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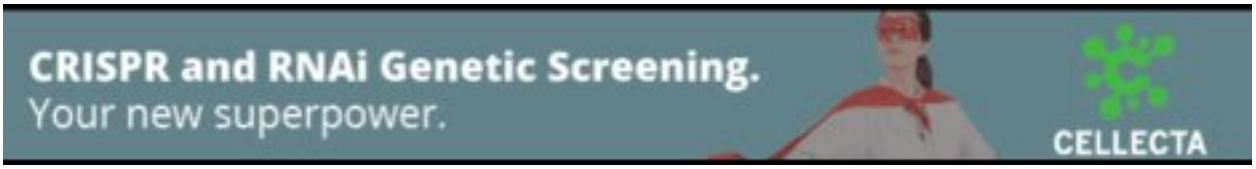

RESEARCH

Conserved Linkage between the Puffer Fish (*Fugu rubripes*) and Human Genes for Platelet-derived Growth Factor Receptor and Macrophage Colony-stimulating Factor Receptor

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We have cloned and sequenced the teleost homologs of the human genes encoding platelet-derived growth factor receptor-β (PDGFRβ) and macrophage colony-stimulating factor 1 receptor (CSF1R) from the puffer fish *Fugu rubripes*. The *Fugu* PDGFRβ and CSF1R genes each consist of 21 coding exons similar to the human CSF1R gene, but are considerably smaller than their human counterparts because of the smaller introns. Furthermore, the two *Fugu* genes are linked tandemly in a head-to-tail array similar to their human homologs with 2.2 kb of intergenic sequence. Amino acid sequences of the *Fugu* and human PDGFRβ and CSF1R genes show an overall homology of 45% and 39%, respectively, with the kinase domains showing a much higher degree of conservation. Dot-matrix analysis revealed several short stretches of conserved sequences in the 3′ untranslated regions of the PDGFRβ genes and the adjacent promoter regions of the CSF1R genes. These conserved sequences may have a role in the regulation of expression of either or both of these closely linked genes.

[The sequence data described in this paper have been submitted to GenBank under accession no. U63926.]

The Japanese puffer fish *Fugu rubripes* has a small genome of 390 Mb which is ~8 times smaller than the mammalian genomes (Brenner et al. 1993). Because teleosts have a body plan similar to that of mammals and possess most of their complex physiological functions, it is not surprising that the *Fugu* genome has a gene repertoire comparable to that of mammals (Brenner et al. 1993). This implies that the gene density in the *Fugu* genome is higher than in mammalian genomes. The compact genome of *Fugu* with small introns and a low abundance of repetitive elements (Brenner et al. 1993; Baxendale et al. 1995; Elgar et al. 1995; Mason et al. 1995; Brenner and Corrochano 1996; Maheshwar et al. 1996; Venkatesh et al. 1996) is an efficient model vertebrate genome for comparative genome analysis. Although teleosts diverged ~400 million years ago from the lineage that gave rise to mammals, the gene order on short stretches of chromosomes might be conserved between fishes and mammals. Conserved linkage of genes between the compact genome of *Fugu* and the expanded human genome would be an advantage in using *Fugu* genomic sequence for positional cloning of human genes. To assess the extent of conservation of linkage between the *Fugu* and human genes, we have been sequencing and analyzing the order of *Fugu* homologs of human genes that are known to be linked closely in the human genome.

Receptors for the human platelet-derived growth factor-β (PDGFRβ) and macrophage colony-stimulating factor 1 (CSF1R) belong to the same subfamily of receptor tyrosine kinases (Yarden and Ullrich 1988). Members of this family have an extracellular ligand binding domain, a single transmembrane domain, and an intracellular tyrosine kinase domain that is interrupted

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by a hydrophilic spacer consisting of 60–140 amino acids known as the kinase-insert (KI) domain. Among all the known kinase receptors, PDGFRβ and CSF1R most closely resemble each other in their amino-acid sequences. Human genes for these receptors have been localized to the long arm of human chromosome 5 and are found to be linked physically in a head-to-tail array, with <500 bp between the polyadenylation signal of the PDGFRβ gene and the transcription start point of the CSF1R gene (Roberts et al. 1988). Mouse homologs of these genes are also linked in a similar way (Eccles 1991). The human CSF1R gene comprises 21 coding exons spread across 33 kb and a short 5′ untranslated exon separated from the first coding exon by a 26-kb intron, and codes for a protein of 972 amino acids (Hampe et al. 1989). The human PDGFRβ gene codes for a 1106-amino-acid protein (Claesson-Welsh et al. 1988). Although this gene is estimated to span ~55 kb of genomic DNA, it has been sequenced only at the cDNA level. The human PDGFRβ and CSF1R genes show a nonoverlapping pattern of expression in different cell types. PDGFRβ is expressed mainly in mesenchymal cells, whereas CSF1R is expressed in blood cells of mononuclear phagocyte lineage and in placental trophoblasts. The promoter sequences that regulate the cell-type-specific pattern of expression of these genes are yet to be identified. In view of the close proximity of the two genes, it has been proposed that the promoter sequence of the CSF1R gene may be located within the genomic sequence of the PDGFRβ gene (Roberts et al. 1988). We have now determined the nucleotide sequences of the *Fugu* homologs of the human PDGFRβ and CSF1R genes and demonstrate that the two genes are linked closely in the *Fugu* genome similar to their human counterparts.

RESULTS AND DISCUSSION

Seven positive recombinant phages that hybridized to the *Fugu* PDGFRβ probe were digested with *Bam*HI, *Eco*RI, *Hin*dIII, *Sac*I, *Xba*I, or *Xho*I, Southern blotted, and reprobed with the *Fugu* PDGFRβ probe. One of the phages, λ6, which hybridized strongly to the probe, was selected for subcloning and sequencing. The complete nucleotide sequence of the insert was determined and assembled to generate a 17-kb contig. A homology search of the contig showed that it contained all the exons of the PDGFRβ and CSF1R genes, except the first three and last two coding exons of the PDGFRβ and CSF1R genes, respectively (Fig. 1). Using the end clones of λ6 as probes, two more phages, λ5 and λ9, which overlapped with the 5′ end and 3′ end of λ6, respectively, were isolated from the *Fugu* genomic library. The nucleotide sequences of the remaining exons of the PDGFRβ and CSF1R genes, and the 3′ untranslated region of CSF1R, were obtained from the subclones of these two phages. A contiguous sequence of 23.2 kb (GenBank accession no. U63926) that includes all the coding exons of the PDGFRβ and CSF1R genes was assembled from these sequences (Fig. 1). The sequences for the PDGFRβ and CSF1R genes are located on the same strand of DNA and are linked tandemly in a head-to-tail array similar to their human homologs (Roberts et al. 1988). The putative polyadenylation signals of the *Fugu* PDGFRβ (AATAAA) and CSF1R (ATTAAA) genes are located 148 and 1300 bases from their respective termination codons. The first codon of the CSF1R gene is separated from the polyadenylation signal of the PDGFRβ gene by 2.2 kb. This distance is ~27 kb in man, but includes a large intron of 26 kb in the 5′ untranslated region of CSF1R gene.

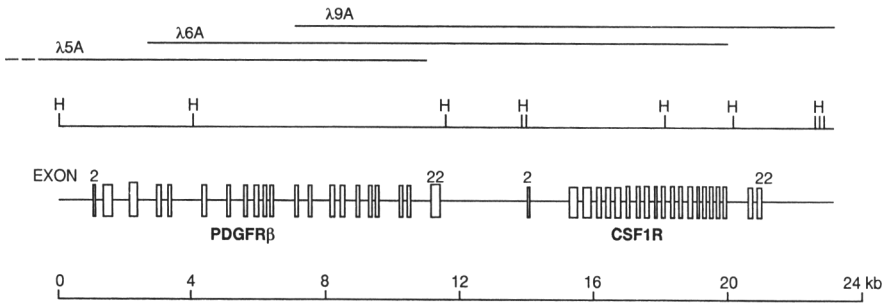

Figure 1 Genomic organization of the *Fugu* PDGFRβ and CSF1R genes. The complete sequence of the coding exons (corresponding to exons 2 to 22 of the human homologs) of the two genes were obtained from three overlapping λ clones (λ5, λ6, and λ9) isolated from a *Fugu* testis genomic library. Sequences of the untranslated exon 1 were not demarcated as the transcription initiation sites were not mapped in this study. Restriction sites of only *Hin*dIII are shown.

The genomic structures of the *Fugu* PDGFRβ and CSF1R genes were deduced by comparison with the amino acid sequences of their

mammalian homologs, the exon–intron structure of the human CSF1R gene, and consensus splice junction sequences. Both *Fugu* genes consist of 21 coding exons each and therefore have a genomic structure similar to that of the human CSF1R gene. In addition to coding exons, the human CSF1R gene has a 5′ untranslated exon which is separated from the first coding exon by a 26-kb intron (Hampe et al. 1989). Because the transcription initiation sites of the *Fugu* genes were not mapped in this study, it is not known whether the *Fugu* genes have the additional untranslated exon. However, it is reasonable to assume that the transcription initiation site of the *Fugu* CSF1R gene lies within the 2.2-kb intergenic region, and if there is any intron in the 5′ untranslated region, it would be <2.2 kb. Although the *Fugu* genes have a genomic complexity similar to that of their human homologs, the overall sizes of *Fugu* genes are smaller than the human genes. The size of the *Fugu* CSF1R gene is <10.6 kb from the translation initiation site to the polyadenylation signal as opposed to 59 kb for the human CSF1R gene (Hampe et al. 1989). Likewise, the coding exons of the *Fugu* PDGFRβ gene span 10.5 kb (from first codon to polyadenylation signal) compared with the estimated size of 29 kb for the human PDGFRβ gene (Roberts et al. 1988). Therefore the *Fugu* genes are considerably smaller than the human genes, and the decrease in size is attributable to their smaller introns. The introns of the *Fugu* PDGFRβ gene range in size from 96 to 959 bp and those of the CSF1R gene range from 60 to 1194 bp (Table 1). The majority of the *Fugu* CSF1R introns are much smaller than their human counterparts with the maximum compression occurring in the first intronic sequence (Table 1). This intron is ~26 kb in the human gene and <2.3 kb in the *Fugu* gene. We now have several examples of "compact" *Fugu* genes, such as the genes for Huntington's disease (Baxendale et al. 1995), glucose-6-phosphate dehydrogenase (Mason et al. 1995), tuberous sclerosis 2 (Maheshwar et al. 1996), and histidyl-tRNA-synthetase (Brenner and Corrochano 1996), which are 2.5–8 times smaller than their mammalian homologs. In all of these *Fugu* genes, the compaction has occurred mainly in the intronic sequences, with dramatic reductions observed in unusually large introns of mammalian genes, such as the first intron of the human CSF1R gene and the second intron of the Huntington's disease gene (12.2 kb reduced to 137 bp in the *Fugu*). Therefore, it is not surprising that human genes that lack "large" introns have undergone little or no reduction in size in the *Fugu*. For instance, the genes for β-cytoplasmic actin, α-cardiac actin (Venkatesh et al. 1996), and growth hormone (B. Venkatesh and S. Brenner, unpubl.) are of similar sizes in *Fugu* and humans.

Table 1. Intron Sizes (bp) of the *Fugu* PDGFRβ and CSF1R Genes

Intron no.	PDGFRβ	CSF1R (Human)
1	–	<2200 (26000)
2	313	1194 (5349)
3	582	103 (430)
4	428	73 (1803)
5	225	79 (676)
6	959	83 (3780)
7	407	253 (2730)
8	420	319 (153)
9	114	93 (118)
10	142	215 (1542)
11	102	92 (6395)
12	641	140 (127)
13	263	153 (517)
14	550	133 (999)
15	100	90 (2107)
16	393	79 (119)
17	193	80 (945)
18	96	82 (81)
19	593	60 (690)
20	104	704 (806)
21	548	100 (97)

Intron 1 found in the 5′ untranslated region of the human CSF1R gene was not mapped in the *Fugu*. Sizes of the human CSF1R introns (Hampe et al. 1989) are given in parentheses. Introns of the human PDGFRβ gene have not yet been sequenced.

The coding sequences of the *Fugu* PDGFRβ and CSF1R genes predict protein sequences with 1048 and 975 residues, respectively (Figs. 2 and 3). The two proteins exhibit similar primary structures comprising an extracellular ligand-binding domain and an intracellular kinase domain separated by a single transmembrane domain rich in hydrophobic amino acid residues. The extracellular ligand-binding domains contain the characteristic pattern of 10 cysteine residues that is found in the extracellular domains of the immunoglobulins. As with their mammalian homologs, the kinase domains of the *Fugu* PDGFRβ and CSF1R sequences are interrupted by a kinase insert domain comprising 103 and 70 amino acids, respectively (Figs. 2 and 3). The two *Fugu* protein sequences exhibit an overall similarity of 32%, with the first half of the kinase do-

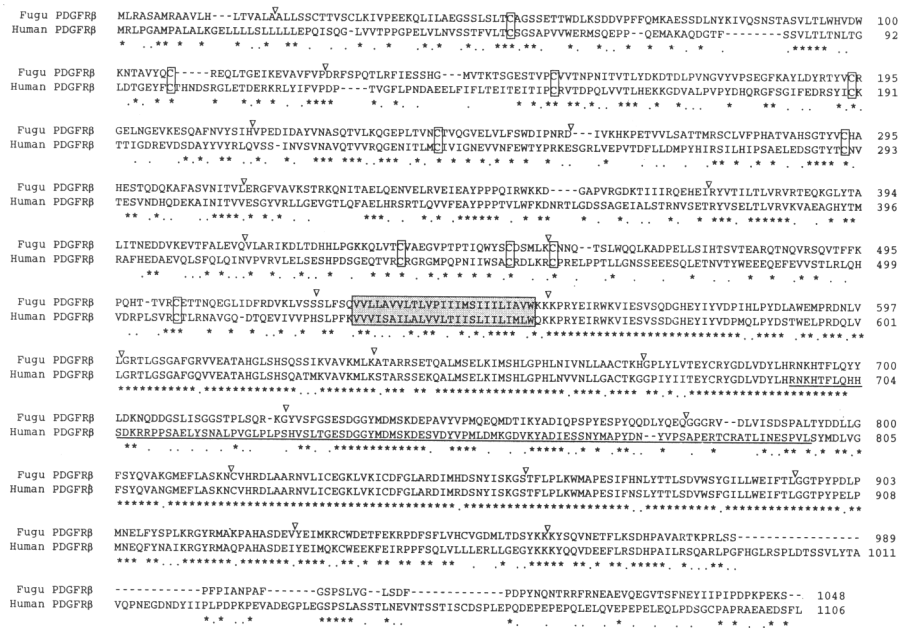

Figure 2 Clustal W alignment of the deduced amino acid sequences of *Fugu* and human (Claesson-Welsh et al. 1988) PDGFRβ genes. Asterisks indicate identical residues and periods indicate conservative substitutions. The conserved cysteine residues in the extracellular ligand-binding domains are boxed. Transmembrane domain is boxed and shaded. Kinase-insert domain that splits the intracellular kinase domain is underlined. Arrowheads indicate intron positions in the *Fugu* gene.

main showing a much higher degree of similarity of 63%.

A comparison of the deduced amino acid sequences of the *Fugu* PDGFRβ and CSF1R genes with their human counterparts (Figs. 2 and 3) shows that most of the significant residues, particularly in the catalytic domain, are conserved. The amino-acid sequences of the *Fugu* PDGFRβ and CSF1R show an overall homology of 45% and 39%, respectively, with their human homologs. The homology is particularly high in the kinase domains that have a crucial role in intracellular signal transduction through kinase activity. The glycine rich motif Gly–X–Gly–X–X–Gly (residues 603–608 in PDGFRβ and residues 591–596 in CSF1R) associated with nucleotide binding, and the predicted ATP-binding site (Lys 630 in PDGFRβ and Lys 619 in CSF1R) are conserved in both *Fugu* genes. The consensus tyrosine residue present in the catalytic domain of all tyrosine kinases is located at residue 852 in the *Fugu* PDGFRβ (Tyr-857 in human) and at residue 813 in the *Fugu* CSF1R (Tyr-809 in human). Although the kinase-insert domains of both *Fugu* receptors exhibit a lower degree of homology compared with the flanking split-kinase domains, most of the tyrosine residues of this domain that are implicated in autophosphorylation and binding of the receptors to cellular substrates in mammals are conserved in *Fugu*. In the murine CSF1R receptor, three autophosphorylation sites, Tyr-697, Tyr-706, and Tyr-721, corresponding to the *Fugu* CSF1R Tyr-700, Tyr-709, and Tyr-724, have been identified in the kinase insert domain and mutation of either Tyr-697 or Tyr-721 has been shown to abrogate signal transduction when expressed in Rat-2 fibroblasts (van der Geer and Hunter 1993). Tyr-721 is crucial for the binding of the receptor to PI 3′-kinase in mammalian cells (Reedijk et al. 1992) and Tyr-697 in murine CSF1R is the binding site for GRB2 (van der Geer and Hunter 1993). Comparison of protein sequences from distantly related species such as teleosts and mammals provides useful clues to identify important functional domains of the proteins. Protein sequence comparisons of *Fugu* and human PDGFRβ and CSF1R have revealed several conserved residues in the extracellular and intracelluar domains whose functions are not known at present. Functional analysis of these residues may throw light on the molecular mechanisms of signal transduction by these tyrosine kinase receptors.

Two separate transcription initiation sites have been reported for the human CSF1R gene transcripts expressed in monocytes and placental cells (Visader and Verma 1989). The transcripts in the monocytes originate within the 26-kb intron in the 5′ untranslated region, whereas those in the placental cells initiate upstream of the noncoding exon located 5′ to the 26-kb intron. Transfection studies in human cell lines and mouse fibroblasts have shown that the promoter elements driving the cell-type specific transcription are located within 1 kb upstream of the independent transcription start sites (Roberts et al.

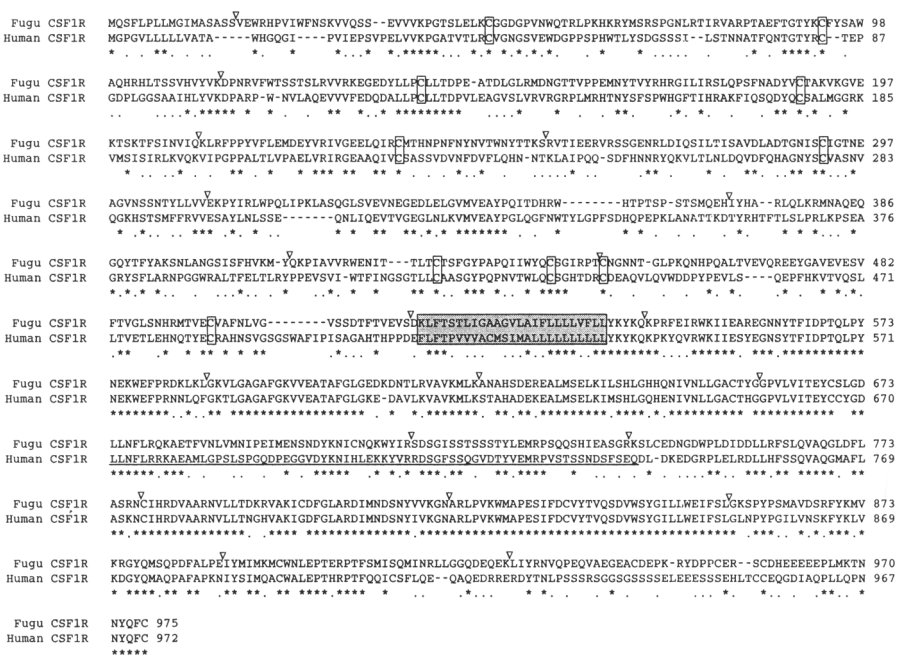

Figure 3 Clustal W alignment of the deduced amino acid sequences of *Fugu* and human (Coussens et al. 1986) CSF1R genes. Asterisks indicate identical residues and periods indicate conservative substitutions. The conserved cysteine residues in the extracellular ligand-binding domains are boxed. Transmembrane domain is boxed and shaded. Kinase-insert domain that splits the intracellular kinase domain is underlined. Arrowheads indicate intron positions in the *Fugu* gene.

1992). Presently only the nucleotide sequence of the CSF1R promoter region upstream of the placental cell transcription initiation site is available and the complete sequence of the 26-kb intron in the 5′ untranslated region is yet to be determined. To identify conserved regulatory elements in the placental cell-promoter region, we compared the promoter sequence of CSF1R (462 bp from the polyadenylation signal of the PDGFRβ gene to the transcription initiation site of CSF1R) with the corresponding sequence from the *Fugu* PDGFRβ–CSF1R loci (500 bp starting from the polyadenylation site of the PDGFRβ gene). The pairwise comparison made by DotPlot analysis in DNASTAR revealed that three stretches of short nucleotide sequences, TGKGTTCCGGC, CTCCACWGA and ATCAKCTGGGACACC (K = T in *Fugu* and G in human; W = T in *Fugu* and A in human) are conserved in the same order. A nine-nucleotide sequence, CAGATTCCA, is found in both *Fugu* and human loci at 88 and 438 bases downstream of the polyadenylation signals of the respective PDGFRβ genes. It is possible that these sequences have some functional significance. A comparison of the 3′ untranslated regions of the *Fugu* and human PDGFRβ genes, which are adjacent to the CSF1R promoter region, showed that two short sequences, CTCTGAGCC and TTTATCAC, are conserved completely. The functional significance of these sequences is not known at present. In view of the close proximity of the PDGFRβ and CSF1R genes, a role for these conserved sequences in the regulation of either or both of these genes cannot be ruled out.

The presence of genes for the PDGF and CSF1 receptors in *Fugu* indicates that these kinase receptors and their ligands had evolved fully in the common ancestors of teleosts and mammals. The conserved genomic organization, similar primary structure of protein sequences, and the close physical linkage of the *Fugu* PDGFRβ and CSF1R genes strongly support the hypothesis that these two genes arose from a common ancestor through gene duplication. Mapping and sequence analysis of the corresponding locus from the most primitive vertebrates such as cyclostomes should help in tracing the evolutionary origin of these genes.

In this paper we have shown that the close physical linkage between the PDGFRβ and CSF1R genes has been conserved in *Fugu* and humans. Random sampling of *Fugu* genomic cosmid clones has shown that there are a number of other examples of conserved synteny between *Fugu* and humans (for review, see Elgar et al. 1996). The most striking of these is the relative order of three genes—cFOS, S31iii125, and S20i15—which are spread across >600 kb in the human familial Alzheimer disease locus (AD3). The order of these genes is conserved in *Fugu* and all three are located within a 12.4-kb region (Trower et al. 1996). These data show that the synteny has been conserved between *Fugu* and

humans on short stretches of chromosomes. Therefore *Fugu* is a model genome of choice for characterizing vertebrate genes rapidly with considerably lower sequencing effort compared with other vertebrate genomes.

METHODS

Isolation of Bacteriophage Clones

To isolate various receptor tyrosine kinase-genes from *Fugu*, we designed several pairs of degenerate PCR primers based on the consensus sequences flanking the catalytic domains of mammalian receptor tyrosine kinases. PCR was carried out in a 60-µl reaction mix containing 0.2 mM dNTP, 1 µM each of forward and reverse primers, 3 units of *Taq* polymerase and 60 ng of *Fugu* genomic DNA. DNA was initially denatured at 95°C for 2 min, then amplified in 35 cycles of 95°C for 30 sec, 50°C for 30 sec, and 72°C for 1 min, followed by a general extension at 72°C for 5 min, in a Perkin Elmer 480 DNA Thermal Cycler. PCR products were end-filled using Klenow Polymerase and subcloned into *Eco*RI-cut pBluescript plasmid and sequenced. The sequences were searched for homology using the BLASTN program against the protein data bases maintained at the National Center for Biotechnology Information (NCBI). One of the cloned PCR products, TM19, which was amplified using the primers TG(CT)AT(ACT)CA(CT)(AC)G(ACGT)GA(CT)GT and CC(AG)TA(AG)CT CCA(ACGT)AC(GA)TC corresponding to residues CIHRDV and DVWSYG of the mammalian kinases, showed very high homology with mammalian PDGFRβ. This clone was used to probe a *Fugu* testis genomic library cloned in λ2001. The probe was labeled by PCR using [α-^{32}P] in the reaction mix. Hybridizing plaques were purified to homogeneity by two more rounds of screening.

Nucleotide Sequence Analysis

Selected positive phages were subcloned and sequenced either after "exo-deletion" by using the Erase-a-Base system supplied by Promega (Madison, WI), or by a combination of "shot-gun" and primer walking methods. The shot-gun technique involved generating sheared random sublcones in the size range of 400 to 1 kb and sequencing up to a three-fold redundancy. The sequences were assembled into contigs by using DNASTAR. Gaps in the contigs were closed by directed sequencing using custom oligonucleotide primers. DNA sequencing was done by the radioactive dideoxy chain termination method (Sanger et al. 1977). Multiple sequence alignment was done by using Clustal W (Thompson et al. 1994).

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