LETTER

Fugu Intron Oversize Reveals the Presence of U15 snoRNA Coding Sequences in Some Introns of the Ribosomal Protein S3 Gene

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We present here the analysis of the genomic organization of the *Fugu* gene coding for ribosomal protein S3 and its intron encoded U15 RNA, and compare it with the homologous human and *Xenopus* genes. Only two of the six *Fugu* S3 gene introns do not contain the U15 sequence and are in fact shorter than 100 nucleotides, as most *Fugu* introns. The other four introns are somewhat longer and contain sequences homologous to U15 RNA; two of these represent functional copies, as shown by microinjections of *Fugu* transcripts into *Xenopus* oocytes, whereas the other two appear to be nonfunctional pseudocopies. Thus *Fugu* turns out to be ideal for the study of intron encoded snoRNAs, partly because of the reduced cloning and sequencing workload, and partly because the intron length per se can be an indication of the presence of a snoRNA coding sequence.

[The sequence data described in this paper have been submitted to the EMBL data library under accession no. X97794.]

The fish *Fugu rubripes* has, among vertebrates, a particularly compact genome, which is approximately eight times smaller than that of mammals (Brenner et al. 1993). This is in part due to the small size of most introns, which have a modal length of less than 100 nucleotides. This seems to be particularly favorable when studying the organization of those genes that host the coding sequences for small nucleolar RNAs in their introns (Seraphin 1993; Sollner-Webb 1993; Maxwell and Fournier 1995; Steitz and Tycowski 1995).

Ever since the intron localization has been revealed for mouse U14 RNA gene (Liu and Maxwell 1990), the number of intron-encoded small nucleolar RNAs (snoRNAs) in various vertebrates has grown to about fifty and is still increasing fast. In general, the host genes code for ribosomal proteins (r-proteins) (Fragapane et al. 1993; Kiss and Filipowicz 1993; Prislei et al. 1993; Cecconi et al. 1994; Qu et al. 1994; Nicoloso et al. 1996) or for other proteins involved in the production and function of the translation apparatus (Liu and Maxwell 1990; Nag et al. 1993; Nicoloso et al. 1994), or appear to have lost function (Tycowski

et al. 1996). All these snoRNAs are produced by processing of the host gene pre-mRNA and present one or two regions complementary to the rRNA (Bachellerie et al. 1995) that have been shown to be implicated, in the case of the fibrillar-associated snoRNAs, in the site-specific 2'-O-methylation of pre-rRNA (Kiss-László et al. 1996). In particular, U15 RNA has been found to be encoded at least in the first intron of the human *rpS3* gene (Tycowski et al. 1993) and in three of the six introns of the *Xenopus* gene for the same r-protein (Pellizzoni et al. 1994) (formerly *rpS1*, here referred to as *rpS3*, adopting the rat nomenclature as the unified system). The multiple copies of U15 RNA coding sequences are quite divergent, conserving well only the two ends of the molecules containing the C and D boxes, the sequences complementary to the 28S rRNA, and a seven-nucleotide loop in the middle of the molecule. The remaining part of the sequence is very divergent, although it is always able to acquire the same secondary structure (Pellizzoni et al. 1994).

We present here the cloning and analysis of the *rpS3* gene of *Fugu* and of the U15 RNA coding sequences hosted in its introns, and a comparison with the *Xenopus* and human counterparts.

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RESULTS AND DISCUSSION

Fugu genomic fragment hybridizing to two *Xenopus* probes, for rpS3 and for U15 RNA, has been isolated and sequenced as described in Methods. The resulting nucleotide sequence with its flanking regions is shown in Figure 1. Exon/intron boundaries and the 5' and 3' ends of the *Fugu* gene have been identified through comparison with the *Xenopus* r-protein S3 cDNA and gene. Transcription start site is located within a pyrimidine tract, as in all vertebrate r-protein genes (Hariharan et al. 1989; Pierandrei-Amaldi and Amaldi 1994; Meyuhas et al. 1996). The gene is made up by seven exons as its *Xenopus* homologs. The position of the six introns is conserved perfectly in the two species and, at least, with that of the first three introns of the human gene. The size difference between the *Fugu* gene (3593 bp) and the *Xenopus* counterpart (12,691 bp) is entirely a result of the smaller size of the introns. Although these are all rather short, only two of them, the second (90 nucleotides) and the third (84 nucleotides) have the typically very small size of most *Fugu* introns. The remaining four introns range between 341 and 891 nucleotides. Two of these (introns 4 and 6) contain a U15 RNA sequence, 76% homologous with respect to each other, and somewhat less (63–71%) with respect to the *Xenopus* and human U15 sequences. The regions corresponding to the C and D boxes, the sequences complementary to 28S RNA and the 7-nucleotide loop mentioned above, are all very well conserved. In spite of the considerable divergence of the rest of the molecule, the computer-derived secondary structure of these *Fugu* U15 RNA (not shown) is substantially identical to the one proposed for the human and *Xenopus* RNAs (Pellizzoni et al. 1994).

Careful inspection of introns 1 and 5, which are also longer than most *Fugu* introns, revealed the presence of sequences (broken underline in Fig. 1) that, though degenerated, can still be recognized as related to the U15 sequence, particularly in the region corresponding to the C and D boxes and to the sequence complementary to 28S rRNA. Attempts to derive a computer secondary structure for these U15 pseudocopies comparable to the canonical one have failed. To verify if these were in fact pseudocopies, we tested their capacity to be properly processed by microinjecting various in vitro transcribed radioactive RNAs, corresponding to different regions of the *Fugu* rpS3 gene, into *Xenopus* oocytes, a system that

CAGCTGT

-360 TTGTGCCAGC AATTGTCTGT CTGTTGGCCA AGCAGGCGAT AAAGAGAAAG AAGAAACTTA
 -300 ACCGTTATTA TTATTATTAT TATTATTATT ATTATTATTA ACTGTTATTA TTACTTATT
 -240 TATTATTATT ACTTGTGTT TGTACACAA ATGAATCTG TAGTATATTA TATCATATA
 -180 GTGATATTAT GATCAGAGTC AACTTCTACT TACCATTTTA AAATACGGTT TAGTGCTCT
 -120 TTTTCTGTA ACTATTACTA ATCAAGCCCT ATATGTAAAA ACTACTGCTC CCGCATGCT
 -60 TGTAAATCGT AACAGAAGTG CAATTTTTGT ATAAATTCAT TCGTATCCG TTTAACCGCTC
 1 CTTTCTCTCT ACGGCTATCG GAAAGATGGC GTTGGCAATC TCCGAAGA GGAAGGTTAG
 61 CATTTGCATAA ACTCGTTTTT TGTAAACAGA CTGGCAGTTA TAGTAAATG GAGCGGGGTC
 121 GGTTAAGATA AAGTATCTTA CGGTCCACTT TATGTCAATT GCGCTTGTC TTCTCCACC
 181 TCGTTGAGGA TCATACCAGA TGCTGTGGC TATGTCTCAT TTGGACAGTT AACATGGCTG
 241 AACCTAGCTA AGGGCCGGG ATGTGACTGA CTACTTACAC GTGTGTGCA TAAGCAGTTT
 301 TCGCAACCGT TATATTGTTG GCTATGAATT ATTTAGTAGT TTGACATTTT AAGCATGTG
 361 CCGGCTGCC AGCGTTGTC TCGTTGATCA GATCCGCTAG AGAAATGAAC ATGGAGCTAC
 421 AGCGAGTTGC GGCTACACTC GGCCTTAAGG CATATTTAAG GTGACGAGAC CACTGTTTGA
 481 GTTGCTTTTC CCAATACATC TGTGGGAGGA ATCAGGGTTA TTAGGCTTC ACTGAAACAA
 541 ACCATTCAAT GAGTGAAGAC TGCTGATGAT CCCCACTGAA ATCTATTCTC TGAGACTTCC
 601 CAGCAAAAT GTGCTCAGT TTGTAGGTCA TGATCTGACC TGTGTAGCTC ACCCTACTCT
 661 ACTCCCTCAG TTTGTCTCAG ATGGCATCTT CAAGGCCGAG CTGAACGAGT TCCTGACTCG
 721 TGAGCTTGCT GAGGATGCAT ACTCTGGGGT GGAGGTACGT GTGACACCAA CCAGGACGTT
 781 AATCATCATC CTGGCTACAA GTAATATTC ATTTCAGT GATCCACAGC ATTTTTGTGA
 841 CATGTTATTT ATGGTTATAG CTTTGTTTGC TGTCATTATTA ACTGCTCCCA GACCCAGCAA
 901 TGCTGTGGGA GAGAAGGGCC GAAGGATCAG AGAGTTGACC GCTGTGGTCC AGAAGAGGTT
 961 TGGCTTCCAG AGGCAAGCAG TCGAGGTAAA TGATTGGGA CTGGATTGT ATTATATATT
 1021 TTATTGTATA TTTGACTCAA TGTTCTTGT GTTGTTCCGC TCACGTGAGC TGTAGCTGTA
 1081 GAAAGTTGGC ACTCGTGGTC TGTGTGCTAT TGCCCAGGCA GAGTCTTGTC GCTACAGGTT
 1141 GCTGGGAGGC CTGGCTGTGC GTAGTAAGC ATGGAACAT CGAGTAATT TATGTTGAAT
 1201 AACCTCAGT GCCTGGCTG TAGACTTTTC CCCTCATTTA TGTAAGGTGC TGGTGTTCCT
 1261 TAACTGCTG ATTGAACCTG TGTTTGTGTT GCATCATCA CGACACATCT CGCAATATTG
 1321 GTAACAACAT GGGTTATCA TACTTTAAA AGGACAGAGC TAATTGACGG AGCTTTGTTT
 1381 CCTATATAAT TCTAGTTTAT TAGCAGCTTT TAGTTTAAA GTGAGAGAAT TGAGCTCTGT
 1441 TTTTTATTC CTCATCAGC CATCAATCT CCCCGTAAA CTGGAGTTGA AGCTTTAAAT
 1501 ATTCCTGCA TGATTGTA TTACATAACT ATTACTTTGA TGATTAGAAA TAACAGACCC
 1561 AAGAAAGGG AAATCATGAA TCACAAGTGA CTAAAGTTTA AAATAAAAG TCCTCTCTGT
 1621 CCACTGTGC ACTCAGTATG GGCTGTATT TGGGAGTAG GTTGACAGAG TGTGGGGGGG
 1681 GGGGGAACAA AGGAAAGAC TGGTCCGGGT GTTCGAATTT GTGTGTGTT GGCCGAATG
 1741 AACCTGGAC TTGTGTTG TAAAGTTCT TTTGGCTTG GTGAAGATA GATGACAGTT
 1801 CGGAATAGGA CAGATCTCTG TTCTGCCGGT CACCTGGTCA GGTCCACGTT CTGTGGGTTT
 1861 CTACCAATTC TTGAGAGTGG GTGCTCCCTC TTCTTTGAAA GACACTGAGG CTATTGCTG
 1921 AGAAGGTTTA AGATGGTAG TGCTCCCTGT TTGGTGGTTC GTGTTCAAAA GAATACCGG
 1981 GAGGCAAAA GGTCCATTTT AATCTTGAC ACATTCACCC AAACATCAAC ATCAAAATGG
 2041 TCTACATTT TACAGGGCGT GCTATGGTGT TCTGAGGTTCT ATCATGGAGA CGTGCCCAA
 2101 AGGCTGTGAG GTGCTGGTGT CTGGTAAGCT GAGGGGTGAC AGAGCCAAGT CCATGAAGTT
 2161 CGTGCAGGC CTGATGATCC ACAGTGGAGA CCGAGTCAAC TATTACGTGC ACACAGCGGT
 2221 CCGCCAGCTC CTGCTGAGGC AGGTGAGTT CACGACGAC AACCTTAACA TCGCAAAAC
 2281 ACGCACATTC TGACCAAGT ATCTGGAATT AGATGTTTT ATTGACAAA ATCTGACGAC
 2341 CACCTCTGT ACATTGATTC TTAGTGGAC TAACTTTAAA TACACACATG CTCAAAAGAT
 2401 TTCAATCGAG ATTACTATG ATGTGATGCC GTGTTCCAA AAGGTATTIA AACAGTGAT
 2461 AATGTGCCG ATTCTCTG ATATAGACGG TTTACACAGC TTTCAATG TTTCAATAT
 2521 ATGGAAGTAT GTGAGGCTAT GAACCTGTAC TCTGACCCAG CTAATCTGCT ATGGGTCCTC
 2581 CATAGGGTGT GCTGGGCATC AAGGTTAAGA TCATGCTGCC CTGGGACCCC ACTGGTAAAG
 2641 TCGGACCCAA GAAGCCCCCT CCTGACCATG TCAGATCTGT GGAGCCAAG GAGGATGCTT
 2701 TTCCACCAAC ACCAATTTCT GAGCAGAAGG GGCCGACGCT AGAGGTGCCA GATGACGCCC
 2761 AAGGAGCACC TGTACCCACT GCATAATCGG GTATGTCACC TGTGTTATGG TTATGTTAGG
 2821 ATGTAAACAC AAACACTAGA GGGTTTTGGT TGCTGCTCTT CAGTGATGAT ACTGATGAGT
 2881 GTGGAACAA AGCCGCTCTG TTCTGGGGGT TGCTGGTTC GTCTACGTT CTGTGTTTCT
 2941 TTTCCAGTTC CTCAGTTCAG GGTCTTTTTT CTTGAAGAC ATAGAGCAT TTTGTGAGA
 3001 AGGGCTTGAA GTGTAAGAT CTACACAGAC AGCAATTCAT TGCATCGGAA AAAACCTAGC
 3061 TGGCTAGTTA TTTTAGCCCC TAATACTGG GTTATGTAAA GGGTGATTC ACGTGACCAA
 3121 TTTCATCAAT AATCATGCA AATGGTGACG ACATACAGCA GACATGGTTC AGTTGTAGTT
 3181 TAAATCTTTT AACTTGTCG GTTTAAGTTA CGTGAAGGT TCAGTCACAT ATTTGTTCAA
 3241 TACAAGTAAA AACTGCAACT TCTATCTCAA ACAGCAGTTA AACTTGAGA CTGATTAAAG
 3301 TGATCACACA ACAAACGGGT TTTGAAGAAA CTTATATTTA GCCTCATTTT CTTGATACAT
 3361 CCAGTGTGG AGGCACCAT GAAGTCACTT CTATTATTTA TTTCCACGAC CAAGCTTGTG
 3421 TCAAATGTAG CAGAATTTAA CTGCGAGTCC TGTGGACTAC AACATAAAAC ACACACTTCC
 3481 TTCTAAAGTG CATACTTTTC TGTTCCAGGG TCTTGATTCA TCCACAGCTG GCAACAGAAC
 3541 CTTCTGTTTT TTGTGTACAA AAACAATAAA ATCTGAAAA TGCCAAGCGC TGTTAACTTT
 3601 GTCTCCATCA CCTTCAGTAA AATAAAAGGT CAAGTGGTGT GATGCCAAGA GTGCGAGAG
 3661 AATGACTCAA AGTAGCCATG AAGTGGTCT GTTGGGGCTG AGCTGGAGAG CAGCTTCATG
 3721 TTTAGCACC ATCAGGTTTT GAGGACCGT ACAACAGATC TGCTGTCTGT GGCAACAAA
 3781 GCAATATTTT GGCTCTCTG GGGCAAGATT GTCTCCACT GGTTTGCA CCAAAGATGG
 3841 GAACAAGTGG AGTCTCTGGA TAATTGATGG CACCTTTATT GGGGGTGTTG GTT

Figure 1 Nucleotide sequence of the *Fugu* S3/U15 gene and its flanking regions. The sequence is numbered relative to the transcription start point. The seven exons are boxed and shaded. Pseudo and canonical U15 sequence copies are underlined (solid and broken lines, respectively) within the introns. The start codon is underlined within the second exon; the stop codon and the polyadenylation signal are underlined within the sixth and the last exon, respectively.

also works interspecifically (Cecconi et al. 1995). While the transcripts of introns 4 and 6 are correctly and efficiently processed to produce mature U15 RNA, the transcripts of introns 1 and 5

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are completely degraded, confirming that the U15 sequences present in these two introns are nonfunctional pseudocopies (examples of these experiments are shown in Fig. 2).

Recently, we have found a similar situation for *Fugu* U17 snoRNA, which is encoded in the introns of the *rpS7* gene. In this case no "empty" intron was found. In fact all six introns were relatively long: four contained canonical U17 sequence copies and two contained pseudocopies (Cecconi et al. 1996).

Figure 3 compares the overall organization of the *Fugu* *rpS3/U15* gene with that of the *Xenopus* counterpart and part of the human gene. Although the coding sequences for U15 RNA have remained hosted in the same *rpS3* gene during the evolution of vertebrates, they have moved from one to another intron. This observation, together with the presence of U15 sequence pseudocopies in other introns, indicates a highly mobile situation for intron nested snoRNA coding sequences, possibly because of sequence duplications followed by divergence.

Interestingly, the two introns that do not contain the U15 RNA sequence, neither canonical nor pseudocopy, present the very reduced size

Figure 3 Comparison of the *S3/U15* gene organization of *Fugu* (F) with that of *Xenopus* (X) (Pellizzoni et al. 1994) and with the available portion of the human gene (H) (Tycowski et al. 1993). Exons are represented by solid boxes. Open and hatched boxes represent, respectively, the canonical and pseudocopies of U15 sequences.

(<100 nucleotides) typical of most *Fugu* introns. This indicates that the presence of U15 sequences is the reason for the relatively large size of the other four introns, and supports the assumption that a specific structural or functional role of intronic sequences can explain the other described exceptions of long introns in *Fugu* genes (Aparicio et al. 1995; Baxendale et al. 1995; Macrae and Brenner 1995; Mason et al. 1995).

The finding that the length of the snoRNA containing introns exceeds the one expected from simple addition of the snoRNA sequence to an average short *Fugu* intron seems to exclude a transposition mechanism neatly involving the repeated sequences, suggesting duplications of more extensive regions that include the snoRNA coding sequences.

METHODS

Cloning and Sequencing

F. rubripes cosmid library (Baxendale et al. 1995) has been probed with a *Xenopus* cDNA specific for r-protein S3 (Di Cristina et al. 1991) and a *Xenopus* genomic fragment containing a U15 snoRNA gene copy (Pellizzoni et al. 1995). The finding of clones hybridizing to both probes suggested that the U15 RNA coding sequence is hosted in the S3 gene of *Fugu* as it occurs in *Xenopus* and in man. The selected cosmid 136B1 has been digested with *Hind*III to generate a 2500-bp

Figure 2 Analysis of *Fugu* U15 snoRNA production by RNA microinjections into *Xenopus* oocyte nuclei. (A) An in vitro synthesized 800-nucleotide radioactive RNA (VI), corresponding to part of the sixth exon, the entire sixth intron bearing the U15f copy, and part of the seventh exon of the *Fugu* S3 gene, was injected in *Xenopus* oocytes. After incubation, at increasing time intervals (30 min to 5 hr), total RNA was extracted and analyzed by gel electrophoresis and autoradiography. Arrows point to the intact injected transcript and to the mature product. A schematic representation of the RNA molecules is shown at right. (B) An in vitro synthesized 537-nucleotide radioactive RNA (Vi), corresponding to part of the fifth exon, the entire fifth intron bearing the Ψ U15e copy and part of the sixth exon of the *Fugu* S3 gene, was used and analyzed as in A. (M) size markers; (S) transcript substrate.

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fragment containing the 5' region and the central portion of the gene and with *HindIII*–*XbaI* to generate a 2400-bp fragment containing the 3' region of the gene. These fragments have been cloned in the pEMBL18 and pBluescript KS(+) vectors, respectively (clones pF-S3.1 and pF-S3.2). A PCR amplification on the 136B1 cosmid was performed to check whether the two clones were contiguous. For sequencing, plasmids pF-S3.1 and pF-S3.2 were digested with various restriction enzymes to generate overlapping fragments and subcloned in the Bluescript KS(+) vector. Analysis and manipulation of DNA and RNA were performed according to standard laboratory manuals (Sambrook et al. 1989). Sequencing was performed by the dideoxy chain-termination method (Sanger et al. 1977) on both strands of overlapping fragments.

In Vitro Synthesis of Radioactive Transcripts

To prepare the transcripts to be used as processing substrates in microinjected oocytes, various genomic fragments encompassing the U15 sequences, derived from plasmids pF-S3.1 and pF-S3.2, were cloned under the T7 promoter in pBluescript: pF-S3.10 and pF-S3.11 (nucleotides 2195–2732 and 2732–3532, respectively, in Fig. 1). For both pF-S3.10 and pF-S3.11 plasmids, 1 µg of DNA was digested with *EcoRI* and transcribed with T7 RNA polymerase, in the presence of 50 µCi of [α - 32 P]UTP, as described by Melton et al. (1984). The 537-nucleotide long (Vi) and 800-nucleotide long (Vli) transcripts were obtained from pF-S3.10 and pF-S3.11 plasmids, respectively. After transcription and DNase digestion, the RNAs were purified by phenol-chloroform-isoamyl alcohol (50:50:1) extraction and ethanol precipitation, and resuspended in H₂O for microinjection.

RNA Microinjections in *Xenopus* Oocytes

Isolation of *Xenopus* stage V–VI oocytes, microinjection of RNA into the germinal vesicle, oocyte incubation, manual isolation of germinal vesicle, RNA extraction, and polyacrylamide electrophoresis analysis were all carried out as described previously (Cecconi et al. 1995).

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