1</i>	1581 (partial)	72 human 74 chicken	Q07954 P98157	12q13

B: New genes of a known gene family

Fugu name	Length of Fugu protein (amino acids)	Closest homolog	Accession no.	Percent identity (amino acids)
<i>Ikaros-like</i>	417	<i>Ikaros/Lyf-1</i> (human)	1289371	85 (zinc finger domains) 50 (full protein)
<i>fugu hedgehog (fhh)</i>	442	<i>shh</i> (human) <i>shh</i> (mouse) <i>ihh</i> (mouse)	663157 A49425 1890097	48 52 52

C. New vertebrate genes

Fugu name	Length of Fugu protein (amino acids)	No. of exons	Significant hits with dbEST entries (acc. no.)	Exons	E-value
<i>R05D3.2-like</i>	497	15	AA307511 AA037301 AA304712 AA307337	3–8 12–15 11–14 8–13	8e-58 1e-39 1e-27 4e-23
<i>138e3.2</i>	255	4	HUMHBC4912 AA905621 AA170127	2 4 3–4	3e-15 2e-06 4e-11
<i>178o23.1</i>	966	23	T27154 H82462 AA312185 AA378749 AA773555 H10578 R88176	3–8 4–9 15–17 9–11 21–23 13–14 1	6e-54 6e-35 4e-29 6e-18 5e-16 7e-13 4e-09
<i>diaphanous-like</i>	1037	24	AA309980 AA224424 T07474 AA459557 U69179 Z41620 AA421752 M78344	17–19 19–22 9–12 5–7, 9–10 17–23 15–16 4–5 23–24	4e-48 3e-46 4e-39 4e-39 9e-32 9e-28 1e-14 1e-09

Homology searches were made with the putative amino acid sequences of the identified Fugu genes using the GAP-BLAST program (Altschul et al. 1997). (A) Fugu genes with highly homologous human counterparts. Genomic location data for human and mouse were obtained from NCBI; <http://www.ncbi.nlm.nih.gov>. (N.A.) Data not available. (B) New Fugu genes belonging to a known vertebrate gene family. (C) Fugu genes with no or only weak homology to any characterized vertebrate gene. Strong homologies to human entries of the dbEST library can be found for all four Fugu genes.

fied as new members of a known gene family. The first gene shows the highest homology to the zinc finger containing transcription factor Lyf-1, encoded by the gene *Ikaros* (Molnar and Georgopoulos 1994). Conserved sequence is almost exclusively found at the six zinc finger domains (exons 3–5 and end of exon 8 in *Fugu*, exon 7 in mouse), where 145 of 171 amino acids are identical. The remainder of the sequence is completely different, except for two stretches comprising 40 amino acids with 75% identities between *Fugu* and human. Human and mouse *Ikaros*, on the other hand, share a high homology of 95% throughout the whole amino acid sequence (Molnar et al. 1996). Therefore, we believe that this *Fugu* gene is only a relative of *Ikaros* and call it *Ikaros-like*.

Evidence for another human family member of Lyf/*Ikaros* exists in the dbEST database of expressed sequence tags (Boguski et al. 1993). Like *Fugu Ikaros-like*, the partially sequenced clone 249118 (GenBank accession nos. H81649 and H81650) shows high homology only to the zinc finger domains of *Ikaros*. *Fugu Ikaros-like* and dbEST clone 249118 do not share any homology outside the zinc finger domains either, indicating that the clone 249118 might be a third member of the Lyf/*Ikaros* gene family.

Interestingly, *Ikaros* has been mapped previously to human chromosome 7p13–p11 and mouse chromosome 11 (Molnar et al. 1996) close to *Egfr*, a member of the *erbB* gene family. It is known that paralogous genes of different families are linked to all four *Hox* clusters (Ruddle et al. 1994). For instance, *HoxC* is linked to *wnt1* and *erbB3*, whereas *HoxB* is linked to *wnt3* and *erbB2*. Genes from additional families, such as aquaporins, integrins, keratins, and collagens are located around the four *Hox* clusters. Therefore, it is possible that the Lyf/*Ikaros* family is another example of such families, with *Ikaros* and *Ikaros-like* linked to *HoxA* and *HoxC*, respectively.

The second gene, *fugu hedgehog* (*fhh*), shows, like all *hedgehog* genes (Zardoya et al. 1996), about 75% identity to human *sonic hedgehog* in the amino-terminal half of the protein, but less conservation in the carboxy-terminal half. In *Fugu*, three more *hedgehog* genes have been isolated (K. Gellner and S. Brenner, unpubl.), one is clearly *sonic hedgehog* (*shh*), the others are homologs to *desert hedgehog* (*dhh*) and *indian hedgehog* (*ihh*). Therefore, it seems that *fhh* is a fourth member of the *hedgehog* gene family.

Group 3: New Vertebrate Genes

We have defined four genes that have no or only weak homology to characterized mammalian genes (Table 1C). One of these is similar to the *Caenorhabditis elegans* gene *R05D3.2*. In the *Fugu* gene *178o23.1* we could identify sequences similar to the C2 domain, found in protein kinase C and Synaptotagmin. The

Fugu gene *diaphanous-like*, shows similarities in the WW domain to the *Drosophila* gene *diaphanous*. All four *Fugu* genes show strong homology to human cDNA fragments from the dbEST database (Table 1C), indicating that these genes also have as yet uncharacterized human counterparts. Defining the exact coding sequence, however, can be difficult. Comparing the predicted *Fugu* protein sequences against the predicted protein sequences of all dbEST database entries with TBLAST gave us some additional clues. Fifty-two of the 66 exons of the four new genes are based on homology with dbEST entries (Fig. 2). In many cases, the homology extends over several exons. Of these 52 exons, 42 are confirmed by XGRAI, and 37 by FGENES. The 14 remaining exons are based only on the two gene prediction programs, and nine of these are predicted by both programs.

Xu et al. (1994) have stated that GRAIL II predicts 93% of the exons in a human genomic fragment, with at least one edge correctly matching. By using the human dataset for the XGRAIL analysis of *Fugu* fragments, we expected a slightly lower accuracy. If we analyze the accuracy of XGRAIL for eight *Fugu* genes classified in this report to group 1, we find that only 80% of the exons are defined by XGRAIL. The exons that GRAIL failed to predict, but that were found by homology searches, are small exons surrounded by small introns. Only one false-positive exon has been predicted. Therefore, the predicted exons of the four genes of group 3 are most likely correct, but we assume that we may have overlooked some small exons, rather than added unreal ones.

Genomic Organization

The overall genomic structure of the analyzed fragment is very compact (Fig. 1). The predicted 15 genes are separated by intergenic regions <5 kb on average, counting from the end of one coding sequence to the beginning (or end) of the next. Only 45% of the sequence is intergenic sequence. Of the analyzed sequence, 26.6% is predicted to be coding. Eighty-four percent of the introns of the genes classified in group 1 and 2 are <200 bp, the majority are <100 bp. Genes of group 3 have larger introns, but we assume that, because of the lack of homology data, some exons have not been predicted and therefore the size of some introns may appear larger than they really are. The only noticeable repetitive areas are some GT or AT dinucleotide repeats, but they never extend >60 bp.

In terms of gene density, the *Fugu* genome resembles strongly that of *C. elegans*. In a 2.2-Mb *C. elegans* genomic fragment of Chromosome III, 29% of the sequence is presumed coding and 52% is intergenic (Wilson et al. 1994). For human it is estimated that only 1%–3% of the genome codes for protein (Jones 1995). If the above predicted numbers were true for the

Figure 2 Predicted exon-intron structure of the four new vertebrate genes of group 3. Exons are indicated by solid boxes or solid arrows (last exon). Shaded boxes indicate regions with significant homology to entries of the dbEST library. Letters above solid boxes show whether exons have been predicted by XGRail (g), FGENES (f), by both (gf), or by neither (–) of these two gene prediction programs.

whole *Fugu* genome, we can calculate that genes are packed eight times tighter in *Fugu* compared to human and, because of the difference in the genome sizes, we can confirm the original estimate (Brenner et al. 1993) that *Fugu* and human have about the same number of genes in their genomes. The size difference between the two genomes is only caused by the expansion of intergenic regions and introns in human.

Comparing Noncoding Sequences

The detection of elements involved in gene regulation is the most challenging area of genome research. It is possible that many processes common to fish and man, such as early development, involve common sets of regulatory elements which may show complete conservation. Comparative sequencing of noncoding regions of two species, which are widely separated in evolution, can uncover these. *Fugu* is very well suited for this approach for three reasons. *Fugu* has small noncoding regions, putative regulatory elements between different vertebrate species are highly conserved in many cases, and the evolutionary distance between teleost fish and mammals offers maximum divergence for mutational changes in nonfunctional sequences, highlighting any conserved sequences.

We carried out a comparison of noncoding flanking sequences for *wnt1* and *ARF3*, for which vertebrate

genomic noncoding sequence information is available. The *Fugu* genomic sequence was specifically screened for a homology to the murine putative regulatory sequence in the 3' flanking region of *wnt1* (Echelard et al. 1994). This led to the detection of a highly homologous sequence in *Fugu* (Fig. 3A), ~200 bp in length and located ~1800 bp downstream of the stop codon. In mouse, it is as much as 5–6 kb downstream of the stop codon and the orientation is reversed. Transgenic studies with mice showed that 110 bp of that homologous region work as an enhancer element and trigger the full *wnt1* expression in the early murine embryo (Rowitch et al. 1998). Furthermore, by DNase I footprinting, Iler et al. (1995) identified some AT-rich elements called HBS1 and HBS2 as binding sites for homeodomain transcription factors that have a role in restricting the expression of *wnt1* to the developing forebrain. Identical elements were found in the *Fugu* sequence within the 200-bp stretch of high homology at the 3' end of *wnt1* (Fig. 3A).

Screening of the 5' noncoding sequence of *Fugu wnt1* in a BLAST search revealed another highly conserved region, in this case with the zebrafish 5' *wnt1* region (GenBank accession no. X58880). The conservation covers 800 bp with a core sequence of ~200 bp, which is 85% identical in both species (Fig. 3B). In zebrafish, several putative TATA boxes were found just

PCR fragments then were used as probes to screen the *Fugu* cosmid library. Positive clones 29P14, 3A8, 186G5, and 178O23 were processed as cosmid 138E3.

DNA Sequencing

Subclones were amplified by PCR, DNA was precipitated with PEG (Rosenthal and Charnock-Jones 1992), and then sequenced with KS and SK primers (Stratagene Inc.) and ABI dye terminator chemistry on an ABI 373 or 377 automated DNA sequencer. Sequences were assembled with the program SEQMAN from the laser-gene package (DNASTAR, Inc.). Sequencing with custom primers was used for gap closure and for completion of both strands. For each cosmid, on average 550 sequences were aligned to obtain one contig. The absence of coligation events in regions covered by only a single cosmid was confirmed by long PCR on *Fugu* genomic DNA with custom primers, spanning up to 13 kb (data not shown). PCR was performed with Bio-X-act (Bioline, UK) under the supplier's suggested conditions. After denaturing for 2 min at 94°C, the following cycle was used 25 times: 10 sec at 94°C, 30 sec at 60°C, 12 min at 68°C.

Sequence Analysis

All subclone sequences were searched with BLAST (Altschul et al. 1997). Prediction of coding sequences was based on the blast results and on the analysis of the entire sequence with the gene prediction programs XGAIL_1.3c (Xu et al. 1994) and FGENES of the BCM Gene Finder package (Solovyev et al. 1995). The XGAIL_1.3c client server has been downloaded via ftp from arthur.epm.ornl.gov and installed on a Solaris 2.5 Sun station. The analysis was made with the human sublibrary on version GRail 2. Highly conserved noncoding regions were found by blast searches or with the alignment programs "bestfit" of the GCG package and CLUSTALW (Thompson et al. 1994). Linkage data were obtained from the Online Mendelian Inheritance in Man (OMIM) database for human and "mousedb" for mouse, respectively. All programs and databases were accessed through the UK Medical Research Council Human Genome Mapping Project Resource Center (<http://www.hgmp.mrc.ac.uk>) or the National Center for Biotechnology Information (NCBI; <http://www.ncbi.nlm.nih.gov>).

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Analysis of 148 kb of Genomic DNA Around the *wnt1* Locus of *Fugu rubripes*

Klaus Gellner and Sydney Brenner

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