Genomic Structure and Comparative Analysis of Nine *Fugu* Genes: Conservation of Synteny with Human Chromosome Xp22.2–p22.1

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The pufferfish Fugu rubripes has a compact 400-Mb genome that is ~7.5 times smaller than the human genome but contains a similar number of genes. Focusing on the distal short arm of the human X chromosome, we have studied the evolutionary conservation of gene orders in Fugu and man. Sequencing of 68 kb of Fugu genomic DNA identified nine genes in the following order: (SCML2)-STK9, XLRS1, PPEF-1, KELCH2, KELCH1, PHKA2, AP19, and U2AF1-RS2. Apart from an evolutionary inversion separating AP19 and U2AF1-RS2 from PHKA2, gene orders are identical in Fugu and man, and all nine human homologs map to the Xp22 band. All Fugu genes were found to be smaller than their human counterparts, but gene structures were mostly identical. These data suggest that genomic sequencing in Fugu is a powerful and economical strategy to predict gene orders in the human genome and to elucidate the structure of human genes.

[Sequence data for this article were deposited with the EMBL/GenBank data libraries under accession nos. A]OII38I and AFO94327.]

Isolation of human genes and their structural characterization is hampered by the fact that >95% of the DNA is noncoding and that intronic sequences can be very large. The Japanese pufferfish Fugu rubripes (Fugu) has a small genome of ~400 Mb, which is 7.5 times smaller than the human genome. Therefore, Fugu has been proposed as a model organism for rapid analysis of vertebrate genes (Brenner et al. 1993). The structure of genes appears to be conserved, because most splice sites reside in identical positions to those found in man (Baxendale et al. 1995; Elgar et al. 1995; Macrae and Brenner 1995; Mason et al. 1995). In regions of the Fugu genome that exhibit conservation of gene order, sequencing would reduce the effort required for isolation of candidate genes in human disease loci and for studying genome organization. However, reports showing conserved linkage over larger distances are scarce. The few available examples include the report of Trower et al. (1996), who have demonstrated that three genes that are linked to FOS in the familial Alzheimer disease locus on human chromosome 14 have homologs in the Fugu genome adjacent to cFOS. Conserved linkage of the genes encoding the plateletderived growth factor receptor and the macrophage colony-stimulating factor receptor (How et al. 1996), of complement C9 and DOC-2 (Yeo et al. 1997), and of

³Corresponding author E-MAIL kalscheuer@mpimg-berlin-dahlem.mpg.de; FAX +49-30-8413-1383. MAP-2, MYL-1, and CPSIII (Schofield et al. 1997) have been described. In contrast, the gene order of the Surfeit genes that map to three separate loci in the Fugu genome is largely different from that found in mammals in which these genes are clustered (Armes et al. 1997; Gilley et al. 1997). These findings gave rise to a controversial discussion on the potential of Fugu as a model organism to accelerate the prediction and discovery of genes by applying comparative sequencing or positional cloning strategies (Aparicio and Brenner 1997; Elgar et al. 1997; Gilley et al. 1997). Important questions concerning the extent and average length of conserved linkage groups and the distribution of conserved segments still wait to be answered. Very recently, Miles et al. (1998) found extensive conservation of synteny between a 1.5-Mb region of human chromosome 11 and <100 kb of the Fugu WAGR region. Here we report on another highly conserved segment in the Fugu genome that encompasses nine different genes from the human Xp22.2-p22.1 region.

RESULTS

Isolation of *Fugu* Cosmids Homologous to Human Xp22.2–Xp22.1

Initially, three partially overlapping human liver cDNA probes encoding *PHKA2* were used to screen a *Fugu* cosmid library (kindly provided by the Resource Center of the German Human Genome Project). One cosmid, ICRFc66A2095Q1.2 (A2095), was entirely sequenced

because it also showed positive hybridization signals with human *PPEF-1* cDNA. Rescreening of this and another *Fugu* cosmid library [kindly provided by Greg Elgar, British Resource Center of the Human Genome Project (HGMP-RC)] with end fragments of A2095 resulted in the isolation of overlapping cosmids ICRFc66L2390Q1.2 and F089J4. Together, these three cosmids encompass 80 kb of contiguous *Fugu* DNA, of which 68 kb has been sequenced.

Sequence Comparison Identified Nine Genes in *Fugu* with Conserved Order of a Five-Gene Cluster and a Two-Gene Cluster

Nine Fugu genes were identified following exon prediction and database searches (Fig. 1; see Materials and Methods). The gene order is as follows SCML2 (related to the Drosophila Sex comb on midleg repressor protein), STK9 (Serine-threonine kinase 9), XLRS1 (Xlinked Retinoschisis), PPEF-1 (Protein phosphatase with EF calcium-binding domain), KELCH2 and KELCH1 (related to the Drosophila Kelch protein), PHKA2 (Phosphorylase kinase 2-subunit), AP19 (Golgi adaptor AP-1 19-kD adaptin), and U2AF1-RS2 (U2 small nuclear ribonucleoprotein auxiliary factor 35-kD subunit-related protein 2). We subsequently compared our results with the gene order in human Xp22 (Kitagawa et al. 1995; Montini et al. 1997; Sauer et al. 1997; Sherman et al. 1997). Very recently, sequencing of overlapping PAC clones dJ757P12 and dJ958B3, which contain the human SCML2 gene, and of dJ245G19 and dJ436M11, which cover the STK9-PPEF-1 region, have been completed at the Sanger Centre, Cambridge, UK (http://www.sanger.ac.uk/), facilitating comprehensive comparison. Human PHKA2 had been mapped to Xp22.2-p22.1 (Davidson et al. 1992) and our YAC and PAC hybridization results confirm its location proximal to *PPEF-1* (not shown), identical to the position in *Fugu*. The human *KELCH* homologs are presently unknown and their isolation is in progress. To map human *AP19*, we performed FISH and Southern blot hybridizations of YAC clones containing human *PHKA2*. FISH assigned *AP19* to Xp22, whereas YAC hybridizations were negative. While this work was in progress, the sequence of a BAC clone was published (AC004106) showing linkage of both human *AP19* and *U2AF1-RS2* genes and two STS markers, located distal to *SCML2*. Therefore, we extended Southern hybridizations of *AP19* to YAC clones CEPHy904D10984 and CEPHy904D11161 and positive signals confirmed its location telomeric to *SCML2* (Fig. 1).

For a more detailed structural analysis of the *Fugu* genes, amino acid sequences were deduced for each putative coding region and were aligned to the sequences of homologous proteins from various species, including human, mouse, rabbit, *Drosophila*, *Dictyostelium*, yeast, and *Caenorhabditis*. Sizes of all *Fugu* genes, protein sequences, and their conservation in other species are given in Table 1.

Fugu SCML2, STK9, and XLRS1

By making use of the computer program NIX (http:// www.hgmp.mrc.ac.uk/NIX/), detailed sequence analysis at one end of our contig revealed the presence of a gene encoding a homolog of the *Drosophila Scm* gene, which is not the ortholog of the recently identified human *SCML1* gene (van de Vosse et al. 1998) and was therefore designated *SCML2* (Fig. 1). The human *SCML2* homolog spans base pairs 69966–107467 of PAC clone dJ958B3 and base pairs 47167–31846 of overlapping clone dJ757P12. BLAST search identified



Figure 1 Scale representation of the *Fugu* genomic region encompassing 80 kb compared with the equivalent genomic region of human Xp22 (not drawn to scale). In total, 68 kb of *Fugu* genomic DNA have been sequenced, of which 62 kb are contiguous. (Broken horizontal line) The region not completely sequenced in both *Fugu* and humans, which, in *Fugu*, spans ~18 kb. (Dotted line) A distance of ~1 Mb in human DNA. (Cross-lines) The borders of conserved gene order. The complete coding region of each *Fugu* gene is represented by aligned solid boxes and the transcriptional direction is indicated by the arrow above each gene. The results of the exon prediction programs FGENES, GENSCAN, and GRAIL 2 are shown (bottom), with each predicted exon shown by a solid box.

	Length					
		amino	Homologs	Amino acid		SwissProt/GenBank
Fugu genes	kb	acid		similarity (%)	identity (%)	accession no.
SCML2	2.0	250	Drosophila SCM	70	63	Q24191
			human SCML2	74	65	Z93023, AL031007
STK9	12.5	1104	human STK9	67	62	Y15057
			yeast SLT2	46	34	Q00772
			Dictyostelium CDK7	49	38	P54685
XLRS1	4.1	280	human XLRS1	78	71	AF014459
			mouse XLRS1	78	71	AJ011381
PPEF-1	5.5	683	Drosophila rdqC	54	42	P40421
			Caenorhabditis PPEF	56	46	AF023454
			human PPEF-1	64	54	AF023455
			human PPEF-2	63	54	AF023456
			mouse PPEF-2	64	55	AF023458
KELCH2	2.1	531	human putative protein Q14145	39	31	AC004021
KELCH1	2.1	518	human putative protein Q14145	45	33	AC004021
РНКА2	10	1229	human PHKA1	74	67	P46020
			mouse PHKA1	73	67	P18826
			rabbit PHKA1	73	67	P18688
			human PHKA2	77	72	P46019
			rabbit PHKA2	78	72	P46018
AP19	1.2	157	human AP19	98	96	AC004106
			mouse AP19	91	86	Q00382
			yeast AP19	67	54	P35181
U2AF1–RS2	1.8	605	human U2AF1–RS1	65	54	D49676
			mouse U2AF1-RS1	69	58	D85430
			human U2AF1–RS2	65	54	D49677
			mouse U2AF1-RS2	68	58	D45205

Table 1. Structural Analysis of Fugu Genes

The nine putative *Fugu* genes are listed together with sets of homologs found by database search. The approximate length of each gene is given in kb. The percent similarity and identity were calculated by the GCG program BESTFIT.

two human ESTs, one of which represents the fulllength cDNA. Its database sequence contains exons 1–4 and part of exon 5 (accesssion no. AA227320). The other EST starts in exon 7 and extends into the 3' UTR (accession no. AA618466). *Fugu* and human *SCML2* splice sites are conserved and both genes contain a total of six coding exons. Human *SCML2* harbors one additional 5' exon that is noncoding and which, by sequence comparison, could not be identified in *Fugu*. Predicted SCML2 proteins consist of 250 amino acids in *Fugu* and 268 amino acids in humans, which are 65 % identical (Table 1).

The same *Fugu* cosmid contains the *STK9* and *XLRS1* genes. Comparison of the predicted *Fugu STK9* protein revealed homology to several serine/threonine kinases of the CMGC group (Hanks and Hunter 1995; Fig. 2). The human homolog has been described recently (Montini et al. 1998) and consists of 20 exons coding for a protein of 1030 amino acids. *Fugu STK9* is composed of 18 exons encoding 1104 amino acids. Gene structures of *Fugu* and human *STK9* are similar with 8 exons having identical length. Amino acids 33–819 of the human protein. Multiple alignment of

the *Fugu* STK9 predicted protein showed high conservation throughout the kinase domain, which, in *Fugu*, consists of the amino-terminal 283 amino acids. All invariant residues found in 60 kinase domains representative of members of the eukaryotic protein kinase superfamily (Hanks and Hunter 1998) are also invariant in *Fugu* STK9. In addition, the *Fugu* protein harbors a polyglutamine stretch that also is present in homologous serine/threonine kinases of *Dictyostelium* and yeast but is absent in the human protein (Fig. 2).

The structures of the *Fugu* and human *XLRS1* genes are identical, except for longer 5' exons in *Fugu* that contain 172 and 142 bp, respectively, compared with 52, 26, and 106 bp in human *XLRS1*. The *Fugu* protein contains 280 amino acids and is 71% identical to the 56 amino acids shorter mouse and human homologs. Sequence identity is mainly confined to the highly conserved discoidin domain at the carboxyl end. The amino-terminal ends of the mammalian proteins contain a secretory leader peptide sequence of 23 amino acids . By use of the program SIGNALP (Nielsen et al. 1997) one cleavage site of the signal peptide in *Fugu* has been predicted also between amino acids 23 and 24 (SQQ↓EK) (not shown).

	· • • • •						
human STK9	mkipnignVMnKFellgvvGEGAYGVVlkcKhKEThEIVAIKKEKDSEENEEVKETTLRELKMLRTLKQeNIVELKEAFR	80					
Fugu STK9	mfnsGsslkskirqgdnEKETnElVAIKKEKDSEENKEVKETTLRELKMIKTIKODNIVELKEAFR	66					
P54685	~~~~~MdKynIealIGEGtYGVVsrAtvKATgqIVATKKirkiliqnqtddginfsaiREiRilqeLKhDNvVnLldiFa	75					
Q00772	adkierhtfkvfnqdfsVdkrFqlikafChQAYGiVcsARFs.EsAedttVAIKKvtnvfsktllokrsLKELKLEhfrghknitclydmdivFy	96					
	— — —						
humon CEVO							
Fuch STRO		174					
254685	bish vyluvelka o kvyletka i i k padi kryke kula (za za z	169					
000772	pdGsingLYLveEIMEcDMhgiIksgor. LtdAhvgSftYQiLcGikviHaaDVJHRDIKPGNIJvABCGIKiCDFGIARGYSPnyaMagfTTEvvar	195					
		100					
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human STK9	RWYRSPELLLGA. PYGXSVDM#SYGCILGELSDGQPLFPGESEIDQLFTIQRVLGPLPsEQMKLFYsNPRFEGLRFPaVnHPQsLERRYIGTINSv1LDL	273					
Fugu STK9	RWYRSPELILGA. FYGRAVDMWSVGCIIGELSDGQPLFPGESEIDQLFTIGKVLGPLPAECMKLFYnNPRFHGIRFPsV+HPQTLERRYqGILSglmidL	259					
P34683	ItTRAPELLIGARSIGDSVDIWSIGCITAELmirtPyiPgtgEIDQDrkIceaLGTPnesnwgqvtgIPNy., IkF. tdHpaTFFkgLftaASdeAiDL						
000112	anagorgannogyygs coordynnygyganganig i gorgirfagatyn genegatyn bran firrygst. NydgyingigirfayPFynIypnANSqAIDL 2						
	▼						
human STK9	MKNLLKLDPadRYLTEQcINEFTFQtqRlldrspsrsakrkFyhveSStlsnrnqagkstalSEhr5nSkdignlsv, gLFR, aDeglpaneSFINGNL	371					
Fugu STK9	MKNLLLINPteRYL_EOsINHPaPOplRqvere.rpappasPnpprSSkrkthhhgentvptrSHskStSrrenskecssLPRhgblhhlgskSFINGNK	35B					
P54685	iskMLLFNPsnRIsaadAlNHPYF.	288					
000772	lequarDPgkRItvdeaLenPY1	318					
human gerra		450					
Fum STER	.eyebber laitettigesseppersanartailisillisillisillisillisillisillisilli	452					
P54685	pppodos zmprodzmow zma prosta v star z know zna v starza z trypera z stara domow z stara prosta stara stara star E sa kteri stara star	458					
000772	siwhdpadePucsekfefSfesVndmedlkQmVjgevOdfrLfvRobl	366					
human STK9		511					
Fugu STK9	qldCQQqQLQQQQlQQQQQQQQQQQQQQQQQQQQQQQQairhffleNKnnTQgggaEKQh8KhtHSmaDsahGemissSksSaSYIslsKSKSALSDaKSVGAUSDgRlhPddP	558					
P54685	aqvD0gL1qk0g000g0Q0Q0Q1ge0PepiqvdNvegTq0aqqvKkvvvvvvvvvvvvvvvvvvvvvvvvvvv	362					
Q00772	reen from a war war war war war war war war war w	464					
human STK9	stsRyFPsSCLDLNsPTsPtptRHSDtrtllSPagRnnrNeqTLD.SrRtttRH.SktmEElklFEhmDsshsHsLs	586					
Fugu SIK9	NantagLgpgaKFFFC5CL5UMaFTgFpgppgspsaRESD:.sgh5FasKssgNirmessTLDsSsKhksKEkSsapExagaFE11DPggaampstHtLp	657					
P54685							
Q00772	NgtlldIøkelaFgldrkyf************************************	484					
human STK9		659					
Fucu STK9	sPHESybYGIGITEPFSSORPGHESMYVRerbRohGyegomagloapGGGiomRAsSLOLLSPOlghttltdaysaaredotddmtreficePtdmt	757					
P54685							
Q00772							
human STK9	varssvketsRegTasFBT.RgksEgGvJHDFBadDGtapKENRhlyndpvERVGSFIRVPSDREDN.SFHennvstRV.sslPasssgtnHSKR	752					
PRASES	npcapikosakonikases qaskinadi inipingengas sakakani tesmirkkvissi ikvisikkinasisinasyqqavpovivqpvanoqaska	857					
000772							
-							
	v v						
human STK9	QpAFDpwkspenishs.EqlKEKEKQCFFFsmKKKKKKSQtVFnsdspdlLtLQkSihSaStpSSRpXewRpekisdlQtqsqplkslRkllhlssa	848					
Fugu STK9	QtAFDwtaaeamvmnplEpvKEKEKQ4FFRaiKKKKKKtQ1VP.gngqehLsLQr5tkSsShhSSRrKnreesRdrhrereQsrdrdrereRererdrer	956					
P54685	~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~						
000772							
	₩						
human STK9	shpassdpVfZLtagqtknsfSeirihplsQasqgssnirgepapkgrPalglPdggcdgrrgRhHsgpgdrrfmlrttaggavfccgdPkkphtsc	948					
Fugu STK9	erergreleRsQPIkslrrllhlSpsssnqrqQspaspapppdlrfqaplDnppqPpskagypetRgHadarghagvseseqkerkasypiDqqiessvl	1056					
P54685	***************************************						
Q00772	***************************************						
human 4750	vnržiji Brizensnum Furzatesc BEI graatdsfeat gasafs ffusium as 14 hesauras 11 subsc-14-5	1030					
human STK9 Fugu STK9	vpnrALhRpisspapypvLqvrgtsmcFTlqvrgtdafsoptqqgfSffvrbwnrealihraqvnqaalltyhenaaltgk hvg.ALoRaecaoftneoLoikocomoFfftasttinnlndlkstal	1030					
human STKS Fugu STKS P54685	vpnrÅlhRpisspapypvEqvrgtsmc%Tlqvrgtdafscptqqggfsffvrhvmrealihraqvnqaalltyhenaaltgk hvs.ÄLgRaegaqFipqdggikpqqng%Tftrastripnlndlketal	1030 1104					

Figure 2 Multiple alignment of the human, *Fugu*, *Dictyostelium* (P54685), and yeast (Q00772) STK9 proteins. The alignment was performed with the GCG programs PILEUP and LINEUP. Amino acids identical in at least two proteins are shown in uppercase letters, shading indicates residues identical in *Fugu* and human and in all proteins, respectively. (\mathbf{V}) Intron/exon boundaries identical in *Fugu* and human *STK9*; (∇) splice sites only present in *Fugu*. The protein kinase ATP-binding region is presented by the first box. The second box indicates the predicted serine-threonine protein kinase active site. The predicted leucine zipper is boxed in bold and the polyglutamine stretch in broken lines. (Asterisks) Amino acid residues invariant in 60 members of the eukaryotic protein kinase superfamily (Hanks and Hunter 1995).

Fugu PPEF-1

Fugu PPEF-1 belongs to a conserved branch of the phosphoprotein phosphatase (PPP) family of serine/ threonine PPPs. In contrast to the 16 exons of the human *PPEF-1* gene (Montini et al. 1997; GenBank accession no. Z94056, partly contained in PAC clone), we discovered 17 putative exons in *Fugu*. Interestingly, 12 of 17 *Fugu* exons are identical in position and length. The deduced protein is 54% identical to human PPEF-1 and PPEF-2 proteins (Table 1). Multiple alignment revealed a high degree of conservation throughout the

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protein, except for a segment that is apparently inserted into the catalytic core and is highly variable in both length and sequence (Fig. 3). In addition, protein phosphatases of the PPEF subtype possess two functional domains. In Fugu the catalytic domain encompasses amino acids 130-473. The second domain is present at the carboxyl end of the protein and encompasses amino acids 595-668. This domain contains two potential Ca2+binding sites, as defined by the highly conserved EF hand motif. Taken together, these data strongly suggest that the PPEF-1-encoded protein is a functional phosphatase (Fig. 3). To assess the degree to which Fugu PPEF-1 is related to other members of the PPEF subfamily, a phylogenetic tree was generated from aligned conserved regions on the basis of nucleotide identity (see Materials and Methods). The unrooted tree shows that Fugu PPEF-1 and human PPEF-1 cluster in the same branch, whereas human and mouse PPEF-2 cluster in another branch (Fig. 4). Lessconserved regions that contained gaps when aligned, like exon 10-11 and the 5' and 3' ends of Fugu PPEF-1, were excluded. We generated 100 trees with the maximal likelihood method and bootstrapped them. Bootstrap values are indicated for each branch.

Fugu KELCH1 and KELCH2

Linked to *Fugu PPEF-1*, we have identified two tandemly arranged genes. Nucleic acid comparison revealed 62% identity. Database searches showed that both genes

encode proteins harboring the so-called kelch domain. The highest score with an expectation value of $P \sim 10^{-70}$, was assigned to the human putative protein Q14145 (Table 1). On the basis of the homology to Q14145 and the *Drosophila* Kelch protein, the *Fugu* genes were designated *KELCH1* and *KELCH2*. The genes are in a head-to-tail orientation and are separated by ~3.5 kb of genomic DNA. For both genes, exon prediction revealed four putative exons that form an identical structure. To confirm the predictions, we performed RT–PCR on total *Fugu* testis RNA with a com-



Figure 3 Alignment of *Fugu* and *C. elegans* PPEF, *Drosophila* RdgC, mouse PPEF-2, and human PPEF-1 and PPEF-2 proteins. The alignment was performed with the GCG programs PILEUP and PRETTY. Amino acids at least identical in two aligned sequences are shown in uppercase letters. Residues identical in *Fugu* and human PPEF-1 and in all proteins, respectively, are shaded. (\mathbf{V}) Identical intron/exon boundaries of *Fugu* PPEF-1 and human PPEF-1; (∇) Position of the putative additional splice site in *Fugu* exons 10–11 are numbered. The catalytic domain and the EF hand motif of the PPEF proteins are boxed. The two positions of the Ca²⁺ chelating side chains are labeled X–Z.

bination of primer sets. Amplification with primer pairs spanning the predicted coding region resulted in the expected products, whereas no transcripts were detectable with a primer set that spans the presumptive intergenic region (not shown). The putative proteins share 48% identity (Fig. 5A). In both proteins the first repeated segment of the Kelch domain contains 48 amino acids, repeat 2-5 contains 47 amino acids, and repeat 6 contains 49 amino acids (Fig. 5B). This is in contrast to the variable length of the repeated segments in other Kelch-containing proteins. Comparison with the consensus sequence of Drosophila Kelch repeats revealed that conservation of the Kelch domain is confined to those amino acids that form the consensus sequence in Fugu (not shown). The first 12 residues at the amino-terminal end of both Fugu Kelch proteins are part of the BTB domain, which encompasses ~24 residues in other homologs.

Fugu PHKA2

The Fugu PHKA2 gene exhibits significant homology to the liver (PHKA2) and muscle (PHKA1) isoforms of the α regulatory subunits of the phosphorylase kinase (Phk) of rabbit, mouse, and humans (Fig. 6). The gene structure for the human homolog has been determined for 11 exons (Hendrickx et al. 1995), corresponding to exons 20-28 in Fugu. Comparison revealed exons with identical length and position. Interestingly, human PHKA2 contains two exons that would reside between exon 27 and 28 in *Fugu*, but in this region splice sites are apparently not conserved. Mammalian PHKA muscle and liver isoforms and Fugu PHKA2 have large stretches of highly conserved amino acid sequences in common, which also include the regions of the hypothetical 5' and 3' calmodulin binding sites and several phosphorylation sites (Fig. 6).

Fugu Clathrin Coat Assembly Protein API9 and *U2AF1–RS2*

The AP19 protein is evolutionarily highly conserved. The high conservation of the *Fugu* protein (Table 1) is reflected even at the nucleotide level showing 80% and 76% identity to the mouse and human homologs, respectively (data not

shown). Intron/exon structure comparison revealed that all splice sites are identical. Comparison of the Fugu and human U2AF1-RS2 genes showed that position and length of Fugu exons 6 and 8-10 correspond to their human counterparts. Because we were not able to assign the intron/exon boundaries at the 3' end of the gene by sequence comparison, we used exon prediction instead. In this way, an unusually long terminal exon 11 was detected, encompassing 875 bp compared with 509 bp in humans. The human U2AF1-RS2 gene is characterized by an increased CG content >50% and by CpG islands surrounding the coding region. The CG content of the corresponding *Fugu* regions is slightly increased (44%) with >60% at the 3' end, but these values do not indicate the existence of CpG islands. A statistical analysis of the protein sequence by



Figure 4 Phylogenetic tree of the PPEF subfamily. The unrooted tree was generated from aligned nucleotide sequences by the DNAML program of the PHYLIP software package. Regions of ambiguous alignment were excluded from the analysis. Scale bar, 0.1 expected substitutions per nucleotide site. Bootstrap values were generated from 100 bootstrap replicates of the alignment and are shown. *Fugu* PPEF-1 and human PPEF-1 cluster in the same branch; human and mouse PPEF-2 cluster in a second branch.

the program SAPS (Brendel et al. 1992) revealed a high usage of arginine (13.2%) and glutamic acid (13.3%), classifying the protein significantly charged.

Intron/Exon Organization, Base Composition, and Repetitive Elements

Comparison of the coding regions of *Fugu* and human homologs revealed 60% to 76% nucleotide identity. Although exon sizes were mostly similar, sizes of *Fugu* introns showed a range from 61 to 2115 bp, giving an average of 308 \pm 343 bp for 82 introns examined. This result concurs with previous observations that *Fugu* introns are at least 50 bp in size and small in average (Brenner et al. 1993). The overall size of the *Fugu* genome is reduced by the factor 6–8 when compared with the human genome. Analysis of individual genes revealed size reductions ranging between factors of 7 and 22 for *Fugu XLRS1* and *PPEF-1*, respectively.

By analyzing the base composition of 164 intron/ exon boundaries, we determined the 5' splice site consensus sequence as $C_{39}A_{61}G_{84}\mathbf{g}_{100}\mathbf{t}_{99}a_{48}a_{51}g_{59}t_{41}$ and 3' splice site consensus sequence as $c/t_{77}c/t_{80}c/t_{80}c/t_{83}g/t_{67}c_{74}\mathbf{a}_{100}\mathbf{g}_{100}G_{44}T_{44}$. Numbers indicate the percentage of the respective nucleotide at this position. The *Fugu* consensus sequences equal those of mammals (Shapiro et al. 1987).

Base composition analysis revealed a GC content of 42.7%, which is slightly less than the 44.2% reported (Brenner et al. 1993). The search for repeated sequences using the program CENSOR (Jurka et al. 1996) identified a few short tandem repeats but neither highly nor moderately repetitive elements.

DISCUSSION

We have sequenced 68 kb of Fugu genomic DNA corresponding to >600 kb of human Xp22.2-p22.1. The comparative analysis of Fugu genes demonstrated the feasibility of gene identification and characterization in silico. Gene structures were deduced by comparing stretches of amino acids with >60% identity to homologous proteins and partly by sequence comparison with Fugu RT-PCR products. High conservation of splice sites were found in evolutionarily conserved domains, however, additional splice sites were detected in regions accompanied by low amino acid identity and in loop regions or protein ends. For the subsequent functional characterization of the putative proteins, we used various methods, including statistical analysis of the amino acid composition, motif and signal search, multialignment, as well as phylogenetic studies.

The gene order in human Xp22.2-p22.1 had been established for the five-gene cluster SCML2-STK9, XLRS1, PPEF-PHKA2 (Sanger Centre; Davidson et al. 1992; Montini et al. 1997, 1998; Sauer et al. 1997) and for the two gene-cluster AP19, U2AF1-RS2 (accession no. AC004106) (Kitagawa et al. 1995) showing conserved linkage and transcriptional orientation. However, in contrast to the order in Fugu, human AP19 and *U2AF1-RS2* are located telomeric to *SCML2*. This result indicates the existence of an evolutionary breakpoint, separating AP19 and U2AF1-RS2 from PHKA2. Other examples of fragmented gene order in Fugu include the Surfeit locus, which is composed of at least six housekeeping genes, whose organization and juxtaposition is conserved between mouse, humans, and chicken, but in Fugu are found at three loci (Gilley et al. 1997). Fugu vasotocin/isotocin locus has undergone a localized reorganization during vertebrate evolution. The simplest model to explain the rearrangement includes a duplication of the ancestral vasotocin gene of cyclostomes, followed by an inversion in the lineage that led to the Fugu (Venkatesh et al. 1997). Compared with the situation in humans, the gene order in mouse is less well defined. STK9, Ppef, U2af1-rs2, and Phka2 map to the telomeric third of the X chromosome (Montini et al. 1998, http://www.informatics.jax.org/).

The *Fugu SCML2* gene is homologous to *Drosophila* Sex comb on midleg and its human homolog has not been published to date other than by database sequences of two overlapping PAC clones, each containing only part of the gene, which may have led its incomplete annotation. Sequence comparison revealed that intron/exon junctions of all coding exons are conserved. These findings strongly sustain the existence of a complete human *SCML2* in human Xp22, which is

Α

- Fagu Fagu	KELCH1 KELCH2	ALFttW&sPdknvyeirDlSFdVtBLTifyAYtstvPvts ALFirWenPeerhfvviDfSPeVmRLTfefAYnnniPlse	tnamklulähdrynvlgivevCCGFLmemLstenCiglvGfsdicyCsGlqeKAfeFric 100 ktirkLirhAgflNimsIsvkCCtFlgnMLcedNCIdIyQltkynfCtGlegNAykFlin 100
Fugu Fugu	Kelch1 Kelch2	NFDqviğskEFnQTsvEelCdIfdhDeLoVknEsiVPeAL NFDlisSgeEFfQtdmEhfCsIissDhLoVssEavVFrAv	okWIsHnserRKrhfAlllpKvRLalLSIEYIithMvtsELmNKNaeCwemvlssiqiKm 200 vrWinHawadRKefchilfsKvRLofLSyEYIrlNMiqhELvNNNslCornievgfniKe 200
		1	▲ 2
Fucu	KELCH1	QmatsiPFLittgdtvAhPRvPKdLLLAiSGWiyedvldv	IZAYnvriquWVsiphhinRpRAYHssVFLNesvYCLGSyDeqEnFssMcRfD1nTctWh 300
Fugu	KELCH2	QldafdPPLtvarnamAyPRyPKnILLAtGGWsgnnpths	vEAYdisthhWVvpgtqLdRzRAYRgvVFLNdnmYCLCCfDrlEtEnlMqRyDfsTgmWs 300
		3	▼4
Fugu	KELCH1	EVAPMHYRRCIASVIVLdqyIIA166YDGtsRqKsAErIt	PdTNQWsLitpHhEkRSDASCTTINNKIYICGSydGeEsvQTgEfYdPEtnQWTmiAsMg 400
Fugu	KELCH2	EAAPMHYRRCYVSVTVLnikIYAmGGYDGfeRlKtAEsYs	PernowtivasMnEqRSDASCTTINNKIVICGGfnGtEclQTcEsInPEvdQWTlfApMs 400
		5 🔻	6
Fugu	KELCHI	toRscheVvAyvghiYAvCCfDCrahlksAEaFNFqtDsW	NFvpnMltaRSNFGyEvierrvFVvGGFsGfrsicsaEcYdadakrWfEveeMetpRfGL 500
Fuga	KELCH2	iQRSGVGVIAaltyvYAiGGsDGnvrLrtAEvYNPdsBik	NF1spMhnpRSNFGiElmdgliFViGGFnGetttrkvEyYnietddWnEaddMdvsRsGL 500
		A	
Fuqu	KELCH1	SCCLisofEdltrkffel	518
Fucu	KELCH2	SCCLvtdlBriinyiyprkilpllktkkgk	530

В

Fugu	KELCH1	repeat	1	illAiGCwiyedvldviEaYnvriqcWvsiphhlnrprayhssVflNe	48
Fugu	KELCH1	repeat	2	svYclGGYDeqEnfsSmcrfDlnTctWhevapMhyrRcyaSvtVldg	47
Fugu	KELCH1	repeat	з	yIYA1GGYDGtsrqkSAErYtEdTnqWslitpMhakRSdaScttlnN	47
Fugu	KELCH1	repeat	4	kIYicGGYDGeEsvqtgEfYDPeTnqWtmiasMgTqRSghgvvayvg	47
Fugu	KELCH1	repeat	5	hTYAvGGfDGrEhlkSAEaYnPqTdsWnpvpnMlTaRSnfgyeVieN	47
Fugu	KELCH1	repeat	6	rvfvvGGfsGfrsicSAEcYDadakrWfeveeMeTpEfglScclisgfp	49
Çonşensus				-IYA-GGYDG-ESAE-YDP-TWM-T-RSSVN-	
-					40
Fugu	KELCHZ	repeat	T	111ACGGWBGNNPENAVKAIGISINNWVVPGCQIGIKIAYNGVVIING	40
Fugu	KELCH2	repeat	2	nmYclGGFDrlEkfnlmqrYdfsTgmWseaAPMhyrRcyvSvtvlnr	47
Fugu	KELCH2	repeat	3	kIYAmGGyDGfErLkTaEsYaPETnqWtlvAsMneqRSdaScttlnn	47
Fugu	KELCH2	repeat	4	kIYicGGFnGtEcLqTcEsYNPEvDqWtlfAPMsiqRSgvgviaalt	47
Fugu	KELCH2	repeat	5	ywYAiGGsDGnwrLrTaEwYNPdsDiWnplsPMhnpRSnfgielmdg	47
Fugu	KELCH2	repeat	6	lIfyiGGFnGetttrkvEyYNiETDdWneaddMdvsRSglScclvtdlp	49
Conse	nsus			-IYA-GGFDG-E-L-T-E-YNPETD-WAPMRSS	

Figure 5 (*A*) Alignment of *Fugu* KELCH1 and KELCH2 proteins. The alignment was performed with the GCG programs PILEUP and PRETTY. Residues identical at each position are shown in uppercase letters and shaded. Intron/exon boundaries are shown by closed arrowheads on either site of the alignment. Each repeated segment is boxed and the beginning of each repeat is indicated by its respective number. (*B*) Alignment of the six repeated segments of *Fugu* KELCH1 and KELCH2 proteins. The alignments were performed with the GCG programs PILEUP and PRETTY. Amino acid residues at least identical in three repeats are shaded and shown in the consensus sequence. Dashes were introduced at less conserved positions.

further supported by two human ESTs, one containing exons 1–5 and the other exon 7 and the 3' UTR. Thus, comparative genomics is a useful tool to establish the genomic structure of new human genes, of which sequences are generated in large scale sequencing projects.

The *Fugu* STK9 protein is a new member of the CMGC group of serine/threonine kinases that are all related by the presence of highly conserved kinase domains consisting of ~250–300 amino acid residues. Highest homology has been found to human STK9 protein. Less homologous proteins include serine/threonine kinases of *Dictyostelium* and yeast, but as in *Fugu*, these contain a polyglutamine stretch that is absent in the human counterpart, suggesting that it might have been lost during evolution.

Fugu PPEF-1 protein belongs to a subfamily of protein serine/threonine phosphatases that are characterized by the presence of at least two EF-hand motifs near the carboxyl terminus, suggesting a regulation of the enzymatic activity by intracellular calcium. Reversible

phosphorylation of proteins on serine and threonine residues plays a crucial role in the regulation of a variety of cellular processes. In the mouse, Ppef-1 transcripts are restricted to primary somatosensory neurons and to the inner ear (Montini et al. 1997) and Ppef-2 transcripts have been found in retinal rods and in the pineal (Sherman et al. 1997) suggesting that the transcripts might play analogous roles in different sensory systems, but their roles seem to be distinct from that described for the Drosophila member retinal degeneration C (rdgC) (Steele and O'Tousa 1990: Steele et al. 1992). The Fugu homolog is closely related to human PPEF-1 as indicated by gene position and phylogenetic tree.

Fugu KELCH1 and *KELCH2* exhibit high nucleotide identity and an identical gene structure. Up to now we could not identify human or mouse orthologs by comparison with databases or by Southern hybridization. Both *Fugu* proteins are members of a family containing at least one Kelch domain. Characteristic for this domain is the presence of up to six repeated segments of ~50 amino acids each. Noteworthy, in *Fugu*, corresponding repeats of both proteins are identical in

length. Although both Fugu proteins share 48% amino acid identity only a few selected residues within the Kelch domain are conserved. These are presumably implicated in the correct folding. Through sequence analysis, Bork and Doolittle (1994) have shown that the six repeats in Kelch proteins form a β-propeller or super-barrel structure. This structural fold is also found in several nonKelch proteins with repeat sequences, including bacterial and fungal, as well as influenza virus enzymes such as neuraminidase, galactose oxidase, or the sialidases. The question remains open whether all propeller folds share a remote ancestor or whether the possibility of structural convergence must be taken into consideration (Bork and Doolittle 1994; Robinson and Cooley 1997). Generating phylogenetic trees from the Kelch region showed various topologies in which only subbranches were reproducible (not shown). Although Kelch proteins form a large family, there are only a few hints toward the biochemical functions of this family. In the case of the *Drosophila* Kelch protein, a simple model suggests that the protein might bind to

MRERSNSGYRLDGTARLYGGTTLohONPYTGLLpASydgrdanvrdnyfstlavnglglatrrnadrdedrakaytlegsvyrlmyglltchirovygve human PHKA1 100 nr sren sgvrid starl v gytilchor svoglods svogkdawvednyt s i lavwglgiatrknad kdedkakateleg svykim tgilhom i rovakve Mesren sgvrid gtarlyng tilchor svoglods svogkdawvednyts i lavwglgiatrknad kdedkakateleg svykim tgilhom i rovakve rabbit PHKA1 100 nouse PHKA1 100 HRSRANSGYRLD GYARLYGGTIL GYONFYTGLLSASD GGRDANYRDWITSILAYWGLGBAYRRNADRDHDKARAYHLBONYYRIM-GLLGCMBROYDRYB human PHKA2 100 rabbit PHKA2 MRSRSNSGVRLDgYARLYqqYYLCygNFYYGLLSASheqRDANYRDN1YSYLAVNGLGmAYRKNADRDEDKAKAYELEgnyyKIMyglLqcmmqydxye MRSRSNSGVRLDgYARLVDeTILgfQNFYTGLLDASvgkKDAWYRDNYISILAVWGLGmAYRKNADRDEDKAKAYELEQsVVKLMqGLLhCMmRQVaKVE Fugu PHKA2 strysqutkdslaakintkicstvygddgwghlgidatsyjlltagwtasginiihsldevnfighightytieakikkadgihergoliselwas strysqutkdslaakintkicstvygddgwghlgidatsyjlftagwtasginiihsldevnfighiytteakkkadgihergoliselwas sfrysqutdslakintkicstvygddgwghlgidats sfrysqutdslakintkicstvygddgwghlgidatsiillergwtasginiihsldevnfighiytteakkkadygwhggiselwas sfrysqutdslakintkicstvygddgwghlgidatsiillergwtasginiihsldevnfighiytteakkkadygwhggiselwas human PHKA1 200 rabhit PHKA1 200 nouse PHKAI human PHRA2 200 ŁYKYŁOSTŁDELIAKTATATCETVYGDDQWGBLQVDATSIFLIELELAQMTASGLEIIFLEUGYAFIQNLVYYTEAAYKVADYGMWERGDKTNGGIPELNAS ŁYKŁŁOSTŁDELMAKYGTETCETVYGDDQWGHLQVDATSIYILMLAQMTASGLEIIENLDEVAFVQNLVYYTEAAYKVADYGMWERGDKYNOGIPELNAS rabbit PHKA2 200 Fugu PHKA2 SVGNAKAALEAIDELDLFSVKGGOQSVIHVLADEVQHCQSILASILERRS 15KEVDALLSVVSFFAFAVEDsQLVelTKQEIITELOGFYGCCRF1RDG human PHKA1 300 EVGKALALEAIDEIDIEG=>COCOQUEVIEVI=DEVGKOGSTI=EILDEASESEE=DAALLS==SEATAVED=>LV=ITKGEITEKLOGSYGCCRFIRDG SYGMAKAALEAIDEIDIEG=>COOPUEVIEVI=DEVGHOGSTI=SILPEASESEE=DAALLS=>SEPATAVED=bLV=ITKGEITEKLOGSYGCCRFIRDG rabbit PHKAl 300 mouse PHKA1 SVGMAKABLEAIDELDLFGANGGIKSVINVLDDEVERCOSILISMLPRASISKEIDAGLLSIISTPATAVEDVNLVNVTKNEIISKLOGRIGCCRFIRDG human PHKA2 300 rabbit WHKA2 SVGMAKAAITERIDEIDLFGabGGrkSVIHVLDDEVeHCOSILFSmLFEASISKEIDAGLLSIISFDAFAVEDanLVnvTKsEIIsKLOGEVGCCEFIEDG Fuon Paka2 SYGMARAALEALDELDIFGehGGpkSVIHVLpDEVeHCQSILGEmLDDASpSKEIDAGLISVISTFAFAVEDadIVtiTKeEIInKLQGRYGCCRFIBDG Yk tREBYRRIYY OFAELKIFENIKCEWPIEPTYFIIDG-FegnacQVQEYEKALOAVIIECKnGvpLIDELYov PpdrVdEEYqWPE tVDKvpmGkiPH human PHKA1 400 XX LFREDFARLYL ERSEXLETENISCENFIFMTYIIDGYFsqaacyygfrefilaevlik6Ka6vplifElysvFpdkydffryDfreudfalfe Ix LfredfqRyyarfalkureniscenfifwrifildgifsgavegyygfrefildsufik6ka6vplifElysvFpdrydfefqMfftyDfvpmgkiff Ix LfrEdfarLydffflifeniscenfvfWTffildsyfsgdavgYQffrEilgsiligka6irlyFlifavFpak/dffyMfftyDrymgkvFf rabbit PEKAI 400 mouse PHKA1 human PHKA2 400 rabbit PHRA2 YL TEREDYNRINY GRAELALFENIECEWF VEWIYF IIDGIFngdal QYGEYGALEGILIFORdGIFLVPELFALEPNLYDRETNPHLYDRYD GLIPH Yngredetriny draelau frifeniecewf vewiti i 10gifaedgy QYGEYFALegylif graf y Belityf dryweet fryfening frifeni 400 Fugu PHKA2 mWggELYIIgsImARGPLApGEIDFLWRRFSTypRFDYVVQYsiLAEteeIKtiLkdkSiyVetiAevyFSTVqpaRiLShIYssLGcnnrmkLSGRPYR 500 human PHKA1 mWqGSLYILgsLmARGFLApGWIDFLWRRFSTwpKYDVWVQVsilAEteelKaiLkdkGinVetiAevyFIrVqpaRiLShlYssIGcnnrmkLSGRPYR mWqGSLYTLgelmAEGFLApGETDFLWRFSTwpKFDVVVQVsilAEteelKaiLkdkGidVetiAevyFirVqpaRiLShlYesIGcnsrmkLSGRPYR rabbit PHKAl 500 ouse PRKA1 INGOLIYILƏ SLIALGFILALGFILDFLARRFS ƏVKPDYVYQY VIAEnnhIKALLırkhövnüqəliydin Piqüqqu LehiyaklörnknamlöğRFYR INGOSLYILƏ SLIARGFILA CETIDFLARRFS TƏVKPDYVYQY VIAEnshIKelirkhövdüqsiAdiyFirüqqqılı ShIYaklörnknaklə GRFYR MƏQSLYIL GLIARGFILA GETIDFLARRFS TƏ KPDYVYQY VIAEs get Kallseqüm Vqt vAeviPirümsakvişqiy vriğnek kisi SGRPYR human PHRA2 500 rabbit PHKAZ Fugu PHKA2 hmGVLGTSK1ZdIRktifTFTFOF1DQqGFTLALDNkMIVENLRtdLs1LcsrWRMTGqFTiTFPisysMLdeDGts1nssiLaalRKmqDGTFqGARyq 600 human PEKA1 rabbit PHKAl hmGVLCTSKIZdIR+tifFFFQGFiDgqqFYLAIDHkHTVEMIRtdLeYLco-WEMFGqFITFFDisqtMIdaDGtslnseiIaalBEmqDGYFgGARiq lmGVLCTSKIZdIRktifFFFGGFiDQqqFYLALDNgMIVEMLRtdLeYLcorWEMFGqFFi7FDishtMLdeDGtslnseiLaalBEmqDGYFgGARiq nouse PHKA1 higvig7sKlyvfAngifTFTFQFtDQhhFYLALDMeMIVEMLKieLaYLotoKNNTGrFTlTFPisttMltnDGsdihsavLstiRKleDGYFgGARvk higvLg7sKlyvIRngifTFTFQFtDQhhFYLALDMeMIVEMLRieLaYLotoKRMTGrFTlTFPithtMltnDGsdihsavLstiRKleDGYFgGARvq higvLg5sKfyeIRnhtyf779QflDQhhFYLALDMeMIVEMLRieLaYLssoREMTgrPTlTFPvtrsMLveDGdavdpoiLstlRKlgDGYFaGARvq human PHKA2 600 rabbit PHKA2 600 600 Fuqu PHRA2 **V**¹⁹ tgkl&sfl#T%rcThLsTmDpgpegkLysedyddny.dylesgnwmndYdatsharcg...DevarYldhLLahTaphpkLaPtsqkggldrfqaAvqt 695 tgkl&sfl#T%rcThLsTmDpgpegkLysedyddny.delesgdwmdgYnstatarcg...DevarYldhLLahTaphpkLaPtsqkgglnrfraAvqt 695 tgkLdefl#T%rcThLsFmDpgpegkLysedydedyeddldsgnwmdsYdstsnarcg...DevarYldrLLahTvphpkLaPtsrkggldrfraAvqt 696 human PHKA1 rabbit PHKA1 mouse PHKA1 human FHKA2 lgnLseFITTFryTyLtFlDpdcdekLfdnasegtfspds..dsdlvgYledtcng...esgDeldhYinhLbgsTslrsyLpPlckntedrhvfsAihs 695 lgmiferlerifyfyftflipdedekifddasegsfspds. dsdlggfleetynqvt.esqDeldkYinhilqsTyskchüpflekkmedhnvfskihs 697 maddstrggTffhfrisglDeehddalleddeegeeeeedkfeddynnigpegnaqveysgDkfdqYltqLibsTtqkchüpflag ...qhhvfsAcht 698 rabbit PHKA2 Fugu PHKA2 21eihlPrDgsGevDfksLVlQLKetsgLQeQADILYm 785 20 TcDlmSlvtkakelihvqnvhKylF.TklfqasRpöfNilDephprqenqvpsvrv.... human PHEA1 ToplmSivtkakeLhvqnvhsylf, TkifqasRpSiNLbsshpaqedqvptvrv....evhlFrhqsGevDfqafVlQTKetasLogQADILIM 785 ToplmSivakakeLhiquvhsylf, TkifqosRpSiNLbspespqdsqvpsvhv.....evhlFrbqsGevDfqaLVsQLKetasLogQADILIM 786 rabbit PHKAl mouse PHKAl human PHRA2 782 rabbit PHRA2 792 Fugu PHKA2 TrbilgfmaqvqqunvpksskylgvplkskhkrglikilDvphpqhgphlkqnkvgtfnsvlaadlhlFrDsqdktDfatfivkgrkacpulgdQADThTi 798 7 22 Z 23 Z 24 polynteżyner. satyreżi tekingkygei rhyginki i sięktych i sięktych i sałyreż tych i dz. 803 **W** 22 human PEKA1 Tartmi LyumKgodWniceLyner..salVreLinBirgkvGsichWEITRIISELINKVXalMACUDLEShqhLirvGFPEREktissEhryGzLiuleEA 683 LyumKgodWnieLyneg..salVreLinBirgkvGsichWEITRIISELINKVXalMACUDLEShqhLirvGEPEPEREtissEhryGzBaliriEEA 883 LyumKgodWnieLyneg..gatVreLisBirkvKdsichWEITRIISELINKVKalMACUDLEShqhLirvGEPEPEREtissEhryGzBaliriEEA 884 LyuKgodWnieLis.gohgvVynLiggUrgkeGingeWEITRIISELINKVKVaLMACUDLEShqhLirvGEPEPEREtissEhryFA 880 LyuKgodWnieLis.gohgvVynLiggUrgkeGingeWEITRIISELINKVKVaLMACUDLEShqhLirvGEPEPEREtissEhryFA 881 LntKGadWlveLsgpggggvSyhtLieEtigaGackeWEITRIISELENKVKVaLMACUDLEShqhCUTVGEPEPEREtissEhryFA 888 rabbit PHKA1 mouse PRKA1 human PHRA2 rabbit PHKAZ Fugu PHKA2 25 secimsisiliysinvilanynsköpölfsanynkleigiliynatelanslycsaskateginnlefsankallehilsekefövkhövretden v.sp. 982 human PHKA1 cegDmisil705TmVYLANTmRt0P0LFacMfRIRIGLI10VMATELANSL=CSaERAEBGLMALSPsaMKNLLHRILSGKEFGVERGVRFLdSnv.SP aegDmsistL705TmVTLNYmRt0p0LfacMfRLBT0LI10VMATELANSL=CSaERAE0LMLSPsaMKNLLHHILSGKEFGVERGVRFLASnv.SD rabbit PHKAl 982 mouse PHKA1 SCODISISVITOEIVYILMEVRAOPSIEVOMIRLEIGITIOVMATELAISLOCSCEERSESIMOLSPECHNNLLHEILSGKEFGVERSVRPIDSstate human PHKA2 sogðisiaviðget vytlant vægðelt vaniriþfellig vartelarsin og seða seina leðr darnilent ledner gynnampi heges se Sogðis varifget þytlant i sogrelfgani Rikrigi megvartelarsin seins seins leftarnilent legnetgynnampi og sets p rabbit PHRA2 Fugu PHKA2 human PHKA1 rabbit PHKA1 mouse PHKA1 human PHEA2 rabbit PHKA2 Fugu PHKA2 ø ē **V** 29 VVPOPFQLVMKvlovCkGLavesCVLPSSTTERMTpgIKTeVhVESVLN=VpOPFXQLIVEs1ULtz1aDieihSIGeIIaVekIvhiAndLFLge 1165 RVFLGFTQLVMKvLoVCkGLavesCVLPSSTTERMTpgIKTeVhVESVLN=VpOPFXQLIVEs1ULtz1aDieihSIGeIIaVekIvhiAndLFLge 1175 RVFLGFTQLVMKvLoVCGCkGLavesCVLPSSTTERMTpgETKFeVhVESVLN=VpOPFXQLIVEs1ULtz1aDieihSIGeIIaVekIvhiAndLFLge 1178 RVFvGFTGrVMKiloVCGSLSidGyVLFSSTTERMTpgETKFeVhVESVLN=VpOPFXQLIVEs1ULtz1aDieihSIGeIIaVekIvhiAndLFLge 1178 RVFvGFTGrVMKiloVCGSLSidGyVLFSSTTERMTpgETKFeVhVESVLN=VpOPFXQLIVEs1UVLt1aDieihSIGeIIAVekIvhiAndLFLge 1178 RVFvGFTGrVMKiloVCGSLSidGyVLFSSTTERMTpgETKFeVhVESVLN=VsOPFXQLIVEsimVL11sDiemsSIGeIINVdIvmAndLFLge 1178 RVFvGFTGrVMKiloVCGSLSidGyVLFSSTTERMTpgETKFeVhVESVLN=VsOPFXQLIVEsimVL11sDiemsSIGeIINVdIvmAndLFLge 1178 human PEKAl rabbit PRKAl mouse PHKA1 human PHKA2 rabbit PHKA2 Fugu PHKA2 human DHKA1 OktloaddhtmlaKDnasGICtllWDSEPSGrfGTMTTLekastuvOefLPhSiCamD 1223 rabbit FHKA1 QktlqAdDimLaNDpasGICtllYDSAFSGrfGTMTYLskAaatyvQefLFhGiCamQ 1237 mouse PHKA1 OttlgAdDtmLaXDpas9ICtllIDSAFSGrfGTMTTLskAaatyvQefLPhSlCamQ 1241 Qvsigåmb.tLokDqatGTChffYDSAPSGayGTNTYLtrÄvasylQellPnSgCqmQ Qistgåmb.tLokDqatGTChffYDSAPSGayGTNTYLtrÄvashlQollPsSgCqtQ 1235 human DUKA? rabbit PHKA2 1235

Figure 6 (See facing page for legend.)

1229

QksyshqDyfLeEDpetGICnffIDSAF5GiyGTMTYLskAavtyiQdfLFsSsCimQ

Fucu PHKA2

ring canal actin filaments through the repeat motif and might cross-link the actin filaments by dimerization through the BTB domain. *Caenorhabditis elegans* genome sequencing revealed at least six hypothetical Kelch proteins. One of these, Spe26, interacts with actin through the repeat motif and is required for a normal actin cytoskeleton during spermatogenesis (Varkey et al. 1995). At present, we cannot make any reliable prediction about the function(s) of the two *Fugu* Kelch proteins. However, our data suggest that the *Fugu* genes represent paralogs that may have originated from a common ancestor and evolved separately after duplication.

Until recently, only one AP19 locus had been reported in humans. Our EST database search identified three distinct groups of homologous transcripts and subsequent fluorescence in situ hybridization with one cDNA highlighted at the known locus on 17q25 and an additional locus on Xp22 (not shown). The presence of an AP19 locus on Xp22 has now been confirmed by the recently published genomic sequence AC004106, in which it is annotated. The AP19 protein is the smallest component of AP-1, the clathrin-associated protein complex found in clathrin-coated vesicles of the Golgi apparatus (Kirchhausen et al. 1991). Disruption of this gene in yeast elicits no detectable mutant phenotype. Fugu AP19 polypeptide shares 96% identity with the human protein. This high conservation suggests that the structure and function of these proteins is under stringent selective pressure. Hence, we consider Fugu AP19 as the true ortholog. Sequence comparison of mouse and Fugu AP19 revealed 86% identity. However, as the mouse gene has not been mapped yet, it is presently unknown whether they are true homologs or only members of the same family.

Taking into account that intergenic regions are compressed in *Fugu*, identification of regulatory elements should be much easier in this species than in mammals. Because sequence comparisons did not identify significantly matching regions, we applied pattern searching and promoter prediction programs (like TESS, PROMOTORSCAN). Depending on the programs used, several possible regulatory elements could be identified, but results were contradictory. In addition, we obtained a long list of putative transcription factor binding sites, the status of which is uncertain because most of the programs available use pattern information derived from mammalian transcription factor binding sites and their degree of conservation in *Fugu* is not yet known. Functional studies with *Fugu* clones that harbor intergenic regions may be a more direct approach toward identifying regulatory elements in the *Fugu* genome.

In summary, we have identified one of the largest regions of conserved gene order between *Fugu* and humans known to date. Given the much smaller size of *Fugu* genes and their generally conserved structure, for which this study provides further evidence, the pufferfish is an excellent model organism to identify and characterize new genes and to predict their order in the human genome. Moreover, the small intergenic and intronic distances should greatly facilitate the detection of regulatory elements once improved sequence recognition software is available or, alternatively, through functional studies involving gene transfer experiments.

METHODS

Probe Generation

Total human liver RNA was reverse transcribed exactly as described previously (Kalscheuer et al. 1993). Amplification of three partially overlapping PHKA2 cDNAs (position 381-1547, 1440-2580, and 2487-3746 bp of accession no. D38616) was carried out with primers Le α 12 + Le α 13 and Le α 15 + Le α 27 with a final MgCl₂ concentration of 1.5 mM and Le $\alpha 6$ + Le $\alpha 4^*$ with a final MgCl₂ concentration of 3 mM (Burwinkel et al. 1996). Initial denaturation was for 3 min at 96°C, followed by 45 cycles each consisting of 94°C for 1 min, 58°C (Le α 6 + Le α 4*) or 56°C (Le α 12 + Le α 13 and Le α 15 + Le α 27) for 2 min and 72°C for 3 min, and a final extension step of 72°C for 7 min. Total human brain RNA was reverse transcribed to generate a 355-bp RT-PCR product of the PPEF-1 cDNA. Primers 41 (5'-GCAGCAATCGAGGAGCT-TAC-3') and 42 (5'-AATGCGGATAATTCTG-GAAGC-3') were used for amplification by PCR in the presence of 3 mM MgCl₂. The amplification conditions were as described above except for the annealing temperature, which was at 60°C.

A 675-bp probe encompassing the coding region of the human *XLRS1* gene was generated by amplifying ~10⁶ PFU of a retina cDNA library cloned in λ gt10 (J. Nathans, Johns Hopkins University, Baltimore, MD) with primers 206 (5'-ATGTCACGCAAGATAGAAGGC-3') and 207 (5'-TCAGG-CACAGTTGCTGACG-3') with a final MgCl₂ concentration of 0.5 mM. Initial denaturation was at 94°C for 10 min, followed by 40 cycles of 94°C for 1 min, 58°C for 1 min, and 72°C.

To generate hybridization probes, PCR fragments were separated by agarose gel electrophoresis. The respective bands were cut out off the gel and the DNA was isolated by centrifu-

Figure 6 Alignment of *Fugu*, rabbit, and human PHKA2 and mouse, rabbit, and human PHKA1 proteins. The alignment was performed with the GCG programs PILEUP and PRETTY. Residues identical at each position are shown in uppercase letters and are shaded. (∇) Intron/exon boundaries of *Fugu PHKA2*. *Fugu* exons 18–29 are numbered. (∇) Known positions of splice sites in the human PHKA2 gene. The PHKA muscle and liver isoforms have two subunit-specific domains (boxed) with comparatively low amino acid sequence similarities of <50%. These domains encompass amino acids 612–788 and amino acids 1027–1055 in *Fugu* with a negatively charged cluster spanning amino acids 620–645. The hypothetical 5' and 3' calmodulin binding sites of all Phk α subunits are highly conserved (boxed in bold) and encompass amino acids 828–855 and amino acids 1056–1087 in *Fugu*. Three autophosphorylation sites, serine 987, serine 1001, and serine 1022, labeled by an P underneath the alignment and their vicinity are conserved.

gal filtration with either an Ultrafree-MC 0.45 µm column (Millipore) or by the Gene Clean Kit (BIO 101), exactly following the suppliers instruction. Labeling was performed with random hexamer priming in the presence of $[\alpha$ -³²P]dCTP.

Total mouse eye RNA was reverse transcribed with random hexamers and amplified with a primer set that spans exons 4-6 of the human XLRS1 gene (209 5'-CAGAATGCCCATATCACAAGCCTC-3' and 210 5'-GCTCC-ATCCGGATGGCAATGCG-3') under the following conditions: initial denaturation for 10 min at 95°C, 40 cycles including 1 min at 94°C, 1 min at 65°C, and 3 min at 72°C and a final extension step of 7 min at 72°C. The resulting product of 465 bp was ligated into the pUAG-Vector (Ingenius) and subsequently sequenced. To complete the mouse cDNA, 5'and 3' RACE were performed on cDNA synthesized by SMART PCR (Clontech) starting with 0.75 µg of total eye RNA. Both amplifications were carried out with primers 210 and 209, respectively, in combination with one adapter primer complementary to part of the SMART linkers. Subsequently, 20 ng of the SMART PCR cDNA were used as template for the RACE experiments in a sample volume of 50 µl containing 50 pmoles of primer 210 or 209 and 10 pmoles of the nested SMART primer. Cycling conditions were as above, except for the decreased annealing temperature of 60°C.

Isolation of Fugu Cosmids

A gridded high-density *Fugu* genomic Lawrist 4 cosmid library (36,864 clones equivalent to 3.7 haploid *Fugu* genomes) was screened with the three human *PHKA2* cDNAs. Filters were hybridized in 0.5 M Na₂PO₄, 7% SDS, 1 mM EDTA at 55°C. Washing was in $2 \times SSC/0.1\%$ SDS for 2×10 min and in $1 \times SSC/0.1\%$ SDS for 1×30 min at 55°C. Exposure was for 6 hr at -70° C. Candidate positive clones exhibiting duplicate hybridization signals were isolated, miniprepared, and digested with *Eco*RI. DNA fragments were separated in an 0.8% agarose gel. Following Southern transfer of cosmid DNA onto Genescreen Plus (NEN), blots were probed as above.

Cosmid Walking

Both end fragments of *Fugu* cosmid ICRFc66A2095Q1.2 were generated by PCR on 80 ng of *Eco*RI-digested cosmid DNA with sequence specific primers E1f (5'-ATGAAGAGCTG-GACTCTTGTG-3') and E1r (5'-TCTCATCGGCGTCGGAGTG-3') amplifying 719 bp and with primers E2f (5'-CTAGT-AGACAGGTTATTGGAC-3') and E2r (5'-ATGAGTAGATA-CAAGAGCAGG-3') amplifying 602 bp. PCRs were performed in the presence of 1.5 mM MgCl₂ with an initial denaturation at 96°C for 3 min, followed by 45 cycles at 94°C for 1 min, 58°C for 2 min, and 72°C for 3 min in case of E1 and 35 cycles with annealing at 56°C for 1 min in case of E2.

Direct sequencing of *Fugu* cosmid ICRFc66L2390Q1.2 with primer Lawrist 4 forward (5'-CGCCTCGAGGTGGCT-TATC-3') enabled us to determine sequence-specific primers 147 (5'-TCGAAACCGAGAGGCCTGTG-3') and 147' (5'-ACCCTGTGATGATGACTGAGG-3') that were used to amplify an end fragment of 758 bp in the presence of 1.5 mM MgCl₂. The starting template was 20 ng of *Eco*RI-digested cosmid DNA, and the PCR cycles consisted of an initial denaturation step of 96°C for 3 min, 35 cycles of 94°C for 1 min, 60°C for 1 min, and 72°C for 1 min, and a final extension step of 72°C for 7 min. The 758-bp long PCR product was used for screening a second *Fugu* genomic Lawrist 4 cosmid library (G. Elgar). Hybridization was performed in PEG buffer (125 mM Na₂PO₄, 250 mm NaCl, 1 mm EDTA, 10% PEG 6000, 7% SDS) at 65°C. Filters were washed in $2 \times$ SSC/0.1% SDS for 10 min and in $2 \times$ SSC/0.1% SDS for 25 min at hybridization temperature. Exposure was for 4 hr at -70° C.

FISH

Human AP19 cDNA (accession no. AA262073) was labeled by nick translation with biotin-16–dUTP. A total of 40 ng of this probe was then coprecipitated with human Cot-1 competitor DNA and herring sperm DNA, followed by resuspension in 50% formamide, 10% dextran sulfate, $2 \times$ SSC. Denaturation for 10 min at 80°C preceeded 1 hr of preannealing. Slides were treated with 100 µg/ml RNase A and 0.01% pepsin, then dehydrated through an ethanol series. Denaturation with 70% formamide/ $2 \times$ SSC at 80°C was followed by dehydration in cold ethanol. The probe was then applied to the slides and allowed to hybridize for 72 hr at 37°C. Slides were washed three times in 50% formamide/ $2 \times$ SSC, once in $2 \times$ SSC, and finally in 0.2 \times SSC at 42°C with little agitation. Detection was performed with fluorescein isothiocyanate (FITC)-conjugated avidin and counterstaining with DAPI.

DNA Sequencing Strategy

Fugu cosmids ICRFc66A2095Q1.2, ICRFc66L2390Q1.2, and F089J4 were digested with *Eco*RI, *Hind*III, and *PstI*, respectively. The resulting fragments were randomly subcloned into appropriately cut and dephosphorylated pBluescript II KS(+) (Stratagene) or pT7T3 18U (Pharmacia) vector. DNA sequences were determined by dideoxy chain termination with the thermo Sequenase fluorescent-labeled primer cycle sequencing kit containing 7-deaza-dGTP (Amersham), primers complementary to the vector arms (5' labeled with IRD700 and IRD800, respectively) on an automated gel reader (LICOR 4000L and LONG READIR 4200, MWG). Gaps were closed by a combination of primer walking and deletion cloning of larger subclones. All sequences were processed and assembled with the Staden package.

Sequence Analysis

Potential coding regions were identified with the exon prediction programs FGENES, GENSCAN, and GRAIL 2. The putative genes were searched for homologies by protein (SWIS-SPROT, PIR) and nucleotide databases (GenBank, EMBL), with the BLAST program. Database entries with a score $>10^{-10}$ were selected and grouped into distinct sets by the criteria homology and position. The putative intron/exon organization of each of the Fugu gene homologs was deduced either from the structure of the corresponding human genes or from sequence comparison to homologs. This procedure was assisted by the PGSEARCH program (Birney et al. 1996). Predicted splice sites were compared with consensus 5' donor and 3' acceptor sequences of mammals (Shapiro and Senapathy 1987) to confirm the results. The sets of homologous proteins were aligned by the program PILEUP, with sequences gapped for optimization. Polyadenylation site predictions were performed by the programs FGENES, GENSCAN, and GRAIL 2 and extended by the program POLYAH.

Phylogenetic trees were generated from the aligned nucleotide sequences with the maximum likelihood method as implemented in the DNAML program, available in the PHYLIP (Felsenstein 1981) computer software package. Regions in which some sequences had alignment gaps or that had ambiguous alignments were excluded. Phylogenetic trees were generated with a different starting parameter. Bootstrap resampling was conducted by the method of Felsenstein (1985). One-hundred bootstrap replicates for the DNAML method were conducted.

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