An analysis of coho salmon chromatin by means of C-banding, AG- and fluorochrome staining, and *in situ* digestion with restriction endonucleases

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The chromosome complement of the coho salmon (*Oncorhynchus kisutch*) has been analysed by means of C-banding, silver and fluorochrome staining, and *in situ* digestion with restriction endonucleases. C-banding shows heterochromatic regions in the centromeres of most chromosomes but not in the telomeric areas. The fifteenth metacentric chromosome pair contains a large block of constitutive heterochromatin, which occupies almost all of one chromosome arm. This region is also the site where the ribosomal cistrons are located and it reacts positively to CMA₃/DA fluorochrome staining. The NORs are subject to chromosome polymorphism, which might be explicable in terms of an amplification of ribosomal cistrons. The digestion banding patterns produced by four types of restriction endonucleases on the euchromatic and heterochromatic regions are described. Two kinds of highly repetitive DNAs can be distinguished and the role of restriction endonucleases as a valuable tool in chromosome characterization studies, as well as in the analysis of the structure and organization of fish chromatin, are also discussed.

Keywords: C-banding, coho salmon, fluorochrome staining, restriction endonuclease banding.

Introduction

The use of restriction endonucleases (REs) is becoming common not only in molecular biology but also as an important tool in molecular cytogenetics. Chromosome banding patterns induced by restriction endonuclease digestion have led to the identification of animal satellite DNA regions (Miller *et al.*, 1983; Bianchi *et al.*, 1985; Mezzanotte, 1986), although certain factors affecting enzyme activity still remain unclear.

Salmonids constitute one of the most extensively analysed fish groups in cytogenetic terms mainly due to their ancestral polyploid origin. Nevertheless, with salmon belonging to the genus *Oncorhynchus*, chromosome differentiation methods have only been used to locate the NOR and the CMA₃⁺ regions (Phillips *et al.*, 1986). Thus, to characterize more accurately the chromatin of one of these species, the coho salmon

(*Oncorhynchus kisutch*), as well as applying conventional banding techniques, we have analysed the mitotic chromosomes using DNA base-pair-specific fluorochromes and *in situ* digestion with restriction endonucleases. Three new observations resulted from the application of these latter techniques to coho salmon chromosomes: (i) extensive chromosome polymorphism affecting the NORs, which we attribute to an amplification phenomenon of the ribosomal cistrons; (ii) certain peculiarities in the NOR-associated heterochromatin compared to the remaining heterochromatin and euchromatin; and (iii) cytogenetical differences between centromeric and telomeric heterochromatin.

Our results show that REs are useful in the characterization of fish chromatin, particularly in fish species of polyploid origin.

Materials and methods

Thirty-six specimens of coho salmon, imported as eggs at eye-stage from the USA (Oregon Aquafood) and reared in a Spanish fresh-water fish farm (Marcultura, SA, Lugo), were employed in this study.

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Cell collections and chromosome preparations

Living specimens were intraperitoneally injected with a yeast solution and 48 h later were colchicined according to the method of Lozano *et al.* (1988). The kidneys were removed and cell suspensions prepared by standard fixation. Other metaphase cells were obtained from lymphocyte cultures following the protocol of Lloyd & Thorgaard (1988) with some modifications (Sánchez *et al.*, 1990). Chromosome preparations were made according to the steps described by Lozano *et al.* (1988) and one slide per individual was stained with 10 per cent Giemsa in phosphate buffer, pH 6.8, for karyotyping.

Chromosome banding methods

The method of Schwarzscher *et al.* (1980) was used to detect constitutive heterochromatic regions. NORs were visualized following the technique of Howell & Black (1980). Chromomycin A₃/distamycin A and DAPI/actinomycin D counterstaining methods (Schweizer, 1976) were performed in order to locate the GC- and AT-rich chromosome regions respectively. *In situ* digestion with various restriction endonucleases was carried out by adding 30 μ l of the specific enzyme solution (suspended in the appropriate buffer) to the chromosome preparations, covering them with 24 $\times$ 32 mm coverslips and incubating the slides for 16 h at 37°C in a moist chamber. The concentration of the endonucleases varied from 0.5 to 1.5 units μ l⁻¹, depending on their activity. After incubation the slides were carefully washed with distilled water and stained with 5 per cent Giemsa in phosphate buffer, pH 6.8. Preparations of the same individuals were simultaneously treated with the buffer alone as a control.

Some of the slides were destained with Carnoy fixative for a few minutes and sequentially stained with fluorochromes or silver nitrate.

Results

Giemsa karyotype

The coho salmon diploid complement is made up of 60 chromosomes (Fig. 1); 42 of them (pairs 1–21) are medium and large metacentric or submetacentric chromosomes and 18 (pairs 22–30) are small telocentric or subtelocentric ones, although pairs 22 and 23 are submetacentric. Two-thirds of the individuals show heterozygosity for the fifteenth pair, characterized by the presence of a metacentric chromosome and one clearly submetacentric one. The remaining individuals were homozygotes with two identical metacentric chromosomes in the fifteenth pair. The NF was 106 in all the cells. Conventional Giemsa staining does not permit the identification of the various chromosome pairs.

C-banding

All the metacentric chromosomes carry heterochromatic centromeric blocks, which vary in size and staining intensity. The fifteenth pair stands out, showing a wide region of constitutive heterochromatin, which occupies almost all of one chromosome arm but not the centromere (Fig. 2). The size of this region differs in individuals heterozygous for the polymorphism mentioned above. A few thin telomeric bands can be seen on some of the pairs, especially the 27th (Table 1 and Fig. 2).

Fig. 1 Giemsa karyotype of a coho salmon ($2n = 60$) homozygous for the fifteenth polymorphic pair. Bar represents 10 μ m.

Fig. 2 C-banded karyotype of *O. kisutch*, heterozygous for the fifteenth pair.

Table 1 Summarized results obtained by means of C-banding and *in situ* digestion with various restriction endonucleases on different kinds of euchromatin in *O. kisutch*

Treatment	Target	Heterochromatin			Euchromatin
		Centromeres	Telomeres	NOR-associated	
C-banding*	—	+†	—	+	—
Alu I/ Mbo I‡	AG/CT /GATC	+†	—†	—	+§
Hae III/ Hinf I‡	GG/CC G/ATTC	+	+	+¶	+
Dde I‡	C/TNAG	+†	—†	+¶	+§
Bgl II/ Hind III‡	A/GATCT A/AGCTT	0	0	0	0

*+ denotes presence and — absence of positive C-bands.

†The majority of the centromeres and/or telomeres.

‡+ denotes digested regions, — undigested regions and 0 denotes homogeneous digestion of the whole karyotype.

§Some intercalary undigested bands also appear.

¶Region differentially digested compared to the rest of the heterochromatin.

NOR silver staining

The silver-stained karyotype of coho salmon, homozygous for the fifteenth pair, shows two metacentric chromosomes with interstitially or subterminally located ribosomal cistrons, depending on the individual (Figs 3a and b and 4a). For this reason the NORs are heteromorphic in position and even in size. In heterozygotes for the polymorphic pair, only one such metacentric chromosome can be seen, together with a subtelocentric one, with NOR activity located at the end of the short arm (Fig. 4b). The NOR-bearing chromosomes correspond to those with the large heterochromatic blocks, i.e. the fifteenth pair.

Fluorochrome staining

Particularly brilliant interstitial CMA₃/DA clusters were found in two medium-sized metacentric chromo-

somes in homozygous individuals (Figs 3c and 6b), while heterozygotes carried fluorescent regions in one metacentric and one subtelocentric chromosome. Chromomycin staining of metaphases previously treated with silver nitrate showed that these chromosomes were the same as those which showed NOR activity, and thus CMA₃⁺ regions flanking the secondary constrictions were observed on these chromosomes (the less condensed the chromosome the more clearly visible the regions). Treatment with the DAPI/AMD combination did not produce any differential staining of the mitotic chromosomes of *O. kisutch*.

Restriction endonuclease banding

Four groups of restriction endonucleases can be described on the basis of the digestion banding pattern that they produce on fixed chromosomes of coho salmon. In Table 1 the effects of these enzymes on the

Fig. 3 Mitotic metaphase of *O. kisutch* shown by silver-nitrate staining (a) and details of the fifteenth, NOR-bearing, chromosome pair (b) showing several positive CMA₃ regions flanking the duplicated NORs (c). Arrowheads indicate the NORs.

Fig. 4 Somatic metaphases of two coho salmon: one carries heteromorphism for the position of the NORs on the fifteenth pair (a) and the other is heterozygous for the NOR polymorphism (b). Details of the NOR-bearing chromosomes (c). Arrowheads indicate the NOR-bearing chromosomes and the bar represents 10 µm.

different kinds of heterochromatic regions (centromeres, telomeres and NOR-associated) and on euchromatin are summarized. It is worthwhile emphasizing that Alu I and Mbo I generate a well-defined banding pattern in which the NOR-associated heterochromatin and most of the telomeres remain undigested (Figs 5, 6a and b), while Hae III and Hinf I digest these areas selectively (Fig. 6d). The cytogenetical behaviour of Dde I falls between these two types of enzymes (Fig. 6c), and no longitudinal differentiation is induced by Bgl II or Hind III.

Discussion

Our conclusions concerning the karyotype of *O. kisutch* concur with those of Uyeno (1972), who describes the existence of a complement composed of 46 metacentric and submetacentric and 14 subtelocentric and telocentric chromosomes (NF=106) in

Japanese coho salmon, but do not coincide with the results of Ueda (1983), working also with Japanese specimens (48 M+SM and 12 ST+T, NF=108). The reasons for these discrepancies may be due to the denomination of one of the smallest chromosome pairs (subtelocentric or submetacentric?) or else to chromosome rearrangements which are frequent in the salmonid group. The possibility that the fish analysed by each author belonged to different chromosome races must also be considered.

The variability in the NOR-bearing pair could be due to in-tandem duplication. If this is the case, the original NORs would be located on the short arms of a subtelocentric pair, just as Phillips *et al.* (1986) found. A progressive amplification involving the ribosomal cistrons would then originate a new chromosome morphology, i.e. a metacentric chromosome with duplicated NORs (several silver-precipitated bands) along one of the arms. We have detected homozygous and

Fig. 5 Karyotype of coho salmon chromosomes after digestion with Alu I restriction endonuclease. This karyotype belongs to a heterozygous individual for the fifteenth pair (underlined).

heterozygous individuals for this new NOR-bearing chromosome type, depending on whether this amplification affects either one or two of the original NOR chromosomes. In the same way, a different activation status in the functional duplicated ribosomal cistrons would be responsible for the previously mentioned heteromorphism. Nevertheless, inversions, translocations, mobile genetic elements, or a combination of any of these processes, cannot be ruled out as possible explanations for these phenomena in *O. kisutch*, just as Wiley *et al.* (1989) have proposed in diploid-tetraploid treefrogs. It is worth bearing in mind that it is quite common in certain fish species for a number of ribosomal genes in the NORs to have increased and various chromosomal rearrangements to have taken place during their evolution (Foresti *et al.*, 1981). In the salmonid group, as well as in other teleostean fishes of tetraploid origin (Sola *et al.*, 1986), a diploidization process involving the rDNA zones must have occurred at a time when many genetic changes affecting the NORs (Robertsonian and others) were taking place. The NORs of this vertebrate group are variable chromosome regions (Mayr *et al.*, 1988; Phillips & Kapuscinski, 1987; Phillips *et al.*, 1988).

Two types of heterochromatin can be distinguished in coho salmon mitotic chromosomes on the basis of their response to conventional banding treatments (C-, Ag- and fluorochrome banding) and *in situ* RE digestion: the NOR-associated heterochromatin and the remaining heterochromatic zones (centromeres and telomeres). At the chromosome level, repetitive DNA

associated with the nucleolar organizer has certain specific characteristics compared to the rest of the DNA: it is GC-rich, as shown by C-banding and its high affinity for CMA3 staining, and it is resistant to endonuclease digestion with Alu I and Mbo I while being very sensitive to treatment with Dde I, Hae III and Hinf I. This differential behaviour demonstrates the characteristic nature of the organization and/or structure of the chromatin in this zone, regardless of its base sequence. Furthermore, *in situ* digestion with Dde I provides some evidence of the peculiarities of the heterochromatin located at the NORs. The NOR-heterochromatin is sensitive to Dde I while the remaining heterochromatic areas resist this enzyme treatment. It would also appear that the structure and/or organization of the chromatin differs between the centromeric and telomeric chromosome areas of salmonids. This idea has also been suggested by Cau *et al.* (1988) in another fish species of the family Muraenidae.

Several authors have found a close correlation between C-banding and restriction banding patterns (see for example Cau *et al.*, 1988; Lloyd & Thorgaard, 1988; Schmid & de Almeida, 1988), although some of them consider that this correlation is not entirely exact (Lloyd & Thorgaard, 1988; López-Fernández *et al.*, 1989). Our findings in *O. kisutch* indicate notable differences between the banding methods. Thus, while C-banded mitotic chromosomes show heterochromatic regions located mainly at the centromeres and only a few telomeric areas appear to be positively stained, the banding patterns generated both by Alu I and by Mbo I

Fig. 6 Metaphase chromosomes of *O. kisutch* digested with different enzymes: Mbo I (a), Alu I and sequentially stained with CMA₃/DA (b), Dde I (c) and Hae III (d). Arrowheads indicate the NOR-associated heterochromatic regions. Bar represents 10 μ m.

produce a clear longitudinal differentiation in the majority of the chromosomes, which permits the identification of different homologous pairs (compare Figs 1 and 5), according to the size and position of interstitial and terminal undigested bands and the presence or absence of digested centromeres. Thus the presence of a positively stained centromeric region distinguishes the eighth pair from the ninth, although both are carriers of intercalary bands near their telomeres. Pairs number 12 and 13 differ in the intensity of their telomeric bands. The seventeenth is one of the most characteristic chromosome pairs due to a clear gap located close to the centromere (Fig. 5). Distinctions can also be made between some of the

pairs that belong to the subtelocentric and telocentric group, e.g. the 23rd, with its large Alu I-resistant region that occupies almost all of the long arm, the 24th with its lack of any region resistant to Alu I or Mbo I, the 27th with a clear heteropycnotic centromere, and the 30th which shows a distinctive telomeric area resistant to RE digestion.

Moreover, the existence of intercalary bands resulting from Alu I or Mbo I digestion, but absent after C-banding, could indicate that although it is not possible to obtain a clear banding pattern on the salmonid euchromatin by means of proteolytic enzyme digestion (G-banding), the euchromatin of this group of fish is susceptible to banding by means of certain

restriction endonucleases. The absence of G-banding in some fish species has been attributed to a deficiency in heavy isochores (homogeneous GC-rich chromosome regions) and the lack of compartmentalization of their genomes (Medrano *et al.*, 1988). Nevertheless, RE treatments lead to reproducible banding of the chromosomes which, when followed by C-, Ag- or fluorochrome banding, makes this technique an important tool for the analysis of chromosome structure and organization, as well as chromosome evolution, in the salmonid group.

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