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Nucleotide sequence organization in the very small genome of a tetraodontid fish, *Arothron diadematus*

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We have investigated the sequence organization of the very small genome (DNA content/haploid cell $c = 0.4 - 0.5$ pg) of a tetraodontid fish, *Arothron diadematus*, by using two main experimental approaches. The first one, renaturation kinetics, showed that slowly reassociating, intermediate, fast and foldback sequences represented 87 %, 7 %, 5 % and 1 %, respectively, of *A. diadematus* DNA, which is, so far, the vertebrate DNA lowest in repeated sequences. The second approach, centrifugation in Cs_2SO_4/BAMD density gradients [BAMD = bis(acetatomercurimethyl)dioxane], showed that *A. diadematus* DNA can be resolved into several components, characterized by buoyant densities of 1.700, 1.704_5, 1.708, 1.712 and 1.723 g/cm^3, and representing 15 %, 73 %, 4 %, 4 % and 2.5 %, respectively, of total DNA. The last component comprised a satellite DNA and ribosomal DNA. A family of interspersed repeats, possibly related to the *Alu*I family of warm-blooded vertebrates, showed an extremely specific genomic distribution, being present in only the 1.708 g/cm^3 component, which it matched in base composition.

Investigations carried out in our laboratory using equilibrium centrifugation of DNA in density gradients containing DNA ligands [1 – 3] have provided novel information on the genome organization of vertebrates and shown remarkable differences between warm-blooded and cold-blooded vertebrates [4 – 6].

Neglecting satellite and minor components, the nuclear DNA of warm-blooded vertebrates can be fractionated into four major components. The two light ones represent about two-thirds of the DNA and range in buoyant density over 1.697 – 1.703 g/cm^3; the two heavy ones, accounting for the remaining third, have densities of 1.704 and 1.708 g/cm^3, respectively; these heavy components are mainly responsible for the asymmetry on the heavy side of CsCl bands exhibited by the DNAs of warm-blooded vertebrates. The major components are families of DNA fragments derived by preparative breakdown from corresponding families of very long (> 300000 bases) DNA segments, the isochores, which are fairly homogeneous in base composition and which may be responsible for chromosomal Giemsa and reverse bands [7]. The physical separation of major components from DNAs of man, mouse and chicken allowed us to show that the sequence organization of these genomes, as judged by the distribution of repeated sequences, is different both in isopycnic DNA components of these three species and in different components of the same genome [8,9]. Moreover, we have shown that the genomic distribution of specific families of interspersed repeats is non-uniform and evolutionarily conserved. For instance, the long repeats of the *Bam*HI family of the mouse are almost exclusively present in the two light components of this genome [10]; likewise, the homologous [11] long repeats of the *Kpn*I family of man are absent from the heaviest component [12]. On the other hand, the short repeats of the *Alu*I family are predominantly present in the two heavy components of the human and mouse genome [12] and almost exclusively present in the heaviest component of the chicken genome [13]. Since there is an increasing body of evidence pointing to the mobility of interspersed repeats in the genome, these results suggest that integration of such mobile repeats is targeted towards specific chromosomal domains. Very interestingly, both repeated sequences and polypeptide-coding sequences show a remarkable match in base composition with the isochores in which they are embedded [10,12 – 15] indicating that sequence homology plays an important role in targeted integration.

In contrast, the DNAs of most cold-blooded vertebrates show, as a rule, a small asymmetry of their CsCl bands, a small intermolecular compositional heterogeneity and buoyant densities within the range of the light components of warm-blooded vertebrate DNAs [4,16,17]. There are, however, exceptions to this general rule, in that modal buoyant densities higher than 1.703 g/cm^3 and/or relatively high asymmetries on the heavy side of CsCl bands have been found in the DNAs from urodeles and reptiles [4] (and unpublished results), from cartilaginous fishes [16,17] and from two orders of bony fishes, Gadiformes and Tetraodontiformes [17]. These exceptions to the rule deserve to be investigated further in order to obtain a better understanding of the origin of heavy components and of genome organization and evolution in vertebrates, in general.

Here we have studied one such DNA, that of *Arothron diadematus*. The main reason for this choice was that the family of Tetraodontidae, to which *A. diadematus* belongs, is characterized by the smallest genome size of all vertebrates. Its c value, the DNA content per haploid cell, is only 0.4 – 0.5 pg, about 14 % of that of a mammal [18]. One could, therefore, hope to obtain a simpler picture from this genome than from the more complex ones mentioned above. Another reason is that the order Tetraodontiformes is one of the two teleost orders (the other one is Gadiformes) comprising families characterized by DNAs exhibiting a wide range of modal

Abbreviations. c, DNA content per haploid cell; BAMD, bis(acetato-mercurimethyl)dioxane; r_t, molar ligand/nucleotide ratio.

buoyant densities (1.700 – 1.705 g/cm^3); in fact, even different species of the family Tetraodontidae exhibit this phenomenon, although to a lesser degree [16,17]. These data suggest the existence of a taxonomical problem, which could hopefully be solved by further DNA homology studies.

MATERIALS AND METHODS

The origin of the *Arothron diadematus* specimen, the DNA preparation and the methodology used in the present work have been described already [7,16]. M_r, the relative molecular mass, was calculated from the sedimentation coefficient, $s_{20,w}$, using the relationship of Eigner and Doty [19]. ϱ_0 is the modal buoyant density, $\langle\varrho\rangle$ the mean buoyant density (for the definition of ϱ_0 and $\langle\varrho\rangle$, see [4]). $\langle\varrho\rangle - \varrho_0$ is the asymmetry of the CsCl band. H is the intermolecular heterogeneity, as defined in [20]. dG + dC content was calculated from ϱ_0 using the relationship of Schildkraut et al. [21] for bacterial DNAs.

Cloning experiments. *Hae*III fragments were tailed with [^{3}H]dCTP using terminal deoxynucleotidyltransferase and annealed with plasmid pBR322 elongated with [^{3}H]dGTP at the *Pst*I site. These plasmids were used to transform competent *Escherichia coli* HB101 cells [10]. Gel electrophoresis, labelling of DNA probes by nick-translation, hybridization experiments after transfer of DNA fragments on nitrocellulose filters and autoradiography were done as previously described [10].

DNA samples to be used in reassociation kinetics were degraded to a weight-average single-stranded length of 150 – 200 nucleotides in a Virtis homogenizer (Gardiner, NY, USA). The experimental procedure used in reassociation kinetics was that already described [8].

RESULTS

The basic properties of the DNA sample studied here are given in Table 1. *Arothron diadematus* DNA has a relatively high modal buoyant density ($\varrho_0 = 1.702_5$ g/cm^3) and a large asymmetry of its CsCl band, which is distinctly non-gaussian and skewed on the heavy side (Fig. 1). Its intermolecular heterogeneity is high, $H = 2.7\%$.

When centrifuged in a preparative Cs_2SO_4/BAMD density gradient at $r_f = 0.14$ (r_f is the molar ligand/nucleotide ratio), the DNA sample was resolved into fractions exhibiting different buoyant densities (Fig. 1 and Table 2). The pelleted DNA (21% of total) had a modal density of 1.700 g/cm^3, but apparently only two-thirds of the pellet had such a density, the rest being centered around 1.703 – 1.704 g/cm^3 (Fig. 1). About 67% of the DNA appeared to be rather homogeneous, and to have a density of 1.704 – 1.705 g/cm^3 (fractions A and B). Finally, the top fraction, C, only representing 11% of DNA, exhibited a main peak centered at 1.708 g/cm^3 skewed on the heavy side, and a satellite peak having a density of 1.723 g/cm^3. These two components represented about 9% and 2%, respectively, of the DNA. When obtained at an r_f of 0.18, the top fraction, C', representing 7% of DNA, no longer contained the 1.708 g/cm^3 peak, but showed instead a 1.712-g/cm^3 peak, apparently responsible for the asymmetry of the 1.708-g/cm^3 peak of fraction C, and an increased relative amount of the satellite peak centered at 1.723 g/cm^3 (Fig. 1). These two components represented about 4% and 3%, respectively, of the DNA. To sum up these results (see Fig. 1 and Table 2), *A. diadematus* DNA appears to be formed by a main component

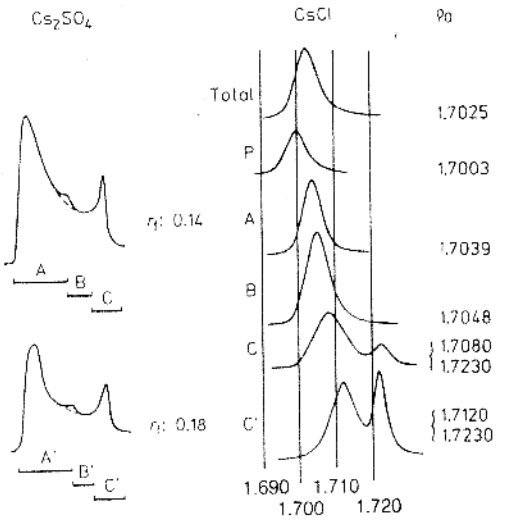

Fig. 1. *Fractionation of* A. diadematus *DNA in* Cs_2SO_4/*BAMD density gradients. A. diadematus* DNA (5 A_{260} units) was centrifuged in 0.1 M Na_2SO_4, 5 mM $Na_2B_4O_2$, pH 9.2, containing BAMD ($r_f = 0.14$ or $r_f = 0.18$) and Cs_2SO_4 ($\varrho = 1.47$ g/cm^3) using a Beckman type 50Ti rotor at 25 °C for 96 h at 35000 rev./min. Transmission at 253.7 nm is shown on the left panel. Letters indicate pooled fractions. The small peak at the border of fractions A and B is an artefact, as shown by its variable amount in different experiments. The right panel shows the analytical CsCl profiles of total, unfractionated *A. diadematus* DNA, of pellet (P) and fractions A – C, as obtained at $r_f = 0.14$, and of fraction C', as obtained at $r_f = 0.18$ (see also Table 2). Values of ϱ_0 are given in g/cm^3

Table 1. *Properties of* A. diadematus *DNA*
The data are taken from [16]; also see Materials and Methods

Property	Value
$s_{20,w}$	19.8 S
M_r	6.7×10^6
ϱ_0	1.702_5 g/cm^3
$\langle\varrho\rangle$	1.703_6
$\langle\varrho\rangle - \varrho_0$	1.1 mg/cm^3
H	2.7%
dG + dC	44.5%
c	0.4 – 0.5 pg

centered at 1.704_5 g/cm^3 and representing about 73% of the total and by about 15% of light DNA (1.700 g/cm^3), 4% of a 1.708-g/cm^3 component and by two heavier components (1.712 and 1.723 g/cm^3), representing about 4% and 2.5% of total DNA. The latter component was characterized by a very sharp peak in CsCl.

The reassociation kinetics (Fig. 2 and Table 3) indicated the presence in *A. diadematus* DNA of only 1% foldback sequences, and of about 5% and 7% sequences reassociating at a fast and at an intermediate rate, respectively. Slowly reassociating DNA represented about 87% of total DNA.

A search for interspersed and clustered repeats, whose presence in *A. diadematus* DNA was indicated by the reassociation kinetic study and by the sharp 1.723-g/cm^3 peak in CsCl, was done by investigating restriction digests. *Hae*III digests of unfractionated DNA revealed, beside the expected smear, a series of three bands, corresponding to repeated fragments of 150, 185 and 190 base pairs (Fig. 3). These bands corresponded to 1 – 1.5% of total DNAs, as estimated by both

Table 2. *Relative amounts of* A. diadematus *DNA in* Cs_2SO_4*/BAMD fractions*

$r_f = 0.14$			$r_f = 0.18$		
fraction	amount	ϱ_0	fraction	amount	ϱ_0
	%	g/cm³		%	g/cm³
Pellet	21 (15)	1.7003	Pellet	50	
	(6)	1.7035			
A	51	1.7039	A′	20	
B	16	1.7048	B′	10	
C	11 (9)	1.7080	C′	7 (4)	1.712
	(2)	1.7230		(3)	1.723
Yield	99			95	

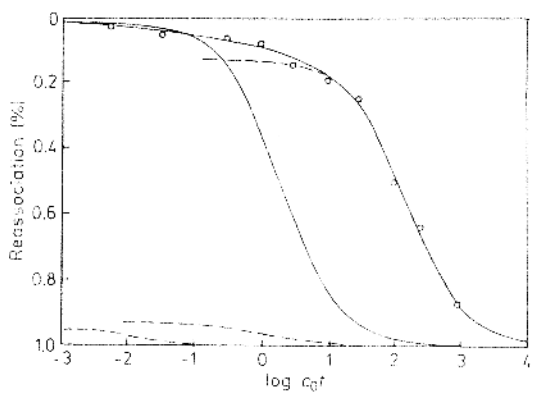

Fig. 2. *Reassociation kinetics of* A. diadematus *DNA*. Results with *Escherichia coli* DNA (– – –) are shown for comparison. The solid line through the experimental points (○) is the overall profile resulting from the analysis of kinetic classes (– – – –) (see [8]). $c_0t_{1/2}$ values are given in mol nucleotide · l⁻¹ · s

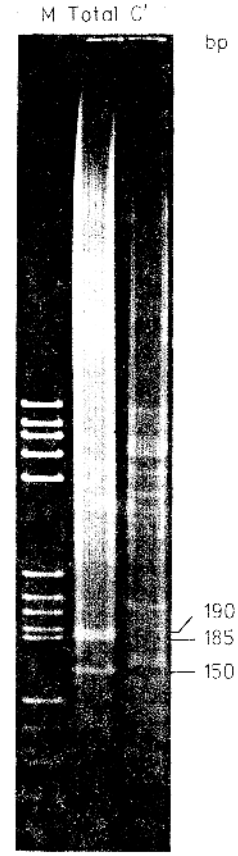

Fig. 3. *Gel electrophoresis of total* A. diadematus *DNA and of fraction C′ as obtained by preparative centrifugation in* Cs_2SO_4*/BAMD gradient,* $r_f = 0.18$ *(see Fig. 1).* DNA samples (5 µg) were degraded by *Hae*III and electrophoresed on a 4–15% polyacrylamide gel. Molecular mass markers (M) are *Hae*III fragments of plasmid pBR322; load of marker DNA was 1 µg. bp = base pairs

Table 3. *Kinetic classes in* A. diadematus *DNA*

Class	Amount	$c_0t_{1/2}$	Frequency	Complexity
	%	M · s		base pairs
Foldback	1			
Fast	5	0.01	16000	1.4×10^3
Intermediate	7	1.1	145	2.2×10^5
Slow	87	160	1	3.9×10^8

microdensitometry and comparison of the intensity of the bands with those of DNA marker bands in the same molecular mass range. On the other hand, *Hae*III digests of fraction C′, the top fraction obtained at an r_f of 0.18, showed a series of bands (Fig. 3), confirming the idea that the satellite peak of fraction C, further enriched in fraction C′, corresponded to highly repeated, clustered sequences. Hybridization experiments with a ribosomal probe from *Xenopus laevis* [22] produced a high-molecular-mass smear in the *Hae*III digest of fractions C and C′ only (not shown).

The repeated fragments of the *Hae*III bands were further studied by eluting them from the gel and by using them as probes on restriction digests of *A. diadematus* DNA (Fig. 4). When the 185-base-pair fragment was used on *Hae*III digests, the probe showed the expected annealing with the repeated fragments, which were, however, not resolved on the autoradiograms. When used on *Alu*I digests, the hybridization band corresponding in size to those seen in *Hae*III digests was accompanied by two additional bands of 380 and 570 base pairs. Finally, when *Msp*I digests were used, a very high-molecular-mass hybridization smear was obtained, indicating either an absence of *Msp*I sites in clustered repeated sequences, or an interspersion of the repeated with non-repeated sequences. Results very similar to those of Fig. 4 were obtained in parallel experiments (not shown) with the 190-base-pair fragment.

Further investigations on the *Hae*III repeats were done using cloned 150-base-pair or 185-base-pair fragments as a probe. When a *Hae*III digest of unfractionated DNA was used, hybridization took place at the level of the three bands (Fig. 5). Under the conditions of long exposure of Fig. 5, individual bands were not resolved, but a minor 380-base-pair band became apparent on the autoradiogram. When *Hind*III digests of total DNA and of the Cs_2SO_4/BAMD fractions were used, smears covering a broad molecular mass region were obtained with total DNA and with fraction C only among the fractions obtained at an r_f of 0.14. In the case of the fractions obtained at an r_f of 0.18, only the pellet showed a hybridization smear. High-molecular-mass smears were also obtained when hybridizing any one of the three cloned fragments on *Eco*R1,

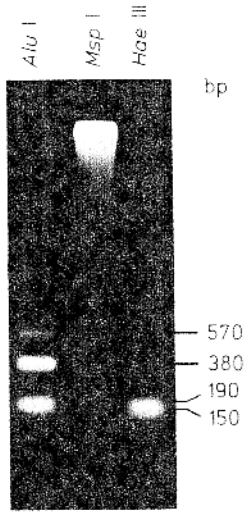

Fig. 4. *Hybridization of the* Hae*III 185-base- pair fragments from the experiment of Fig. 3 on three restriction digests from the* A. diadematus *DNA. Alu*I, *Msp*I and *Hae*III digests of 5 µg each of DNA were electrophoresed on a 0.8% agarose gel, transferred on nitrocellulose filters and hybridized with the probe. bp = base pairs

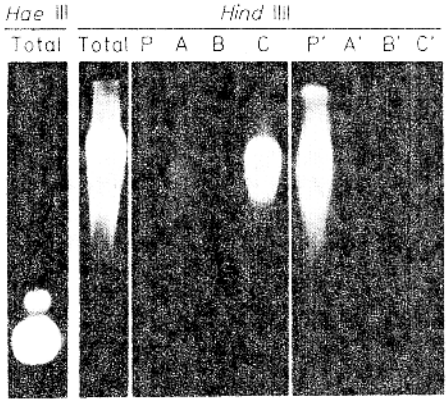

Fig. 5. *Hybridization of cloned* Hae*III 150-base-pair repeated fragments from* A. diadematus *DNA on* Hae*III digests of total DNA and on* Hind*III digests of total DNA and* Cs_2SO_4*/BAMD fractions (see Fig. 1).* Total DNA: 5 µg; Cs_2SO_4/BAMD fractions: quantities proportional to their relative amount in the genome; see Table 2

*Bam*HI, *Hind*III + *Bam*HI, *Xho*I, *Pst*I, *Hind*III, *Bgl*II digests of total DNA (not shown).

The main conclusions of these experiments are that the repeated sequences revealed by the probes are interspersed in the *A. diadematus* DNA; they are concentrated in the 1.708-g/cm^3 component present in fraction C, as obtained at an $r_f = 0.14$, since this component disappears from fraction C', being shifted to the pellet at an $r_f = 0.18$ (see Fig. 1 and Table 2). The non-clustered nature of the repeats was confirmed by the fact that partial *Hae*III digests failed to produce any band ladder, but produced instead a smear whose intensity increased as the intensity of the three bands decreased (results not shown).

Finally, hybridization experiments of cloned *Hae*III fragments of 150, 185 and 195 base pairs with each other were negative (not shown), suggesting the existence of a repeat unit at least 525 base pairs in size. The 380-base-pair *Hae*III band of Fig. 5 might well correspond to a subclass of 185 – 190-base-pair repeats lacking the internal *Hae*III site. Likewise, the 380-base-pair and 570-base-pair *Alu*I fragments (Fig. 4) might correspond to repeats lacking one or two internal *Alu*I sites. Detailed mapping experiments are required to provide further information on the repeat units under consideration.

Table 4. *Complexity of* A. diadematus *slowly reassociating DNA*

DNA	Genome size	$c_0t_{1/2}$	Complexity of slowly reassociating DNA from	
			c	$c_0t_{1/2}$ ratio
	base pairs	M · s	base pairs	
E. coli	4.25×10^6	2		
A. diadematus	4.06×10^8	160	3.4×10^8 [a]	3.5×10^8 [b]

[a] This value is 87% of genome size.
[b] This value is obtained by multiplying the $c_0t_{1/2}$ ratio *A. diadematus*/*E. coli* by the genome size of *E. coli*.

The $c_0t_{1/2}$ value of the slowly reassociating class of *A. diadematus* DNA is 80-times larger than that of *Escherichia coli* DNA. Using a value of 4.25×10^6 base pair for the genome complexity of *E. coli* [23], the $c_0t_{1/2}$ ratio indicates a complexity of 3.4×10^8 base pairs for the slow fraction of *A. diadematus* DNA. This value is in excellent agreement with the value one can calculate for 87% of the *A. diadematus* genome (using a c value of 0.45 pg), namely 3.5×10^8 base pairs (Table 4), indicating that the slowly reassociating fraction of *A. diadematus* DNA is indeed single-copy DNA.

A comparison of the $c_0t_{1/2}$ value for the slowly reassociating fractions of *A. diadematus* DNA with those of mouse, man and chicken DNA [8,9] indicates that the former is smaller than the latter, as expected from the smaller genome size of the former. If one corrects for this, however, the $c_0t_{1/2}$ values for the slowly reassociating fractions of warm-blooded vertebrates are still too high by a factor of 2.4 (chicken), 2.9 (man) or 3.8 (mouse), indicating that those fractions correspond to sequences present 2 – 4 times in these haploid genomes (Table 5).

DISCUSSION

In the small *Arothron diadematus* genome, about 87% of the DNA is slowly reassociating, the highest value reported so far for a vertebrate genome. This figure is remarkably higher than that (60% on the average) which we found previously in two mammalian genomes [7], but not far from that (84%) found in the chicken genome [8].

Foldback sequences only represent 1% of *A. diadematus* DNA, an amount much lower than those we found previously [8,9] in man (12%), mouse (5%) and chicken (3%). Repeated sequences comprise 5% of rapidly renaturing DNA and 7% of intermediate repetitive sequences. As already mentioned, these values are much lower than those found in man and mouse, but not far from those of chicken.

The Cs_2SO_4/BAMD fractionation experiments provide valuable information on the *A. diadematus* genome. The main DNA component represents about 73% of total *A. diadematus* DNA (fractions A and B plus a third of the DNA pelleted at $r_f = 0.14$) and is centered at 1.704 g/cm^3. The rest appears to be formed by a light fraction, 1.700 g/cm^3, representing 15% of

Table 5. *Copy number of slowly reassociation sequences from warm-blooded vertebrates*
The expected $c_0t_{1/2}$ value is calculated by using the *A. diadematus* value as a reference single-copy $c_0t_{1/2}$ (see text)

DNA	Genome size	Slowly reassociating sequences			
		complexity	$c_0t_{1/2}$		
			observed	expected	obs./exp.
	pg	pg	M · s		
Mouse [8]	3.2	1.9	3000	780	3.8
Man [8]	3.2	2.0	2400	820	2.9
Chicken [9]	1.25	1.05	1050	430	2.4
A. diadematus	0.45	0.39	160		

the DNA and by three heavy components. A 1.723-g/cm^3 component, corresponding to 2.5% of the DNA, comprises both a satellite DNA and ribosomal DNA. The other two components, representing 4% of the DNA each, have densities of 1.712 and 1.708 g/cm^3, respectively. The latter contains all the family of interspersed repeats studied here and is shifted to the pellet when centrifugation is carried out at $r_f = 0.18$ instead of 0.14; this is, in all likelihood, due to a sequence-specific binding of BAMD.

The preceding paragraphs lead us to discuss the correlations between fractionation and reassociation kinetics data, particularly as far as repeated sequences are concerned. (a) The satellite DNA sequences should belong in the rapidly renaturing material for which they would account to the extent of about 50%. The *Hae*III banding pattern (Fig. 3) does not correspond, however, to a simple ladder, as found in many satellite DNAs, but indicates a more complex organization. (b) We have no information on the correlation between the 1.712-g/cm^3 component and its kinetic behavior. (c) The ribosomal genes should belong in the intermediate class of reassociating sequences; the frequency of this class, about 150, is in the right order of magnitude. (d) As far as the family of interspersed repeats is concerned, several points should be made. Firstly, if our estimate of the relative amount of repeats (1–1.5% of total DNA) is correct, these repeats would represent a quarter to a third of the DNA present in the 1.708-g/cm^3 component (which corresponds to 4% of total DNA) and would be interspersed with single-copy DNA. Secondly, if the repeat length is assumed to be 600 base pairs (see the end of Results), the number of repeats present in the *Arothron* haploid genome would then be 6500–10000; since this value is not far from the frequency estimated for the rapidly renaturing DNA, 16000, it is likely that the repeats belong in this kinetic class. Thirdly, the genomic distribution of the interspersed repeats, limited as it is to a 1.708-g/cm^3 component only representing 4% of total DNA, is an extreme case of the non-uniform genomic distribution already found in other families of interspersed repeats from the genomes of warm-blooded vertebrates [10, 12, 13]. Fourthly, the base composition of the cloned 150-base-pair repeats (45% dG + dC, unpublished results) is close to that of the 1.708-g/cm^3 component (49% dG + dC) in which the repeats are embedded; this finding parallels our previous results on the other families of interspersed repeats and in particular of the *Alu*-like sequence of chicken [13]. Fifthly, sequence data on the cloned 150-base-pair repeats suggest a possible relationship with the *Alu*I repeats of warm-blooded vertebrates; other data (unpublished) indicate the presence in heavy components of DNAs from the genera Trematomus and Dissostichus of interspersed repeats definitely homologous to *Alu*I repeats, confirming the idea that these repeats are very ancient and conserved in genomic distribution. Finally, use has been made of the *A. diadematus* interspersed repeats to assess the phylogenetic relationships among families and genera of the order Tetraodontiformes; these results will be published elsewhere.

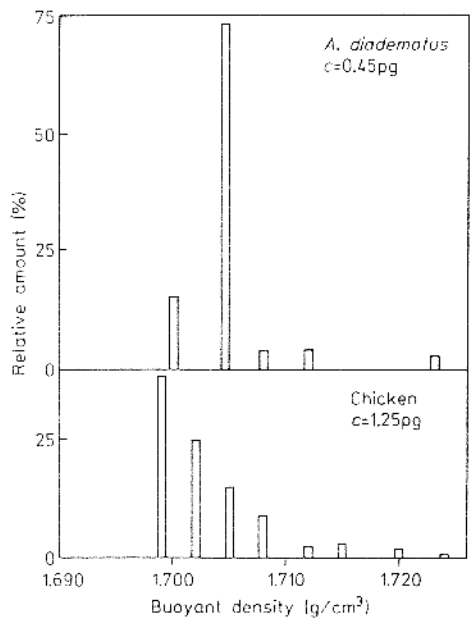

Fig. 6. *A comparison of the relative amounts and buoyant densities of the DNA components found in* A. diadematus *(present work) and in chicken [6, 9]*

The last point to be discussed is the presence of distinct DNA components in a fish genome and their significance. Although the study of the *A. diadematus* DNA components has not been pushed as far as that of warm-blooded DNA components [7–9], the gaussian analysis of Cs_2SO_4/BAMD fractions leaves no doubt as to the existence of one light, one main and at least two heavy components, neglecting the satellite-ribosomal DNA peak. One should, therefore, conclude that the genomes of at least some cold-blooded vertebrates contain discrete DNA components. As shown in Fig. 6, even in such cases, however, the relative amounts of the components do not compare with those found in warm-blooded vertebrates, in that a very homogeneous main com-

ponent is always very predominant. This is not only true in the case of *A. diadematus*, but also in the other cases we have met so far: *I. iguana* and *T. graeca* among reptiles, *P. waltlii* and *Xenopus laevis* among amphibians [4] *Trematomus borchgrevincki* among fishes [16]. Concerning the evolutionary meaning of these findings, the main questions which are raised are the following. (a) What are the causes for the increase in modal buoyant density above the general level of 1.699 – 1.702 g/cm^3 found in most fishes? (b) What are the causes for the formation of heavy components, like those seen in *A. diadematus* DNA? (c) Is there any correlation between the presence or absence of such components and the presence or absence of a Giemsa and reverse chromosomal banding, as we have previously suggested for warm-blooded vertebrates [7]? (d) Finally, is there any correlation between some components found in cold-blooded and warm-blooded vertebrates? The latter question is raised by the finding of repeated sequences, possibly of the *Alu*I type, in a component having the same buoyant density as the warm-blooded DNA component with the highest concentration in *Alu*I sequences. Studies currently under way should provide an answer to some or all of these questions.

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