

Complete sequencing of the *Fugu* WAGR region from WT1 to PAX6: Dramatic compaction and conservation of synteny with human chromosome 11p13

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ABSTRACT The pufferfish *Fugu rubripes* has a genome ≈ 7.5 times smaller than that of mammals but with a similar number of genes. Although conserved synteny has been demonstrated between pufferfish and mammals across some regions of the genome, there is some controversy as to what extent *Fugu* will be a useful model for the human genome, e.g., [Gilley, J., Armes, N. & Fried, M. (1997) *Nature (London)* 385, 305–306]. We report extensive conservation of synteny between a 1.5-Mb region of human chromosome 11 and <100 kb of the *Fugu* genome in three overlapping cosmids. Our findings support the idea that the majority of DNA in the region of human chromosome 11p13 is intergenic. Comparative analysis of three unrelated genes with quite different roles, *WT1*, *RCN1*, and *PAX6*, has revealed differences in their structural evolution. Whereas the human *WT1* gene can generate 16 protein isoforms via a combination of alternative splicing, RNA editing, and alternative start site usage, our data predict that *Fugu WT1* is capable of generating only two isoforms. This raises the question of the extent to which the evolution of *WT1* isoforms is related to the evolution of the mammalian genitourinary system. In addition, this region of the *Fugu* genome shows a much greater overall compaction than usual but with significant noncoding homology observed at the *PAX6* locus, implying that comparative genomics has identified regulatory elements associated with this gene.

The pufferfish *Fugu rubripes* has a compact genome of 400 Mb, some 7.5 times smaller than that of mammals but with a similar number of genes. This compaction is caused by intron sizes being smaller as well as much reduced intergenic distances, i.e., distances between transcription units. For this reason, *Fugu* has been proposed as a model organism to study genes both at the level of the individual gene and in a genomic context (1, 2). The *Fugu* genome exhibits homology with other species across critical regulatory segments of some genes (3), and it has been established that synteny is conserved across a number of small regions between pufferfish and mammals. To date, linkage of two or three genes has been demonstrated for certain synteny groups such as FOS/S31iii125/s20i15 (4), PDGFR β /CSF1R (5), MAP-2/MYL-1/CPSIII (6), and TSC2/PKD1/SSTR5 (7) although these examples consist of a limited number of genes covering relatively small distances in terms of human DNA. Additionally, these studies did not provide complete *Fugu* DNA sequence. It is becoming apparent that not all regions of the human genome are suitable for modeling in *Fugu*; for example, even the tightest mammalian gene cluster, that of the surfeit genes, does not exhibit complete conservation of

synteny between mammals and *Fugu* (8). With accelerating progress in the Human Genome Project, the biological importance of long range genomic organization is now beginning to emerge, and it is important that conservation of synteny, genomic organization, and noncoding sequence homology be identified across vertebrate species to explore genomic function.

We chose to investigate possible conservation of synteny between *Fugu* and the 11p13 region of the human genome for several reasons: (i) The human region contains two well studied disease genes, *PAX6* and *WT1*, each of which is essential for normal mammalian development. Their heterozygous deletion gives rise to WAGR syndrome - Wilms' tumor, Aniridia, Genitourinary abnormalities, and mental Retardation (9). *PAX6* and *WT1* are conserved through evolution, can generate several different protein isoforms, and have complex regulatory systems, which orchestrate tissue-specific and temporally regulated expression patterns. Certain forms of aniridia arise as a result of a translocation outside the *PAX6* gene presumably through a "position effect" (10) and it is thought that both *WT1* and *PAX6* use long-range genetic regulatory elements. Therefore, a study of this region should lead to a greater understanding of the structure and regulation of these complex, developmentally important human disease genes; (ii) There are several well characterized mouse mutants in this region, both at specific gene loci (11, 12) and deletions up to several megabases (13, 14), which will facilitate *in vivo* functional analyses by gene replacement/complementation studies in the mouse; (iii) There is extensive coverage of this region in both human and mouse with YAC/PAC contigs (15) although the associated gene maps are not complete. This will allow the degree of synteny to be accurately assessed while allowing the assembly of better gene maps across this region; (iv) Other human disease-associated loci, e.g., those associated with loss of heterozygosity in breast and bladder cancers (16), mapping to this region may be defined through homology studies. In addition, the cause(s) of mental retardation associated with WAGR deletions has yet to be determined. (v) Sequencing of 225 kb of the human *PAX6* region has been completed [http://www.sanger.ac.uk/cgi-bin/hummap?map = Chr.11 misc] and that of the *WT1* region is underway, allowing direct "base-by-base" comparisons to be made.

The *PAX6* and *WT1* genes are separated by approximately 750 kb in the human genome. Recently, a human homolog of reticulocalbin (*RCN1*) was identified at a CpG island site between *WT1* and *PAX6* (14), thereby providing another

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Abbreviations: WAGR syndrome, Wilms' tumor, Aniridia, Genitourinary abnormalities and mental Retardation; HGMP-RC, Human Genome Mapping Project Resource Centre.

Data deposition: The sequence reported in this paper has been deposited in the GenBank database (accession no. AL021531 for the sequence of cosmid 151-J19).

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reference point with which to compare *Fugu*. If this region shows synteny conservation and the expected level of compaction for the *Fugu* genome, a search for new genes and for long distance regulatory elements within 11p13 could be undertaken by using comparative genomics in addition to allowing detailed studies of the structure and regulation of individual genes.

METHODS

The *Tetraodon fluviatilis* probe was generated by degenerate PCR of 50 ng of genomic DNA, by using the following oligonucleotide primer pair (5'-3'): exon 8 (sense) CAG TGT GA(C/T) TT(C/T) A(A/C)(A/G) GAC TG and exon 10 (antisense) GT(T/C) TCT (C/T)TG (G/A)TG CAT (G/C)TT CTG.

The reaction profile of the PCR consisted of an initial 5-min denaturation at 94°C, followed by 30 cycles of 10 s at 94°C, 45 s at 50°C, 2 min at 72°C with a final extension step of 10 min at 72°C.

PCR products of ≈900 bp were subcloned and sequenced to verify clones corresponding to *WT1*. A gridded cosmid library of *Fugu rubripes* genomic DNA [Greg Elgar, Human Genome Mapping Project Resource Centre (HGMP-RC)] was probed with the pufferfish (*Tetraodon fluviatilis*) *WT1* genomic fragment (exon 8-exon 10) at high stringency.

Sequence analysis of cosmid 151-J19: Cosmid DNA was sonicated and ligated into *EcoRV* digested pBluescript. Recombinants were PCR amplified with limiting dilutions of dNTPs and primers. PCR products were sequenced by using dye terminator chemistry on an Applied Biosystems 377 sequencer. Gaps were filled in by using walking primers. All

coding sequence has been sequenced in both directions, and all regions have been covered by at least two independent clones. Assembly was performed by using SEQMAN (DNASTar). Sequence analysis was performed by using CLUSTAL W (17) alignments, and DOTTER for *Fugu*:human genomic DNA comparisons (18).

PCR sequencing was used to verify the presence of amino acids KPS in multiple cosmid clones and independent samples of *Fugu rubripes* genomic DNA. The probes used for this were: sense oligo (5'-3') CTG TGA AAC CTC CAC CAC and antisense oligo (5'-3') GAG TGA TTT CAG CTC CAG.

Reaction conditions were 5-min denaturation at 94°C, followed by 30 cycles of 5 s at 94°C, 30 s at 58°C, 30 s at 72°C, and a final 5-min elongation step at 72°C. The resulting 240-bp PCR products were directly sequenced by using the Sequenase kit as directed by the manufacturer.

RESULTS AND DISCUSSION

To provide an entry point for analysis of the *Fugu* homolog of the WAGR region, we screened the HGMP *Fugu* cosmid library (Greg Elgar, HGMP-RC) by using a PCR-generated genomic *Tetraodon WT1* probe. One cosmid, 151J19, was selected for further analysis because it contained the largest *Fugu* insert. Sequence scanning demonstrated, in addition to *WT1*, the presence of *Fugu* homologs of both *RCN1* and *PAX6* on this cosmid. As the gene content of this *Fugu* cosmid corresponded well to the WAGR region, we decided to sequence 151J19 in its entirety to allow comparison with human and other species data for the region. Cosmid 151J19 contains 45,565 bp of *Fugu* DNA with the 5' end of the *WT1* exon 1 at base 191 and of *PAX6* exon 12 at base 44,736. As in

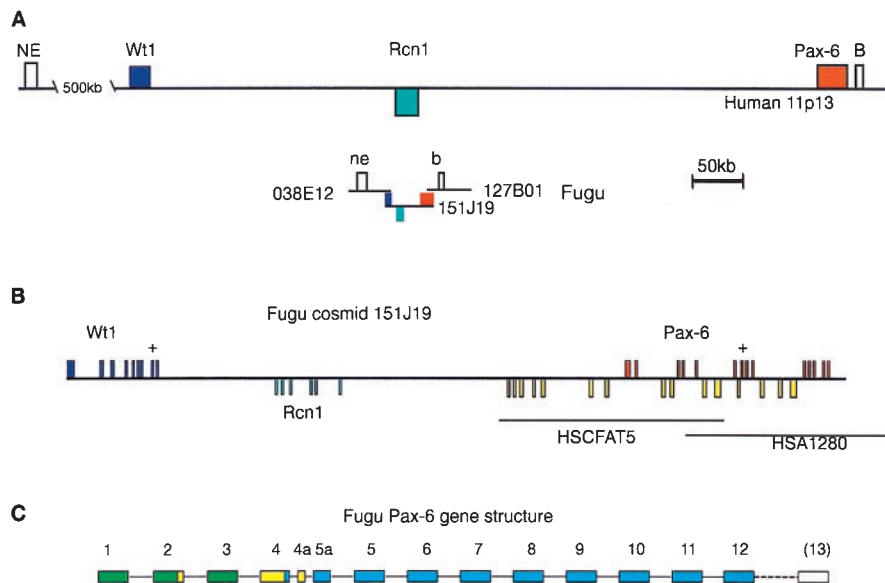


FIG. 1. (A) Scale representation of the human chromosome 11p13 region encompassing the *WT1*, *RCN1*, and *PAX6* genes compared with the equivalent genomic region in *Fugu* cosmids. Colored boxes indicate the positions of the three genes relative to each other in the two genomes. Genes in the reverse orientation are marked below the sequence line. *Fugu* cosmid 151J19 has been completely sequenced (see text), and the approximate positions of two overlapping *Fugu* cosmids are indicated. The two overlapping cosmids have been sequence scanned. Cosmid 038E12 contains a sequence (ne) homologous to a *Not1* end clone (NE, GenBank accession no. HSMFRAI) lying 500 kb from the *WT1* gene in man. There is an expressed sequence tag cluster (B) 6 kb 3' of the human *PAX6* gene, which has a *Fugu* homolog (b) found on cosmid 127B01. (B) Detailed diagram of *Fugu* cosmid 151J19. *Fugu* genomic DNA (45,565 kb) is represented. *WT1* and *PAX6* are transcribed in the forward direction whereas *RCN1* is transcribed from the reverse strand. The complete coding sequence of the *WT1* gene is represented by the nine blue exons, *RCN1* by six green exons, and *PAX6* by the first 12 exons in red. The last exon of *PAX6* (exon13) is not present on this cosmid clone. A "+" on top of an exon indicates that this exon is alternatively spliced in man. Human genomic sequence is available across the human *PAX6* gene, and this is represented by *HSCFAT5* and *HSA1280*. Additional regions of DNA similarity between human and *Fugu* is indicated by yellow boxes. (C) Exon structure of the *Fugu* *PAX6* gene. The human *PAX6*-coding region (represented in blue) starts toward the end of exon 4, with exons 1–3 being entirely noncoding. Fish (including the blind cavefish and zebrafish) have additional coding sequences located in exon 2, exon 4, and exon 4a (yellow), which combine with the blue exons to form a series of additional isoforms. Note that the beginning of the *Fugu*-coding sequence results from the splicing together of exons 2 and 4, skipping exon 3.

the human genome, the *Fugu RCN1* gene is transcribed in the opposite direction to its neighbors, demonstrating conservation of both gene order and orientation between *Fugu* and mammals. Screening of the HGMP *Fugu* cosmid library with probes isolated from 151J19 resulted in the isolation of overlapping cosmids at each end (Fig. 1). Analysis of the PAX6 overlapping cosmid has located part of the *Fugu* homolog of a putative gene (expressed sequence tag contig) telomeric to PAX6. The *WT1*-overlapping cosmid has revealed homology with a human CpG island expressed sequence tag (19) mapping ≈ 500 kb centromeric to *WT1*. The size reduction of this region in *Fugu* compared with man is 15-fold, with *Fugu* sequences in three contiguous cosmids covering ≈ 1.5 Mb of the human genome. More importantly, the relative positions of five genes has been conserved between *Fugu* and man thereby fixing these loci and enabling the intervening sequences to be directly compared as they become available. These data predict that *RCN1* may be the only gene between *PAX6* and *WT1* in the human genome, leaving >600 kb of DNA between transcription units. Until the complete sequence of human 11p13 becomes available, the presence of other genes between *WT1* and *PAX6* remains a possibility. To date, however, despite intense study (15, 19–21), no other genes have been found in this region in man. It is interesting that the *Fugu* homolog of this human region, which exhibits a low density of CpG islands, shows greater than average size reduction, consistent with *Fugu* counterparts of gene-poor regions exhibiting the greatest compaction (2). The complete sequencing of this *Fugu* region will facilitate studies probing the function of noncoding DNA in gene sparse regions of the human genome. Given that known gene order and orientation across 1.5 Mb has been conserved, this example provides the most compelling evidence to demonstrate the validity of the *Fugu* genome project away from the context of gene families.

The *Fugu WT1*, *RCN1* and *PAX6* genes show very high similarity to their well-characterized human orthologs. The intron/exon organization of both the *WT1* and *PAX6* genes is conserved and that of the reticulocalbin gene has been predicted (Fig. 2) to be the same as in the mouse. The *Fugu PAX6* gene also contains a homolog of the alternatively spliced human exon 5a (Fig. 2), although this exon in *Fugu* consists of 12 amino acids instead of the 14 amino acids found in other vertebrates. Comparisons with *PAX6* cDNA sequences from zebrafish (22) and the blind cavefish *Astyanax* (23) suggest that the fish proteins can initiate upstream of the mammalian ATG site to generate an N-terminal extension, which would involve skipping the *Fugu* sequence homologous to the noncoding mammalian exon 3 (Fig. 1C).

The human *WT1* gene has the potential to generate 16 different protein isoforms by a combination of alternative splicing, RNA editing, and alternative translational start site usage (24). We predict that the *Fugu WT1* gene can generate only two different isoforms by the introduction of three amino acids between the two most C terminal zinc fingers (Fig. 2). The retention of this alternative splice implies that *WT1* could play a dual role in both transcription and RNA processing in nonmammalian, as well as mammalian, vertebrates (25). In *Fugu*, this amino acid triplet consists of KPS (verified in several independent cosmid clones and samples of *Fugu rubripes* genomic DNA, not shown) in contrast to all other known *WT1* homologs where it is KTS. The change from threonine to proline converts a noncharged polar amino acid to a non-

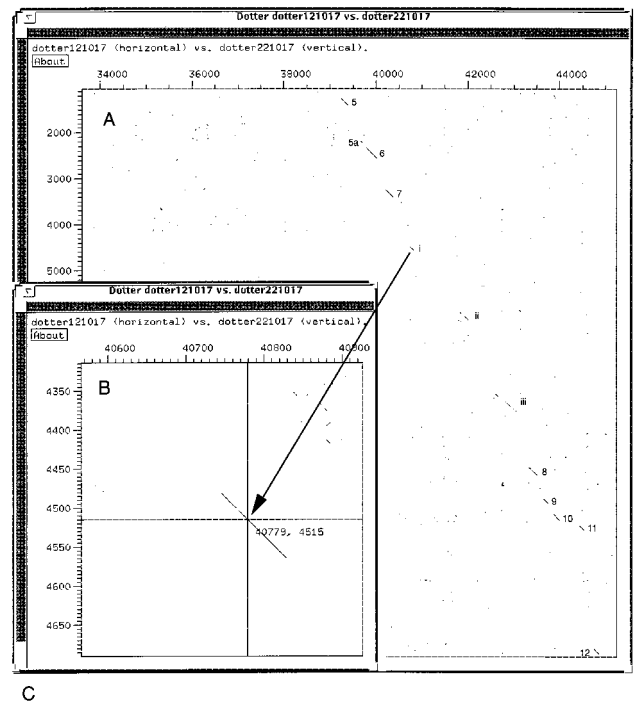


FIG. 3. (A) Dot plot alignment of cosmid 151J19 (x axis) with human genomic sequence HSA1280 (y axis) from the human PAX6 region. The program DOTTER was used to align the sequences with a window size of 21. Regions of similarity are identified as a concentration of dots (identical bases) generating a line. Exonic regions of similarity are marked by numbers. Three other regions of high DNA similarity also are marked (i–iii). (B) The weakest of these is magnified by using the DOTTER program, and a nucleotide alignment across this region is represented in C, which illustrates the lack of third base “wobble” associated with these noncoding segments of homology between *Fugu* and man.

charged nonpolar amino acid. Given the general level of amino acid conservation of the zinc finger region of *Fugu WT1* and the conservation of the alternative splice donor and acceptor sequences, it would appear that the ± 3 amino acid alternative splice is functional in *Fugu* and that this function is not strictly reliant on KTS. The role of the \pm KPS alternative splicing could be to generate protein isoforms with different target-binding abilities by altering the spacing of zinc fingers 3 and 4. *Fugu WT1* lacks the 17 amino acid exon 5, consistent with this feature being mammalian-specific (26). Mammalian *WT1* translation can initiate at a CTG codon, adding 68 amino acids to the N terminus of the protein (27). The sequence upstream of the ATG codon in *Fugu* shows no evidence of an alternative start site resembling that found in mammals, suggesting this may represent another mammalian specific isoform. The final element creating the diversity of human *WT1* proteins is RNA editing (28), which results in an amino acid change at position 281 from leucine to proline. Intriguingly, the *Fugu* genome encodes a proline at position 281 (Fig. 2). The cloning of the *Fugu WT1* gene opens up the possibility of investigating the evolution of genitourinary development at a functional level in terms of both evolving protein structure and the regulation of

(B) Clustal alignment of mouse, human, and *Fugu RCN1* genes. Intron/exon boundaries are marked for *Fugu* as these are not published for the mouse or human genes. It is likely that the mammalian genes have a very similar or identical genomic organization. (C) Clustal alignment of *PAX6* genes from *Fugu*, blind cavefish, zebrafish, *Xenopus*, mouse, and human. In the three fish species shown, there are additional coding sequences at the 5' end of the gene giving rise to a variety of transcripts. The most 5' transcripts in fish are formed by splicing together exons II and IV, skipping exon III. There is also an additional exon found in cavefish and (by homology) *Fugu* that has not been found in higher vertebrates and does not appear to be present in human genomic sequence for this region (HSCFAT5 and HSA1280). Exon 13 has not yet been sequenced in *Fugu*.

gene expression, i.e., to what extent the evolution of WT1 protein isoforms and genetic regulatory elements have contributed to the evolution of the mammalian genitourinary system.

To test the usefulness of *Fugu* for identifying conserved regulatory elements, we performed dot plot analyses between *Fugu* PAX6 and the corresponding human genomic sequence (Fig. 3). Several noncoding, potential regulatory, sequences were identified, which are represented in homologous genomic positions along the genome in *Fugu* and in man. The first two clusters of these regions are >5 kb 5' of the noncoding exon 1 of human PAX6. The third, most highly conserved, region is the proposed retinal specific enhancer (29) in intron 4, while a fourth conserved noncoding cluster lies in intron 7 (Fig. 3), the largest intron in both *Fugu* and man. Human intron 7 has the features of a CpG island, suggesting the possible presence of another gene; however, none of the sequences conserved between *Fugu* and man, nor any neighboring tracts, reveal ORF. The lack of "wobble" at what might be the third position of the triplet codon also suggests a regulatory function for these sequences. We are considering the possibility that these sequences may constitute an alternative start site, which would give rise to a PAX6 isoform lacking a paired domain. A similar paired domain lacking PAX6 product has been found at the genetic locus *mab-18* (male abnormal) in *C. elegans* (30) as an alternative form to the full length protein observed in *vab-3* chemotactic mutants (31). N-terminally truncated forms of PAX6, lacking the paired domain, also have been reported in quail (32). By comparing genomic data from species as divergent as *Fugu* and man in this way, it has been possible to identify a number of conserved regions, with characteristics of regulatory elements, which may now be studied further to assess their role in gene expression and function.

In conclusion, we have demonstrated conserved synteny between *Fugu* and 1.5 Mb of human chromosome 11p13—a region where the identified genes are not members of gene families and yet their order and orientation is not scrambled compared with *Fugu*. There are important implications of such a finding for the comparative analysis of a large genomic region, given that the full human chromosome 11p13 sequence of ≈15 Mb may be available soon and excellent biological resources exist for functional studies in several animal species as well as in cell culture. Despite near complete coverage of this region with human YAC/PAC contigs, the identification of genes remains difficult, although there is some evidence (such as relatively low CpG island density) that there may be few genes in this region. This study suggests that it will now be possible to use comparative genomics between *Fugu* and man to assist the designation of new genes and the identification of regulatory elements and, more fundamentally, increase our understanding of the nature of large segments of intergenic DNA in the human genome.

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