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Genome Res. 1996 6: 1227-1231

Access the most recent version at doi:[10.1101/gr.6.12.1227](https://doi.org/10.1101/gr.6.12.1227)

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LETTER

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

Claudia Crosio,¹ Francesco Cecconi,¹ Paolo Mariottini,²
Gianni Cesareni,¹ Sydney Brenner,³ and Francesco Amaldi^{1,4}

¹Department of Biology, University of Rome, "Tor Vergata," 00133 Rome, Italy; ²Department of Biology, Terza University of Rome, 00154 Rome, Italy; ³King's College, Cambridge CB2 1ST, United Kingdom

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 (Bachelierie 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.

⁴Corresponding author.
E-MAIL amaldi@utovrm.it; FAX 39-6-72594316.

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 rRNA 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

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-360 TTGTGCCAGC AATTGTCTGT CTGTTGGCCA AGCAGGGCAT AAAGAGAAA AAGAAACTTA
ACCCTTATTA TTATTATTAT TATTATTATT ATTATTATTA ACTGTATTA TTACTAAT
-240 TATTATTATT ACTTCTGTGT TGTACACAA ATGAACTGTG TATGATATTA TATCACAATA
GTGATATTAT GATCACAGTC AACTTCTACT TACCATTITTA AAATACCGTT TAGTGCTCT
-180 TTTTCTGTGA ACTATTACTA ATCAAGCCCT ATATGTAAA ACTACTGCTC CGGCCATGCT
-120 TGTAATACGT AACAGAAGTG CAATTTTTGT ATAAATTCAT TCTGTATCCG TTTAACCGCTC
-60 CTTTCCTTCT ACGGTATCG GAAAGATGGC GTGGCAATC TCCAAAGAA GGAAGGTTAG
1 CATTGCATAA ACTCGTTTTT TGTAAACAGA CTGGCAGTTA TAGTAAAATG GAGCCGGGTC
61 GGTTAAGATA AAGTATCTTA CGGTCCAATT TATGTCATTG GGCCTTGTGC CTTCCACC
121 TCCTGTGAGG AATACACAGA TGCTGTGGC TATGTCATCT TTGGACAGTT AACATGGCTG
181 AACCTAGCTA AGGGCCGGG ATGTGACTGA CTACTTACAC GTGTGTGCA TAAGCAGTTT
241 TCGCAAACCG TATATTGTTG GCTATGAATT ATTTAGTAGT TTGACATTTT AAGCATGTG
301 CCGGCTGCC AGCGGTTGTC TCGTTGATCA GATCCGCTAG AGAAATGAAC ATGGAGCTAC
361 AGCGAGTTGC GGCTACACTC GGCCTTAAGG CATCTTTAAG GTGACGAGAC CACTTGTGTA
421 GTTGTCTTTC CCAATACATC TGTGGGAGGA ATCAGGTTTA TTAGGCTTC ACTGAAACAA
481 ACCATTCAAT GAGTGAAGAC TGCTGATGAT CCCCAGTGA ATCTATTCTC TGAGACTTCC
541 CAGGAAAT GTGCTCAGT TGTAGTCA TGATCTGACC TGTGTAGCTC ACCCTACTCT
601 ACTCCCTCAG TTTGCTCAG ATGGCATCT CAAGGCCGAG CTCGAACGAG TCCTCAGCTG
661 TGAGCTTGTG GAGGATGAT ACTCTGGGGT GGAGTACGTG GTGACACCAA CCGAGAGTTA
721 AATCATCATC CTGGCTACAA GATAATTCTT ATTTGCAATT GTCCACAGC ATTTTITGTA
781 CATGTTATTT ATGTTTATAG CTTGTTTTC TGCCATTITA ACTGCTCCA GGCACAGAA
841 TGTGCTGGG GAGAAGGCC GAAGGATCAG AGAGTTGACC GCTGTGGTCC AGAAGAGTT
901 TGGCTCCAG AGGCAAGCAG TCGAGGTAAA TGATTTGGGA CTGGATTGT ATTATATTT
961 TTATTGATA TTGACTCAA TGTTCTTGT GTGGTCCGC TCACGTGAGC TGTAGCTGA
1021 GAAGGTTGG ACTCGTGGTC TGTGTCCAT TGCCAGGAGA GAGTCTTGC GCTACAGTT
1081 GCTGGGAGC CTGGCTGTGC GTAGTAAAG ATCGAAACAT CGAGCTAAT TATGGTGAAT
1141 AACTTCAGT GCCTGGCTG TAGACTTTTC CCCTCATTTA TGAAGGTGC TGGTGTAACT
1201 TAAGCTGTG ATGAACTGC TGTTTTGGT TGACTCATCA CGACACACT CGCATGTTT
1261 GTAACAACAT GGGGTATCA TTACTTAAA AGCCACAGC TAATTGACGG ACCTTTGTTT
1321 CCTATAAAT TCTAGTTTAT TAGCAGCTTT TAGTTTTAAA GTGAGAGAAT TGACTCTGT
1381 TTTTATTTT CTCATCAGC CATCAATCT CCCCCTAAA CTGGAGTTGA AGCTTTAAAT
1441 ATTCCTGCA TGATTGTA TTACATAACT ATACTTGTGA TATTAGAAA TAACAGACC
1501 AAGAAAGGG AATACATGAA TCACAGTGA CTAAGTTTA AAATAAAAAG TCCCTTCTGT
1561 CCACTGTGC ACTCAGTATG GCGCTGTATT GTGGGAGTAG GTTGACAGAG TGTGGGGGG
1621 GGGGAAACAA AGGAAAGAC TGGTCCGGT GTTCAATTT GTTGTGTGT GGCCCGAATG
1681 AACTTGGAC TTGTGTTTCA TAAAGTTCT TTGGGCTTG GTGAAGATAA GATGACAGT
1741 CGGAATAGGA CAGATCTGCT TCTGCGGGT CACTGGTTCA GGTCACAGT CTGTGGGTT
1801 TTACCAATTC TTCAGAGTGG GTCGTCCTC TTCATTGAAA GACATGAGG CATTGTCTG
1861 AGAAGGTTA AGATGGTGA TCGTCCCTG TTTGGTGTTC GCTTCAAAA GAAATCCGG
1921 GAGGCAAAA GGTCCATTTT AATCTTTCAC ACATTCACC AACATCAAC ATCAAAATGG
1981 TCTACACTT TACAGGGCGT GCTATGGTGT TCTGAGGTTT ATCATGGAGA GCGTGCCCAA
2041 AGGCTGTGAG TCTGTGGTGT CTGGTAAGCT GAGGGGTCAG AGAGCCAAGT CCATGAAGTT
2101 CGTCGACGGC CTGATGATCC ACAGTGGAGA CCGGCAAGC TATTACCTGC ACACAGCGGT
2161 CCGCCAGCTC CTGCTGAGGC AGGTGAGTT CACCAGCAGC AACCTTAAAC TCGGAAAAC
2221 ACCGACATTC TGACCAAGT ATCTGGAATT AGATGTTTTT ATTCAGAAA ACTTGACAGC
2281 CACTTCTGT ACATTGATTC TTAGTGGAC TAACTTTAAA TACACACATG CTCAAAAGT
2341 TTCAACGAG AITTAATATG ATGTGATGCC GTGGTTCAA AAGGTAITTA AACAGTGGAT
2401 AATGTGCCG AITTTTCTG ATATAGACGG TTTCACCAG TTAGTAAGT TTTGCATAC
2461 ATGGAAGTAT GTCAGGCTAT GAACCTGTAC TCTGACCCAG CTAATCTGCT ATGGTCTCTC
2521 CATAGGGTGT GCTGGGCATC AAGGTTAAGA TCATGCTGCC CTGGGACCCC ACTGGTAAGA
2581 TCGGACCCAA GAAAGCCCTC CTTGACCATG TCAGGATGCT GGAGCCAAG GAGGAGATCC
2641 TTCCACCAC ACCAATTTCT GAGCAGAAGG GCGCCAAAGC AGAGGTGCCA GTCATGCCCC
2701 AAGGAGCACC TGTACCCTAT GCATAATCGG GTATGTCACC TGTGTATGG TTAGTATAG
2761 ATGTAACAC AACACACTAGA GGGTTTTGGT TCGTCTTCTC CAGTGATGAT ACAGTACGA
2821 GTCCGAAACA AGCCCTCTG TCTGGGGGTT TCGTGGTTCA GCTCTACGTT CTGTGTTCT
2881 TTCCAGTTC CTCAGTTTCA GGTCTTTTTT TCTTGAAGAC ATAGAGGCAT TGTCTGAGA
2941 AGGCTTGAAT GTGTAAGAT CTCACCAGC AGCAATTCAT TGCATCGGAA AAAACCTAGC
3001 TGGCTAGTTA TTTTAGCCCC TAATACTGG GTTATGTAAA GGGTGCATTC ACCTGACCAA
3061 TTTCAAT AATCATGCA AATGGTGAG ACATAACA GAACATGGTC AGTTGTAGTT
3121 TAAATCTTT AAACCTGTGC GTTTAAGTTA CGTGAAGGT TCAGTCACAT ATTTGTTCAA
3181 TACAAGTAAA AACTGCAACT TCTATCTCAA ACAGCAGTTA AACTTGA GA CTGATTAAGA
3241 TGATCACACA ACAACCGGTT TTTGAAAGAA CTTATATTTA GCCTCATTT CTGTGACATC
3301 CCAAGTGTG AGGCACCCAT GAAGTCACTT CTATTAAATG TTTCCACGAC CAAGCTTGT
3361 TCAAATGTAG CAGAATTTAA CTTGAGTCTC TGTGGACTAC AACATAAACA ACACACTTCT
3421 TTTAAAGTG CATACTTTTC TGTTCCAGCG TCTTGTATTCA TCACACAGT GCAACAAGAC
3481 CTTCTGTTTT TGTGTACAA AAACAATAAA ATCTGAAAA TGCCAAGCG TGTTAACTTT
3541 GTCTCCATCA CCTTCAGTAA AATAAAGGT CAAGTGGTTG GATGCCAAGA GTGCAGAGAG
3601 AATGACTCAA AGTAGCCATG AAGTGGTCT GGTGGGGCTG AGCTGGAGAG CAGCTTCATG
3661 TTTTAGCACC ATCAGGTTTT GAGGGACCGT ACAACAGATC TGCTGTCTGT GCAACAACAA
3721 GATTATTTT GGCCTCTCG GGGCAAGATT GTCTCCACT GGTTTGTGCA CCAAAGATGG
3781 GAACAAGTGG AGTCTCTGGA TAATTGATGG CACTTTTATT GGGGGTGTG GTT
3841

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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 (Ceconi 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 sequences 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

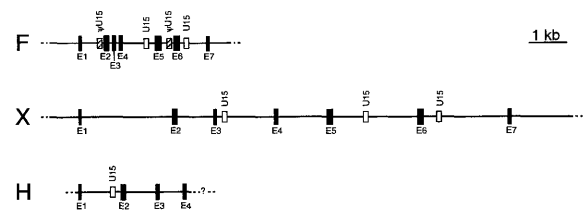


Figure 3 Comparison of the *S3/U15* gene organization of *Fugu* (F) with that of *Xenopus* (X) (Pellizzoni et al. 1994) and with the available portion of the human gene (H) (Tycowski et al. 1993). Exons are represented by solid boxes. Open and hatched boxes represent, respectively, the canonical and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

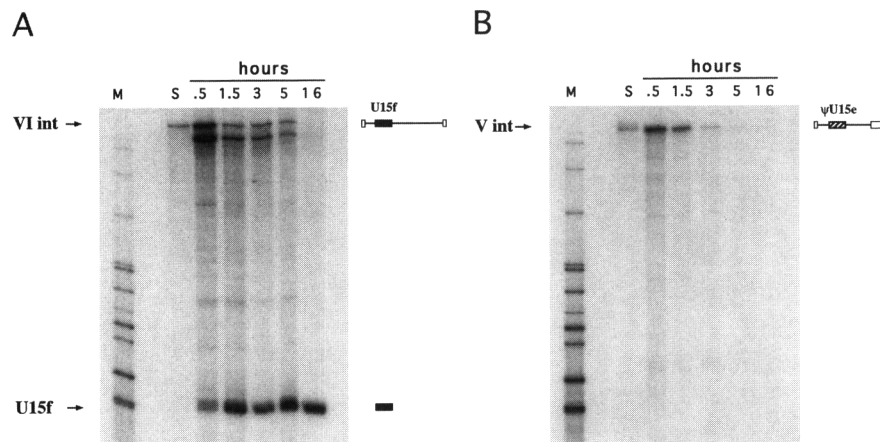


Figure 2 Analysis of *Fugu* U15 snoRNA production by RNA microinjections into *Xenopus* oocyte nuclei. (A) An in vitro synthesized 800-nucleotide radioactive RNA (VIi), corresponding to part of the sixth exon, the entire sixth intron bearing the U15f copy, and part of the seventh exon of the *Fugu* S3 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 μ g of DNA was digested with *EcoRI* and transcribed with T7 RNA polymerase, in the presence of 50 μ Ci of [α - 32 P]UTP, as described by Melton et al. (1984). The 537-nucleotide long (Vi) and 800-nucleotide long (VI) 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 (Ceconi et al. 1995).

ACKNOWLEDGMENTS

We thank M. Giorgi for expert technical assistance. This work was supported by grants from Progetto Finalizzato Ingegneria Genetica, C.N.R., and from Ministero Università e Ricerca Scientifica e Tecnologica.

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Received August 2, 1996; accepted in revised form September 9, 1996.