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Identification of tandem organization of soluble guanylyl cyclase α_1 and β_1 subunit genes in the Japanese pufferfish (*Fugu rubripes*) genome: comparison with their human homologues.

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Abstract We determined the complete nucleotide sequences of the genomic structure of the α_1 subunit gene (*FrGCS-α_1*) and the β_1 subunit gene (*FrGCS-β_1*) of Japanese pufferfish (*Fugu rubripes*) soluble guanylyl cyclase (GC). In the *Fugu* genome, the two genes are only 161 bp apart and span approximately 16 kbp in the order of *FrGCS-α_1* and *FrGCS-β_1*. The *Fugu* genomic structures were compared with those of human soluble GC α_1 and β_1 subunit genes constructed by the Human Genome Database.

Teleosts have all the complex physiological functions found in higher vertebrates and a gene repertoire similar to that in mammals. Comparative vertebrate genome sequencing has been demonstrated to be a useful method for detecting conserved regulatory sequences. The Japanese pufferfish, *Fugu rubripes*, has the smallest known vertebrate genome (Venkatesh *et al.*, 2000). The homology between *Fugu* and mammalian genes is high enough to facilitate easy identification through database comparison. This forms the basis of the suitability of *Fugu* for use in comparative genomics. Soluble guanylyl cyclase (GC) is a heme-containing heterodimer consisting of α_1 and β_1 subunits and activated by nitric oxide (NO), suggesting that it is a key enzyme in the NO/cGMP signalling pathway participating in various physiological phenomena (Koesling and Friebe, 1999; Lucas *et al.*, 2000). Increasing evidence has demonstrated that both α and β subunits are required for basal and NO-stimulated activity of soluble GC. The α_1 and β_1 subunit genes are colocalized in human, rat and mouse chromosomes (Azam *et al.*, 1998; Giuili *et al.*, 1993; Sharina *et al.*, 2000). These facts imply that the expression of both genes is coordinated. However, little is known about the genomic structure of soluble GC. As a first step in investigating whether the expression of both genes is coordinated, we determined the genomic structure of the α_1 subunit gene (*FrGCS-α_1*) and the β_1 subunit gene (*FrGCS-β_1*) of Japanese pufferfish. The *FrGCS-α_1* and *FrGCS-β_1* genes are organized in tandem in the pufferfish genome separated by a 161 bp spacer sequence.

A Japanese pufferfish (*Fugu rubripes*) genomic library in EMBL3 SP6/T7 vector (Clontech Laboratories, Inc., Palo Alto, CA) was screened by hybridization using cDNA fragments of medaka fish soluble GC subunit genes, *OlGCS-α_1* (nucleotides 1911-2534) and *OlGCS-β_1* (nucleotides 12-402), as probes. The probes were labeled with digoxigenin (DIG)-dUTP using a DIG-High Prime (Roche Diagnostics GmbH, Mannheim, Germany) or [^{32}P]dCTP using a Random Primer DNA labeling Kit ver.2 (Takara Shuzo Co., Ltd., Otsu, Japan). Phage DNA obtained from positive clones was purified by using a QIAGEN lambda kit (QIAGEN, Valencia, CA), and was subcloned into pBluescript II (Stratagene Inc., La Jolla, CA). The nucleotide sequence of the insert DNA was analyzed by an ABI Prism 377 or genetic analyzer 3100 with a BigDye Terminater cycle sequencing kit (Applied Biosystems, Japan). Total RNA was prepared from the *Fugu* brain and ovary according to the method of Chomczynski and Sacchi (1987). For reverse-transcription polymerase chain reaction (RT-PCR), the first strand cDNA was synthesized using an oligo(dT) primer by SuperScript

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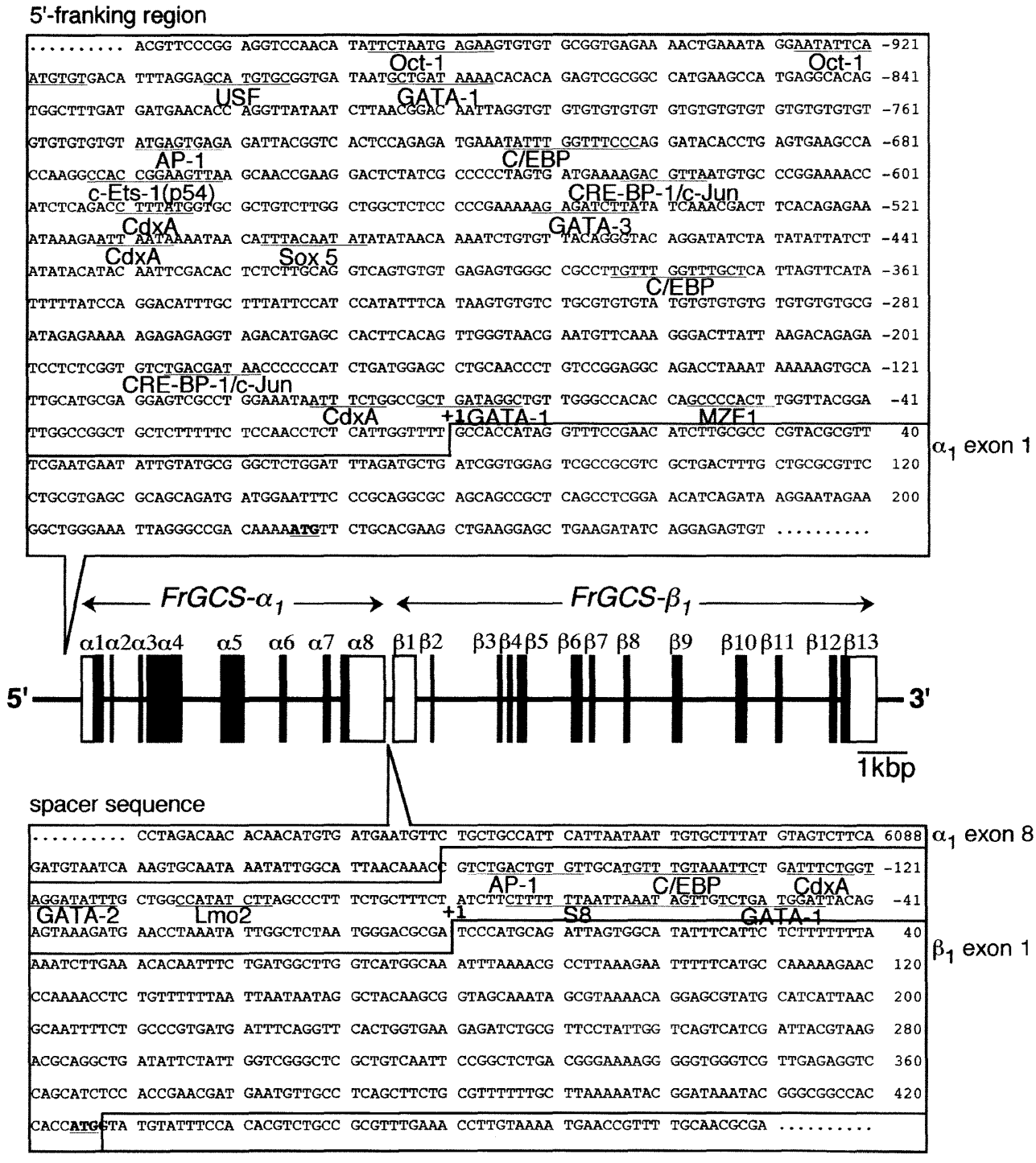

Fig. 1. Genomic structures of the *Fugu* soluble GC subunit genes, *FrGCS-*α_1 and *FrGCS-*β_1. Exons are indicated by open-boxes (non-coding region) and closed boxes (coding region) with numbers. The transcription initiation site is indicated by +1. The 5'-franking region of *FrGCS-*α_1 and *FrGCS-*β_1 is shown in the upper and lower panels with several known *cis*-regulatory elements. The nucleotide sequences of the 5'-franking region of the human soluble GC α_1 and β_1 subunit genes are from NCBI and the Celera Human Genome Database, respectively.

II reverse transcriptase (Life Technologies, Inc., Rockville, MD). Poly(A)$^+$RNA was isolated using an Oligotex-dT30 <Super> mRNA Purification kit (Takara Shuzo Co., Ltd.), according to the manufacturer's protocol. The transcription start site was determined by the primer extension method using the oligonucleotide primers, 5'-GATATCTTCAGCTCCTTC-3' for *FrGCS-*α_1 and 5'-CCCGACCAATAGAATATCAGC-3' for *FrGCS-*β_1, according to the previously described method (Mikami *et al.*, 1999).

The genomic structures of *FrGCS-*α_1 and *FrGCS-*β_1 are shown in Fig. 1. In the *Fugu* genome, *FrGCS-*α_1 and *FrGCS-*β_1 are 161 bp

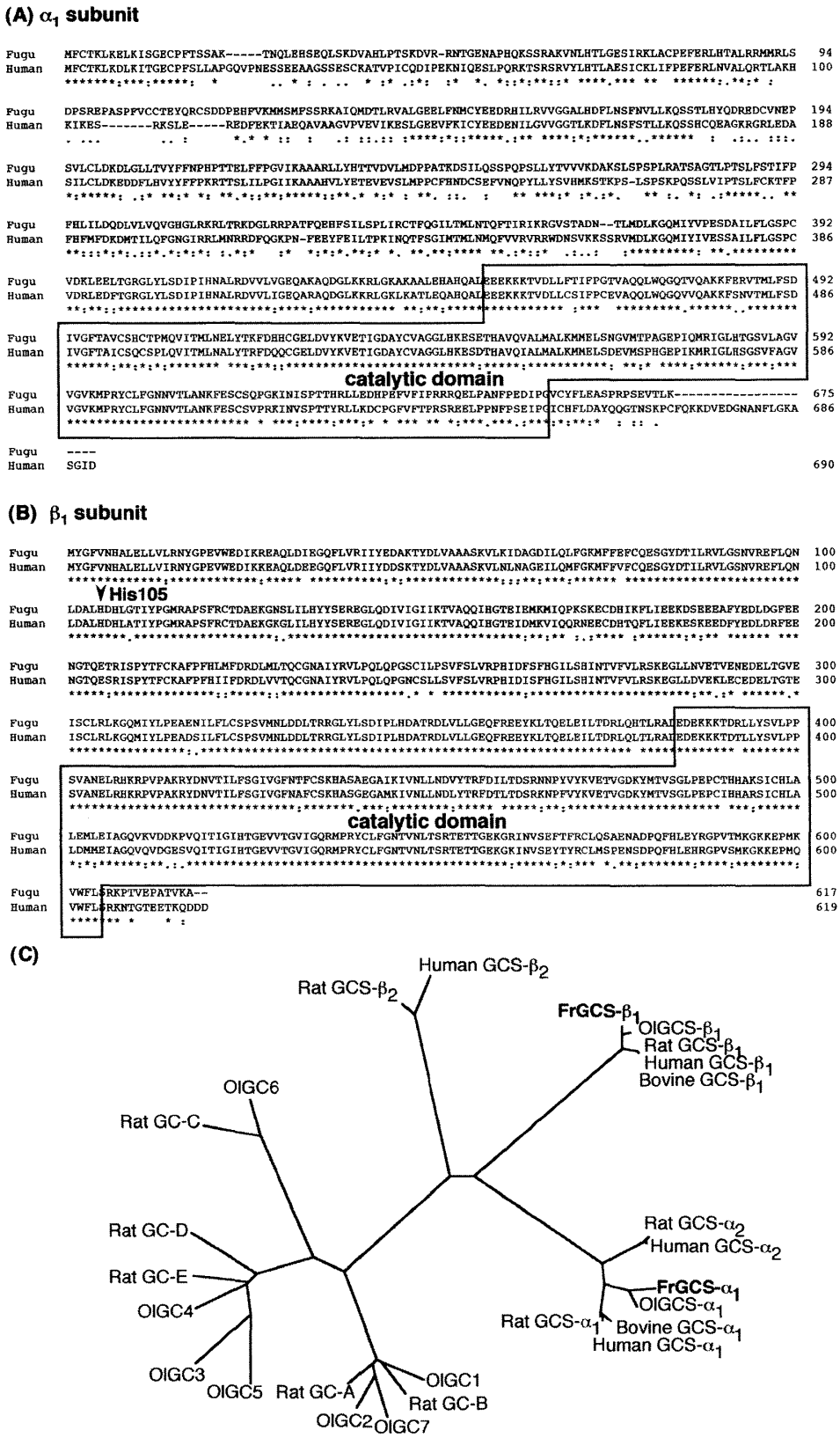

Fig. 2. Alignment of the amino acid sequences of the α_1 (A) and β_1 (B) subunits of *Fugu* and human soluble GC, and molecular phylogenetic analysis of various GCs (C). The deduced amino acid sequence of *FrGCS-α1* or *FrGCS-β1* was compared with that of the corresponding human soluble GC subunit (accession numbers; CAA75738 for α_1 and CAA47144 for β_1). Amino acids identical between the two soluble GCs are indicated with asterisks (*) below the residues. Gaps in the sequences are indicated by a dash (–). The conserved histidine residue in the β_1 subunit, which is necessary for heme binding, is indicated by an arrowhead. The putative catalytic domains are boxed. Aligned amino acid sequences of the catalytic domain of soluble GC α_1, β_1, α_2 and β_2 subunits (human, bovine, rat and medaka fish) and membrane GCs (rat and medaka fish) were subjected to phylogenetic analysis. An unrooted phylogenetic tree was constructed by the neighbor-joining method. Branch lengths were proportional to evolutionary distance.

Table 1. Exon and intron sizes in *Fugu* and human soluble GC α_1 and β_1 subunit genes. Exon and intron lengths are in bp. Human exon/intron sizes were derived from the Celera Human Genome Database.

α_1 subunit gene (6127 bp)				
	Exon size (bp)		Intron size (bp)	
Number	*Fugu*	Human	*Fugu*	Human
1	462	240	130	380
2	68	74	497	670
3	89	94	77	28577
4	710	367	773	6814
5	486	62	702	4247
6	144	59	726	2261
7	155	710	200	1846
8	908	486		3581
9		144		4735
10		155		7824
11		597		
Total	3022	2988	3105	60935

β_1 subunit gene (9601 bp)				
	Exon size (bp)		Intron size (bp)	
Number	*Fugu*	Human	*Fugu*	Human
1	447	82	290	643
2	74	74	1264	15106
3	101	101	89	2455
4	119	119	98	12072
5	198	198	879	>3596
6	231	231	141	1253
7	117	117	566	919
8	134	134	850	3367
9	198	198	1063	2267
10	238	238	569	1044
11	141	141	949	827
12	155	155	74	355
13	616	127		1159
14		519		
Total	2769	2434	6832	>45063

FrGCS-α_1 and *FrGCS-β_1* are organized in tandem and span over 15889 bp. The spacer sequence between the two genes is 161 bp.

apart and span approximately 16 kbp in the order of *FrGCS-α_1* and *FrGCS-β_1*. *FrGCS-α_1* consists of 8 exons and *FrGCS-β_1* consists of 13 exons. The GT-AG rule was maintained for all splice sites. Although a number of known *cis*-regulatory elements are present in the 5'-upstream regions of each gene, there is no TATA box consensus sequence. Comparison of the deduced amino acid sequences of *FrGCS-α_1* and *FrGCS-β_1* with those of the corresponding human soluble GC indicated that the amino acid sequences of the putative catalytic domain of FrGCS-α_1 and FrGCS-β_1 are 81% and 88% identical to those of human soluble GC, respectively (Fig. 2 (A) and (B)). Furthermore,

phylogenetic analysis of known soluble GC α_1, β_1, α_2, and β_2 subunits and membrane GCs demonstrated that the catalytic domains of α_1 and β_1 subunits are highly conserved between *Fugu* and mammals (Fig. 2 (C)).

Comparison of exon and intron sizes of *FrGCS-α_1* and *FrGCS-β_1* subunit genes with those of the human soluble GC α_1 and β_1 subunit genes based on data from the Celera Human Genome Database indicated that, in the coding region of the β_1 subunit gene, all the exon/intron boundaries are the same in both species except for the last exon, and exons 4-7 in *FrGCS-α_1* correspond to exons 7-10 in the α_1 subunit gene of human soluble GC (Table 1). The database also revealed that the human soluble GC α_1 and β_1 subunit genes are organized in tandem in the order of α_1 and β_1 with a 28 kbp spacer sequence on chromosome 4. Thus, the genomic structures of the soluble GC subunit genes are very similar in both species. These results suggest that the expression of these genes is regulated in the same manner. We referred to the Human Genome Database of Celera and NCBI for detection of the regulatory elements and found that various known *cis*-elements in the 5'non-coding region of both genes were conserved in *Fugu* and humans (Fig.1).

Sequence comparisons between *Fugu* and mammals have identified several conserved non-coding sequences (Venkatesh *et al.*, 2000). A comparison of non-coding sequences between the evolutionary distant *Fugu* and human genomes is useful for detecting conserved regulatory elements. The function of such conserved elements can be tested in cell lines or transgenic systems. Our study should help to clarify the transcriptional regulation of soluble GC. Further studies are in progress in our laboratory.

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