(TTAGGG)_n telomeric sequence in selachian chromosomes

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The distribution of telomeric sequence (TTAGGG)_n in the genomes of Chondrichthian species at different stages of evolution was investigated both by DNA genomic hybridization, and by fluorescence *in-situ* hybridization (FISH) of metaphase chromosomes. The sequence is highly conserved in all the species examined. FISH revealed a label uptake only by the telomeres in *Raja asterias*. However, in *Torpedo ocellata*, we revealed pericentromeric and interstitial sequence localization on some chromosome pairs in addition to the hybridization signal on telomeres. These findings confirm that the karyotype evolution of these fish began by Robertsonian fusion.

Keywords: Chondrichthian evolution, FISH, telomere, repeated DNA.

Introduction

All vertebrates display particular hexanucleotide repeats (telomeres) that cap their chromosomes. Telomeres are essential for chromosome function. They offer chromosomal stability and contribute to the replication of linear DNA. The DNA sequences that, together with specific proteins, make up the nuclear protein complexes of telomeres are relatively simple: these are 5–6 nucleotides rich in Guanine (G), tandemly repeated from 10 to 50 kb in different organisms (Blackburn, 1991). The telomere sequences are synthesized by an enzyme, telomerase, that recognizes the G-rich polymer of an existing telomere and lengthens it in a 5′–3′ direction by using a sequence of RNA within the enzyme as a template.

The telomere sequence studied in humans (Moyzis *et al.*, 1988) and in many other vertebrates, consists of hexanucleotide repeats (TTAGGG)_n that are not only ubiquitous in vertebrate genomes, but also show highly conserved chromosomal localization through the phylogeny of vertebrates (Kipling, 1995). However, additional interstitial localization has also been observed. These additional sites more commonly appear in pericentromeric positions, but in a variety of species they are found within, or at the borders of, constitutive C-positive heterochromatin blocks (Meyne *et al.*, 1990). Therefore, the human telomere stretch has often been used as a probe to explore problems regarding chromosome evolution during speciation and is even

more interesting in species with recognized structural rearrangements or changes in chromosome number. According to Meyne *et al.* (1990) the presence of additional interstitial sites can be correlated with the evolutionary state of the species. The most primitive species, or the species displaying a karyotype believed to be primitive, possess exclusively telomeric (TTAGGG)_n sequences, while the taxa undergoing active evolution have additional nontelomeric sites that are inclined to fuse. Highly evolved species are endowed with (TTAGGG)_n telomeric stretches and additional nontelomeric sites. During chromosome rearrangement the unnecessary nontelomeric (TTAGGG)_n sequences could have been lost and therefore reset the chromosome number with an exclusive telomere localization. Such a localization simply indicates a functional telomere, but eventual interstitial sites are interesting because they could be directly linked to the evolutionary process of the telomeric fusion of chromosomes starting with those from ancestral species (Meyne *et al.*, 1990).

The Chondrichthyes are probably the least studied class of vertebrates from the systematic/evolutionary and genome organization viewpoints. The literature provides scarce and inconclusive evidence on chromosome evolution and genome arrangement for these species.

In order to study the organization of (TTAGGG)_n repeats in the selachian genome, the human telomeric sequence was hybridized on the DNA of six species belonging to two different superorders, the Batoidea and the Galeomorphii, and on metaphase chromosomes from two species of Batoidea at different evolutionary

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stages: *Raja asterias* and *Torpedo ocellata*. The former, belonging to the Rajiformes order, considered primitive among the Batoidea, has 98 chromosomes, of which only three pairs are bi-armed. The latter has a diploid number of 66 chromosomes with six meta- and sub-metacentric pairs and belongs to the more evolved torpediniform order (Stingo & Rocco, 1991).

The present study is the first in the examined literature to assay telomeric repeats in the genome of a cartilaginous fish, and, in addition, to use *in situ* hybridization for chromosomal localization of these sequences in selachians.

Materials and methods

The present study was carried out on four species of Batoidea (*Torpedo marmorata*, *Torpedo ocellata*, *Raja asterias*, *Raja montagui*) and two species of Galeomorphii (*Mustelus asterias*, *Scyliorhinus stellaris*). *Torpedo marmorata*, *T. ocellata* and *S. stellaris* specimens were kindly provided by the 'A. Dohrn' Zoological Station of Naples. *Raja asterias*, *R. montagui* and *M. asterias* specimens were collected from the sea off Naples and in the Pozzuoli gulf.

Genomic DNA from the six species was extracted from whole blood. This was repeatedly washed in a solution of 3.2% sodium citrate, then in the same solution at 0.5%, and incubated in 1 × TE, supplemented with proteinase K (100 µg mL⁻¹) and 1% SDS at 37°C overnight. DNA was extracted with a solution of chloroform and isoamyl alcohol. The pellet obtained after precipitation of the supernatant with absolute ethanol was dissolved in 1 × TE.

The oligonucleotides (GGGTTA)₇ and (TAACCC)₇ were 3'-labelled by terminal transferase. The reaction mix included 4 µL of 5× concentrated buffer (1 M potassium cacodylate, 0.125 M Tris-HCl, 1.25 mg mL⁻¹ Bovine Serum Albumin; pH 6.6), 4 µL of 25 mM CoCl₂, 1 µL of 1 mM Dig-ddUTP, 100 pmol oligonucleotide, 50 units of terminal transferase and enough redistilled H₂O to make a final reaction volume of 20 µL. The reaction was incubated at 37°C for 15 min and then stopped with 2 µL of a glycogen-EDTA mixture (0.2 M EDTA, 20 mg mL⁻¹ glycogen solution).

Extracted DNA (5 µg) was digested with *EcoRI* restriction enzyme at 37°C overnight at a concentration of 2 U µg⁻¹ and then run on a 1.4% agarose gel in 1 × TBE and transferred onto positively charged nylon membranes in 20 × SSC.

The filter was prehybridized at 55°C in 5 × SSC, 0.02 × SDS, 0.1% N-lauroylsarcosine, 1% blocking reagent (Roche) for 4 h, and then hybridized at 50°C overnight. It was washed twice in 2 × SSC, 0.1 × SDS for 5 min at room temperature, then in 0.5 × SSC,

0.1 × SDS for 15 min at room temperature and then developed as described by the DIG System User's Guide for Filter Hybridization (Roche).

Chromosomes were obtained from testis, spleen and gills by a slightly modified method for selachians of scraping + air drying 18 h after an injection of colchicine at a concentration of 0.5 mg mL⁻¹ (0.1 mL/10 g body weight). The tissues were immersed in a hypotonic solution (0.075 M KCl + 0.5% NaCl) for 45 min and fixed in Carnoy for 30 min; they were then sieved (100 mesh sieve) and the cellular suspension obtained was centrifuged at 1000 g for 10 min. The pellet was resuspended in fresh fixative, placed on a slide and air-dried. Chromosome preparations were aged at least 1 week at room temperature or 12 h at 60°C before hybridization.

Slides containing a considerable number of metaphase plates were treated with RNase A (100 µg mL⁻¹ in 2 × SSC) and dehydrated through an ice-cold ethanol series. Chromosomal DNA was denatured at 95°C for 30 s and dehydrated in an ice-cold ethanol series. Hybridization was carried out in 60, 40 and 30% v/v formamide at 37°C in a humid chamber for 16 h. The post-hybridization washes were performed both in low (20% formamide, 2 × SSC, at room temperature) and in high stringency (20% formamide, 0.1 × SSC, at 42°C). Hybridization sites were visualized using fluorescein labelled anti-DIG antibody as described by the Boehringer Mannheim DIG-System.

Chromosomes were counterstained with DAPI (0.5 mg mL⁻¹) and propidium iodide (0.2 mg mL⁻¹), mounted in a DABCO solution, viewed under a ZEISS AXIOSCOP with the appropriate filter combination and photographed on Kodak Ektachrome 400ASA film.

Results

The telomeric probe was able to hybridize to genomic DNA from all the species analysed (Fig. 1). A probably conserved band of about 3 kb was present in almost all the selachians examined, but different intense bands, varying in length from 0.5 to 2 kb, were visible in rays. One of these, about 850 bp, was common to both rays. In torpedoes, three bands in *T. ocellata* with high molecular weight (between 2 and 3.5 kb) were more evident, while in *T. marmorata* they were less intense. Moreover, the peculiar arrangements of hybridization signals were almost absent in *M. asterias*, whereas in the other galeomorph, *S. stellaris*, three high molecular weight bands were visible (from 1.7 to 2.1 kb).

In order to study the 'human' telomeric repeats on *T. ocellata* and *R. asterias* chromosomes, from 10 to 20 metaphases per individual were analysed by FISH, for a

Fig. 1 Genomic hybridization of the (TTAGGG)₇ sequence in selachian species. M, λ /EcoRI-HindIII; Ma, *Mustelus asterias*; Ra, *Raja asterias*; Rm, *Raja montagui*; Ss, *Scyliorhinus stellaris*; Tm, *Torpedo marmorata*; To, *Torpedo ocellata*.

total of two specimens for the former and five for the latter.

With this probe, FISH produced fluorescent signals on the telomeres of both uni- and bi-armed elements of almost all chromosomes in the two species, (Fig. 2). In particular the acrocentric chromosomes revealed stronger signals on the short arm.

In *T. ocellata*, unlike the ray, there was also some interstitial and/or paracentromeric label on bi-armed chromosomes; on pair 1 an interstitial localization of the hybridization signal was visible on one of the two arms. On pairs 2, 4 and 5, a label was present on the paracentromeric site (Fig. 2 a,c).

The interphase nuclei showed different signals of varying size, generally with dense grains.

The reaction was highly specific. Any change in either hybridization conditions (from 30 to 60% formamide), or stringency washes (with or without formamide and at low or high temperature) produced no variation in the distribution of label.

Discussion

The cartilaginous fish are among the least studied vertebrates phylogenetically, both within their class

and in relationship to other vertebrates. Although investigations have been carried out in palaeontology (Estes & Sanchiz, 1982) and in comparative anatomy (Schaeffer, 1967; Compagno, 1973), our cellular and molecular understanding especially regarding the genome characteristics is scarce and fragmented.

The selachian literature reports few studies on genome size (Hinegardner, 1976; Stingo, 1979; Stingo *et al.*, 1980) and organization (Olmo *et al.*, 1982; Stingo *et al.*, 1989a,b; Stingo & Rocco, 1991; Rocco *et al.*, 1996). Taken together, these findings reveal peculiar characteristics of the selachian genome: large amount of DNA (up to 34 pg/N) and karyotypes with numerous elements (up to 104) with many microchromosomes.

The analyses carried out herein on the presence and chromosome localization of telomere sequences in the genome of cartilaginous fish extend the investigations to another class of vertebrates.

The (TTAGGG)_n sequence occurs in all examined selachian species, as seen from findings on hybridization of the probe on genomic DNA. The hybridization of the probe was first performed on the genomic DNA of *R. asterias* digested with six restriction endonucleases: *AluI*, *HindIII*, *EcoRI*, *PstI*, *TaqI*, *RsaI*. Afterwards, because only *EcoRI* yielded appreciable hybridization signals (data not shown), the probe was hybridized to the DNA of the other species digested with endonuclease *EcoRI*. Although the hybridization results obtained in all of the species show the presence of interspecific polymorphisms, no correlation can be proposed between the complexity of the bands and the chromosome number, on one hand, and the presence of telomeric interstitial sequences, on the other.

The localization of the hallmark indicator on the telomeres of many chromosomes lends further support to the hypothesis made by Meyne *et al.* (1989) according to which the sequence can be ubiquitously distributed in the telomeres of vertebrates.

The absence of nontelomeric signals in *R. asterias* chromosomes even in low stringency conditions likely indicates that there are no interstitial (TTAGGG)_n sequences on chromosomes in the ray, but it is possible that they might exist in a very low number of copies, below the resolving power of the present method.

The finding of a paracentromeric and interstitial label in *T. ocellata* on bi-armed elements and on a pair of small acrocentric chromosomes is in agreement with data in the literature on other vertebrates. According to Meyne *et al.* (1990) and Nanda *et al.* (1995), species that underwent a chromosome rearrangement as a result of Robertsonian fusion also have nontelomeric sites showing the TTAGGG sequence. This finding therefore strengthens the hypothesis (Stingo & Rocco, 1991; Stingo *et al.*, 1995) that karyotype evolution in

Fig. 2 *In situ* hybridization of digoxigenin labelled (GGGTTA)₇·(TTACCC)₇ oligomers to (a) *Torpedo ocellata* and (b) *Raja asterias* metaphase chromosomes and (c) *T. ocellata* karyotype.

cartilaginous fish occurred by a progressive fall in the diploid number because of a Robertsonian event (Robertson, 1916). In fact, the most widespread and primitive species in the different taxa of the Chondrichthyes have high diploid numbers with many telocentric elements. Conversely, as the species has become more advanced and evolved, a progressive reduction in the diploid number and an increase in the number of the biarmed chromosomes can be observed. This phenomenon appears more evident in Batoidea, especially if the karyotypes of the primitive rays and the advanced Myliobatiformes are compared. However, Robertsonian rearrangements are not unusual in vertebrate speciation (White, 1969, 1973; John & Freeman, 1975).

The more intense signals on the acrocentric chromosomes short arms revealed by FISH, may have resulted

from longer stretches in the marked sites. This interpretation is supported by studies performed on mice by Starling *et al.* (1990), showing that TTAGGG sequences are hypervariable.

The presence of additional interstitial sites of the TTAGGG sequences in *T. ocellata* and the absence in *R. asterias* indicates that, from a karyological point of view, the torpedo, unlike the ray, is in an evolutionary phase. Furthermore, according to Meyne *et al.* (1990) the rearrangement and the amplification of interstitial sites of the telomeric sequences confer greater flexibility to the karyotype in the face of chromosomal shuffling, in that they furnish alternative sites to form telomeres. Whatever the case, the interstitial sites with the (TTAGGG)_n sequences are involved in recombination events; moreover interstitial localization at aberrant

chromosomal break points has been observed and it has been proposed that telomeric sequences behave like fragile sites (Bouffler *et al.*, 1993).

Because the literature contains no mention of the sequence location in other Chondrichthyes, it is not possible to compare the findings from this report with those on other cartilaginous fish. A look at another class of fish, the Osteichthyes, however, is interesting (Table 1).

Telomeric sequences (TTAGGG)_n have so far been studied in 10 species of teleosts belonging to four orders: Anguilliformes (Salvadori *et al.*, 1995), Salmoniformes (Abuin *et al.*, 1996; Perez *et al.*, 1999), Cyprinodontiformes and Cypriniformes (Meyne *et al.*, 1989) and in five species of Chondrostei belonging to the Acipenseriformes order (Fontana *et al.*, 1998; Tagliavini *et al.*, 1999). Interstitial telomeric sites have been observed in two salmon species and in a sturgeon: in *Salmo salar* (Abuin *et al.*, 1996), within interstitial heterochromatin blocks in some large chromosomes and in *Oncorhynchus mykiss* (Abuin *et al.*, 1996), on the

short arms of a NOR-bearing chromosome pair. In *Acipenser gueldenstaedti* the sequence hybridization signals are also localized on a GC-rich heterochromatic chromosome pair (Fontana *et al.*, 1998).

Conclusions

This is the first evidence of chromosomal localization of telomeric repeats in Chondrichthyes. Telomeric sequences (TTAGGG)_n are present in all the examined selachian species. These belong to four genera of two different superorders and it is therefore likely that these stretches could be ubiquitous in cartilaginous fish.

The interstitial sequence revealed in *T. ocellata* strengthens the hypothesis already advanced by Stingo *et al.* (1989b, 1995) on the presence of Robertsonian fusion during the karyotype evolution of these vertebrates. Further studies should elucidate the chromosomal location of these sequences both in congeneric species and in other Chondrichthyan superorders.

Table 1 *In situ* localization of telomeric sequence (TTAGGG)_n in fishes so far studied

Class Order Species	(TTAGGG) _n sequence localization		References
	Telomeric	Interstitial	
Osteichthyes			
Anguilliformes			
<i>Anguilla anguilla</i>	+		Salvadori <i>et al.</i> (1995)
<i>A. rostrata</i>	+		Salvadori <i>et al.</i> (1995)
<i>Muraena helena</i>	+		Salvadori <i>et al.</i> (1995)
Cyprinodontiformes			
<i>Gambusia affinis</i>	+		Meyne <i>et al.</i> (1989)
Cypriniformes			
<i>Eigenmannia virescens</i>	+		Meyne <i>et al.</i> (1989)
<i>Eigenmannia</i> sp.	+		Meyne <i>et al.</i> (1989)
Salmoniformes			
<i>Salmo salar</i>	+	+	Abuin <i>et al.</i> (1996)
	+		Perez <i>et al.</i> (1999)
<i>S. trutta</i>	+		Abuin <i>et al.</i> (1996)
<i>Oncorhynchus mykiss</i>	+	+	Abuin <i>et al.</i> (1996)
<i>O. kisutch</i>	+		Abuin <i>et al.</i> (1996)
Acipenseriformes			
<i>Acipenser naccarii</i>	+		Fontana <i>et al.</i> (1998)
<i>A. ruthenus</i>	+		Fontana <i>et al.</i> (1998)
<i>A. gueldenstaedti</i>	+	+	Fontana <i>et al.</i> (1998)
<i>A. baeri</i>	+		Fontana <i>et al.</i> (1998)
<i>A. sturio</i>	+		Tagliavini <i>et al.</i> (1999)
Chondrichthyes			
Torpediniformes			
<i>Torpedo ocellata</i>	+	+	This paper
Rajiformes			
<i>Raja asterias</i>	+		This paper

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