An Analysis of the Bovine Genome by Density Gradient Centrifugation: Fractionation in Cs₂SO₄/3,6-Bis(acetatomercurimethyl)dioxane Density Gradient

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The fractionation of calf thymus DNA by centrifugation in density gradients of Cs₂SO₄/BAMD, where BAMD = 3,6-bis(acetatomercurimethyl)dioxane, is described. A large-scale separation of (dG+dC)-rich DNA fractions has been obtained, allowing the relative amounts of minor and satellite components in the bovine genome to be precisely assessed.

Density gradient centrifugation is one among several possible experimental approaches to the study of sequence heterogeneity in DNA. As such it is potentially very useful for analyzing and fractionating eukaryotic DNA, particularly when applied to DNA complexes with sequence-specific ligands. Centrifugation in Cs₂SO₄/Ag⁺ density gradients, for instance, has proved to be a very powerful tool not only to separate simple-sequence ('satellite') DNA from mainband DNA [1], but also to fractionate the latter [2−4].

In the present series of investigations we have pushed much further our previous analysis [2,3] of the bovine genome, by using two density gradient centrifugation steps. In a first step, described in this paper, we fractionated calf DNA in Cs₂SO₄/BAMD density gradients. BAMD, or 3,6-bis(acetato-mercurimethyl) dioxane, preferentially interacts with (dA+dT)-rich DNA [5] and increases its density (H. Bünemann, personal communication); this new fractionation technique is particularly suited for the large-scale purification of (dG+dC)-rich DNA components, which are present in small amounts in eukaryotic genomes. The fractions obtained in the first step were analyzed by analytical centrifugation in CsCl in order to assess the buoyant densities and the relative amounts of the DNA components they contained. The second step, to be presented in a future paper, consisted of applying the Cs₂SO₄/Ag⁺ density gradient centrifugation technique to the fractions obtained from the Cs₂SO₄/BAMD step, in order to prepare the DNA components recognized here.

MATERIALS AND METHODS

DNA Preparation

Calf (*Bos taurus*) thymus DNA was preparation CTR2, already described elsewhere [6], and characterized by Filipski *et al.* [2]. The sedimentation coefficient of this preparation was re-determined at different concentrations by band centrifugation using split-beam optics and found to be equal to 26 ± 2 S; this value is higher than that (22.6 S) previously reported [2] and corresponds to a molecular weight of 13.10^6, if the s vs M_r relationship of Eigner and Doty [7] is used.

DNA · BAMD Complex Formation

The complex between DNA and 3,6-bis(acetato-mercurimethyl)dioxane (BAMD) was formed by mixing at room temperature, under vigorous mechanical stirring, equal volumes of DNA and BAMD solutions in 0.1 M Na₂SO₄, 0.005 M Na₂B₄O₇, pH 9.2 [5], of such concentrations as to obtain the desired final DNA concentration and BAMD/DNA-P molar ratio (r_f). The DNA-P concentration was calculated from the absorbance at 260 nm (A_{260}), using a mean molar absorption coefficient of 6600 M⁻¹ cm⁻¹. When the calf thymus DNA preparation was used at high concentrations, precipitation due to local concentration effects occurred upon complex formation; to avoid this, the complex was formed in dilute solution and then concentrated in a rotary evaporator, under reduced pressure. The synthesis of BAMD was described by Edsall *et al.* [8].

Centrifugation Experiments

Preparative Centrifugation of DNA · BAMD Complexes. Solid Cs₂SO₄ (Suprapur, Merck, Darmstadt) was added to the DNA·BAMD complex solution to a final density of 1.47 g/cm³. The following relationship was experimentally established between refractive index n and density ϱ in 0.1 M Na₂SO₄, 0.005 M Na₂B₄O₇, pH 9.2: $n_D^{25\,°C} = 1.2636 + 0.07346\,\varrho$. Polyallomer tubes were two-thirds filled with the DNA · BAMD solution avoiding contact of the solution with the tube cap; paraffin oil was added to fill the tubes completely. Centrifugations were carried out at 25 °C in a Beckman L2-65B preparative ultracentrifuge, using a type 30 rotor at 25000 rev./min for 110 h, or types 65 (or 50, Ti) rotors at 35000 rev./min for 60 h. After centrifugation, the tubes were emptied using a stainless steel needle [2]. Care should be taken not to touch the complex pelleted at the bottom of the tube (see Results) with the needle, in order to avoid contamination of the fractions. The solution was pumped out with a peristaltic pump; according to tube size, the flow rate was 10 or 20 ml/h, and fractions of 0.3 ml or 0.8 ml were collected. The absorbance was continuously monitored at 253.7 nm with an LKB Uvicord (Stockholm, Bromma, Sweden).

Analytical CsCl Density Gradient Centrifugation. Fractions from preparative gradients were exhaustively dialyzed against 2 M NaCl to remove BAMD and then against 0.005 M NaCl, 0.01 M Tris-HCl, pH 7.6. Solid CsCl was added to the solutions to a density of 1.70 g/cm³. Measurements were made on a Spinco model E analytical ultracentrifuge equipped with a monochromator, mirror optics, electronic scanner and multiplexer, modified so as to allow the simultaneous study of nine DNA samples (G. Macaya and M. Grosjean, unpublished results). Phage 2C, $\varrho = 1.742$ g/cm³ [9], was used as a density marker and a value of 1.19×10^9 cm⁵ g⁻¹ s⁻² [10] was taken for the buoyancy gradient constant β_B.

RESULTS

OPTIMAL CONDITIONS FOR THE FRACTIONATION OF CALF THYMUS DNA IN Cs₂SO₄/BAMD DENSITY GRADIENTS

Influence of r_f and Tube Loading on Fractionation

Preliminary analytical experiments had shown that an increasing fraction of calf thymus DNA becomes heavier as the r_f, the molar ratio of BAMD to DNA-P, increases. At $r_f = 0.18$, about one half of the complex banded very near, or pelleted at, the bottom of the centrifuge cell. These results indicate that in a preparative run, the amount of DNA complex per ultracentrifuge tube should be calculated on the basis of
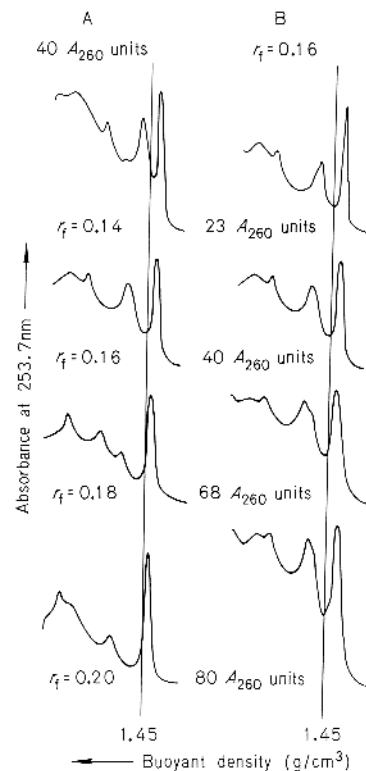

Fig. 1. *Influence of r_f and tube load on fractionation in Cs₂SO₄/BAMD density gradients.* (A) Calf thymus DNA (40 A_{260} units) in 0.1 M Na₂SO₄, 5 mM Na₂B₄O₇, pH 9.2, and Cs₂SO₄ ($\varrho = 1.47$ g/cm³) was centrifuged in the presence of BAMD at increasing values of r_f in a Beckman type 65 rotor at 25 °C for 60 h at 35000 rev./min. (B) Increasing amounts of calf thymus DNA were centrifuged in the presence of BAMD ($r_f = 0.16$) under the same conditions as in A. Profiles were aligned at $\varrho = 1.45$ g/cm³; comparisons are qualitative since the gradient slope was not always the same; density increases from right to left

the material banding near the center of the tube, and not of the total DNA load.

Experiments were done to analyse the influence of r_f and tube load on the fractionation of calf thymus DNA in Cs₂SO₄/BAMD density gradients. The effect of pH was not considered in this work, all centrifugations being performed at pH 9.2 [5].

To study the influence of r_f on fractionation, a load of 40 A_{260} units of DNA per rotor 65 tube was chosen; this load is 5−10 times larger than the load currently used in our laboratory for Cs₂SO₄/Ag⁺ density gradient experiments [2−4]. Fig. 1 A shows the results of centrifugation to equilibrium in Cs₂SO₄ of 40 A_{260} units of calf thymus DNA · BAMD complexes at four r_f values ranging from 0.14 to 0.20. In this range the lightest peak (furthest to the right) remains at about the same buoyant density; the best resolution is observed at $r_f = 0.18$, where four distinct peaks are obtained. The complete analysis of this particular profile is given in the following section.

Fig. 1 B shows the effect of a 4-fold change in total amount of complex loaded at an $r_f = 0.16$ in a 65 rotor.
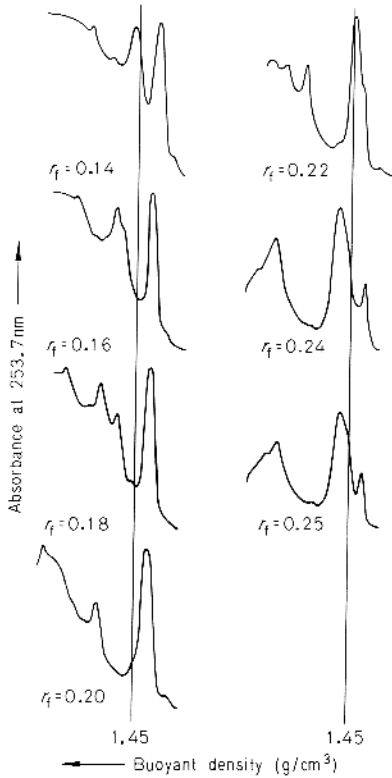
Apart from a widening of the peaks due to increased concentration, the resolution of components and the shape of the profile appears not to change in the load range studied.

Influence of r_f on Resolution at High Tube Load

Fig. 2 shows the influence of r_f on peak resolution at a load of 210 A_{260} units per rotor 30 tube. The profiles obtained in Fig. 2 for r_f from 0.14 to 0.20 are qualitatively comparable to those of Fig. 1 A; the best resolution is obtained, as in Fig. 1 A, at $r_f = 0.18$. The lightest peak, which remains at a constant buoyant density up to $r_f = 0.20$, begins to increase in density with r_f from 0.20 to 0.25; a new peak is resolved at $r_f = 0.24$ and a shoulder appears at $r_f = 0.25$; at the same time, the heavier peaks are shifted to higher densities and merge into the pellet at the bottom of the tube.

FRACTIONATION OF CALF THYMUS DNA COMPONENTS

On the basis of the experiments of Fig. 1 and 2, three conditions for fractionation were chosen: $r_f = 0.18$ and high or low load and $r_f = 0.23$ and high load. The description of the results obtained follows.
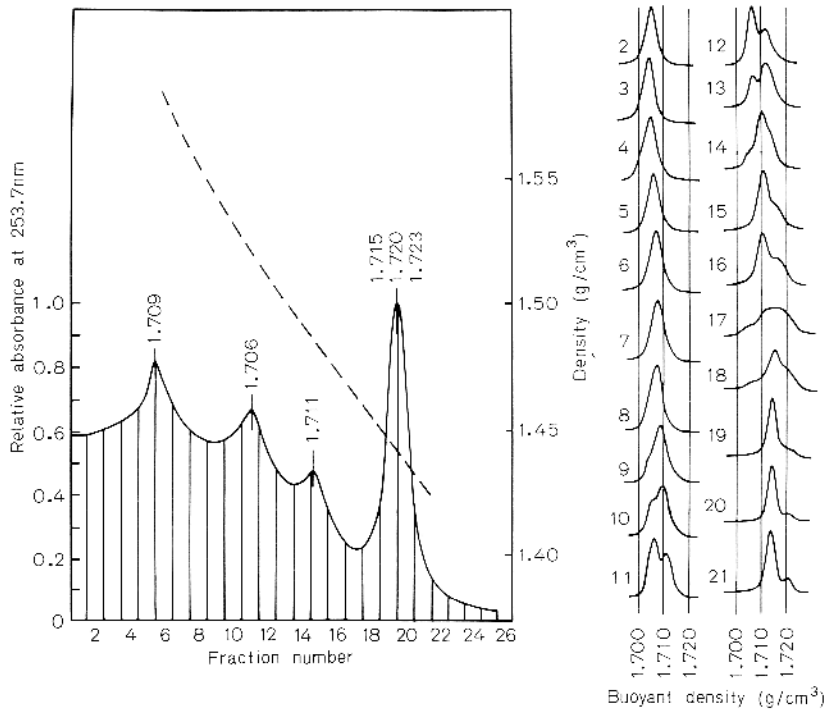
Fig. 2. *Influence of r_f on fractionation in $Cs_2SO_4/BAMD$ density gradients at high tube load*. Calf thymus DNA (210 A_{260} units) in 0.1 M Na_2SO_4, 5 mM $Na_2B_4O_7$, pH 9.2, and Cs_2SO_4 ($\varrho = 1.47$ g/cm³) was centrifuged in the presence of BAMD at increasing values of r_f in a Beckman type 30 rotor at 25 °C for 110 h at 25000 rev./min. Other details as in Fig. 1

Fig. 3. *Analysis of calf thymus DNA in Cs_2SO_4 density gradient in the presence of BAMD at $r_f = 0.18$*. Calf thymus DNA (40 A_{260} units) in 0.1 M Na_2SO_4, 5 mM $Na_2B_4O_7$, pH 9.2, containing BAMD ($r_f = 0.18$) and Cs_2SO_4 ($\varrho = 1.468$ g/cm³) was centrifuged in a Beckman type 65 rotor at 25 °C for 60 h at 35000 rev./min. The broken line indicates the density gradient. The CsCl buoyant densities of the major satellite components present in each peak are indicated. Analytical CsCl density gradient centrifugation profiles of the fractions of the preparative profile are shown on the right
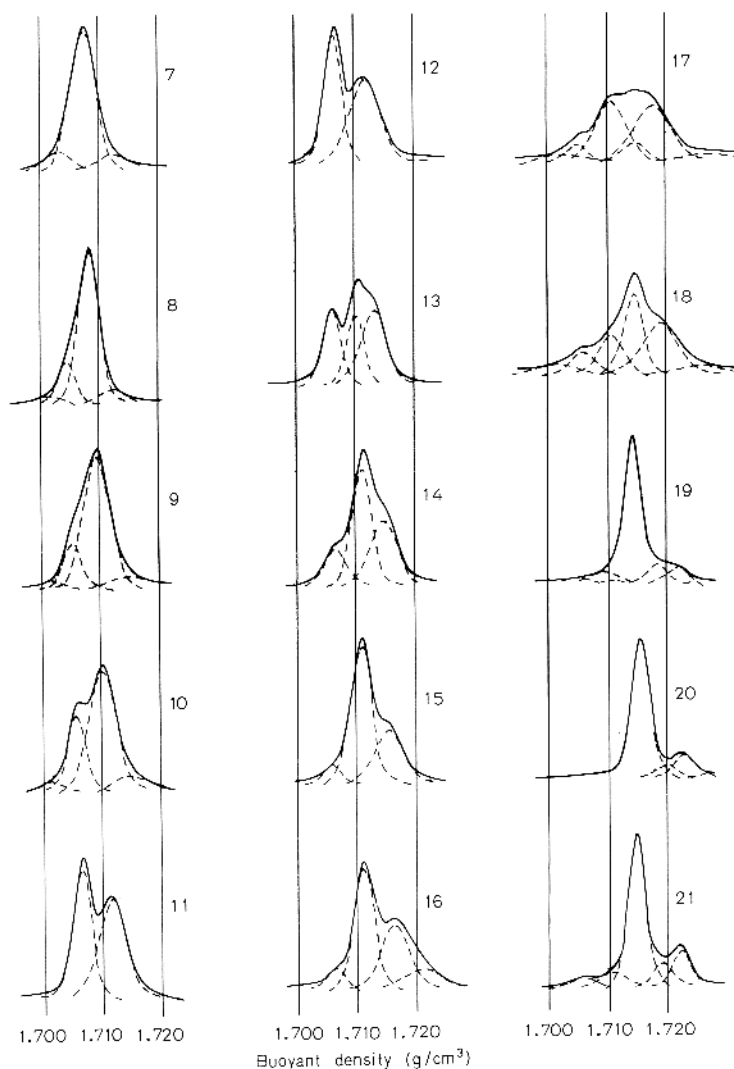
Fig. 4. *Gaussian analysis of analytical CsCl density gradient centrifugation profiles.* The CsCl profiles of fractions 7−21 of Fig. 3, as analysed in terms of gaussian components using a Dupont 310 curve resolver (see text), are presented

Cs₂SO₄/BAMD Preparative Density Gradient Centrifugation at $r_f = 0.18$ at Low Load

Fig. 3 shows the fractionation of calf thymus DNA in a Cs₂SO₄/BAMD preparative gradient at $r_f = 0.18$ and a load of 40 A_{260} units in a rotor 65 tube (Fig. 1A). The material present in fractions 2−25 represented 40% of the total DNA loaded, the rest being present as a pellet at the bottom of the centrifuge tube. The CsCl density profiles of the fractions, also shown in Fig. 3, provided information on the DNA components present in them. A number of components could be recognized at first glance in the profiles; several more could, however, be pinpointed by the detailed gaussian analysis of the profiles [4] presented in Fig. 4, which provided, in addition, quantitative estimates of the amount of each component.

The results of the gaussian analysis of Fig. 4 are given in Table 1, where they are compared with the previous results obtained in our laboratory [2,3] using the Cs₂SO₄/Ag⁺ fractionation procedure. As in previous work, components are classified into 'satellite' components, or simple-sequence components, characterized by very sharp CsCl bands [4], and major and minor components, both of which show broader CsCl bands, the latter being present in relative amounts lower than 5%.

As far as major components are concerned, relatively little new information was obtained in the present work, since they were mostly pelleted to the bottom of the tubes under the experimental conditions used. It is, however, very significant that their fractionation can be obtained in Cs₂SO₄/BAMD, as pointed out by the separation of the 1.7045-g/cm³ and 1.7085-g/cm³ components (see the very clear

Table 1. *Bovine DNA components*

Components	Buoyant density and relative amount found by					
	Filipski *et al.* [2]		Thiery *et al.* [3]		this work	
	ϱ	proportion	ϱ	proportion	ϱ	proportion
	g/cm³	%	g/cm³	%	g/cm³	%
Major		85		81		77.2
	1.697	50 ⎫	1.697	25 ⎫	1.697 ⎫	
		⎬75	1.700	32 ⎬72	1.700 ⎬	68ᵃ
	1.704	25 ⎭	1.704	15 ⎭	1.7045 ⎭	
	1.709	10	1.709	9	1.7085	9.2
Minor		1		2.8		8.8
	1.699	—			1.7065	0.4
					1.7110	0.8
			1.712	2.5	1.7115	4.3
	1.714	—	1.714	0.15	1.7150	2.2
	1.716	—				
	1.719	—	1.719	0.1	1.7190	1.0
					1.7280	0.1
Satellite		14		14.5		13.9
	1.705	4	1.705	4	1.706	4.1
					1.709	—ᵇ
	1.710	1.5	1.710	3	1.711	3.3
	1.714	7	1.715	7	1.715	5.4
					1.720	0.5
	1.723	1.5	1.723	0.5	1.723	0.6

ᵃ This value represents 60% of non-analyzed material (pellet) plus the 8% of 1.7045-g/cm³ component present in the gradient (see text).

ᵇ The amount of this component will be given in a future paper.

discontinuous distribution of densities in fractions 2 − 9 in Fig. 3), the latter component being recovered quantitatively under the experimental conditions used.

Finally, a new satellite was discovered in the present work. It has a buoyant density of 1.720 g/cm³ (Fig. 4, fraction 20). Another new satellite, having a buoyant density, 1.709 g/cm³, identical to that of the heaviest major components, was revealed by the Cs_2SO_4/Ag^+ analysis of the first peak of Fig. 3; this satellite, which remains hidden even in the $Cs_2SO_4/BAMD$ gradient, is in fact, responsible for the first peak of Fig. 3 (unpublished results). Six minor components were revealed by the detailed analysis shown in Fig. 4. The main ones, having buoyant densities of 1.7115 g/cm³ and 1.715 g/cm³, can be easily distinguished from two satellite components of identical density, because of their localization in the $Cs_2SO_4/BAMD$ gradient.

$Cs_2SO_4/BAMD$ Preparative Density Gradient Centrifugation at $r_f = 0.18$ and High Load

Fig. 5 shows the fractionation obtained at $r_f = 0.18$ and a load of 210 A_{260} units per rotor 30 tube. Such fractionation at high load is as good as that obtained under the conditions used in Fig. 3. The CsCl profiles of the pooled samples shown on the right indicates that the peaks equivalent in position to those of Fig. 3 are indeed due to the same satellite components. Two new minor peaks become evident at this higher
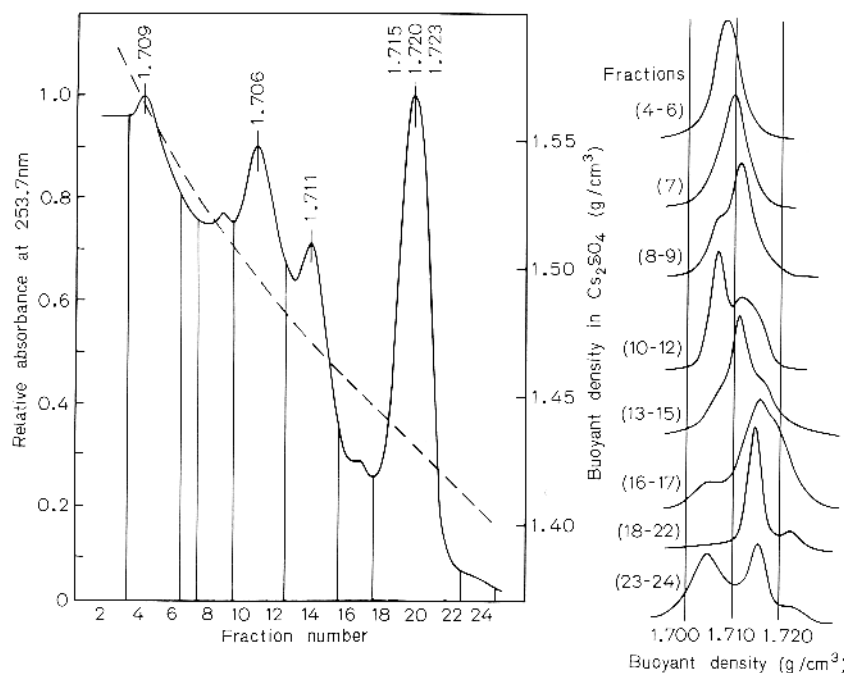

Fig. 5. *Profile of calf thymus DNA in Cs_2SO_4 density gradient in the presence of BAMD at $r_f = 0.18$.* Calf thymus DNA (210 A_{260} units) in 0.1 M Na_2SO_4, 5 mM $Na_2B_4O_7$, pH 9.2, containing BAMD ($r_f = 0.18$) and Cs_2SO_4 ($\varrho = 1.460$ g/cm³) was centrifuged in a Beckman type 30 rotor at 25 °C for 110 h at 25000 rev./min. Other details as in Fig. 3
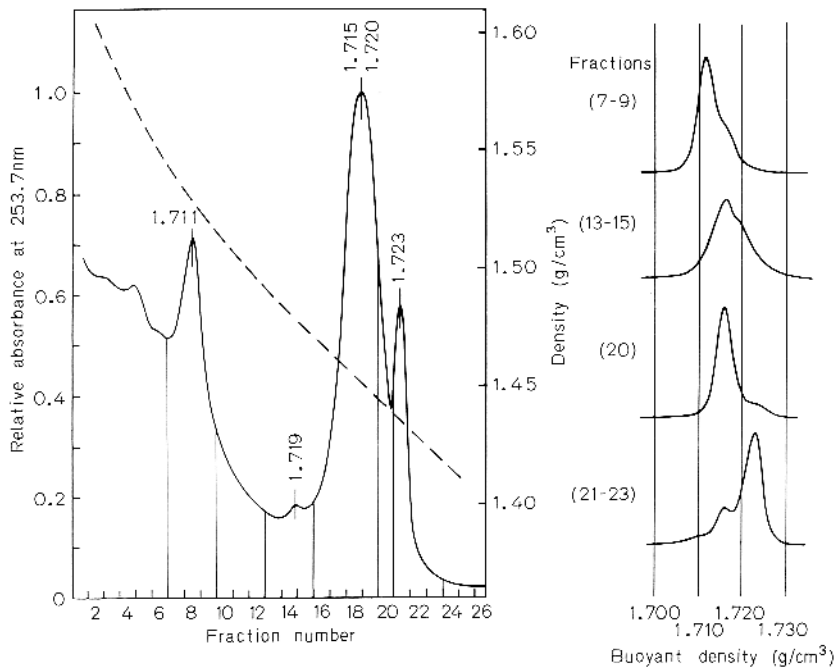
Fig. 6. *Profile of calf thymus DNA in Cs₂SO₄ density gradient in the presence of BAMD at* r_f *= 0.23.* Calf thymus DNA (210 A_{260} units) in 0.1 M Na₂SO₄, 5 mM Na₂B₄O₇, pH 9.2 containing BAMD (r_f = 0.23) was centrifuged under the same conditions as in Fig. 5. Other details as in Fig. 3

gradient load; in view of the complexity of these two fractions (8−9 and 16−17), it is very difficult to associate a particular component with each peak. This was, however, made possible by the Cs₂SO₄/Ag⁺ analysis to be presented in a future paper.

Cs₂SO₄/BAMD Preparative Density Gradient Centrifugation at r_f = 0.23 and High Load

Fig. 2 showed progressive changes in the Cs₂SO₄/BAMD centrifugation profiles upon increasing r_f. Of particular interest was the resolution of a new peak in the light region at r_f = 0.24. In order to characterize this new peak and to analyse the general shift to higher densities of the components, 210 A_{260} units of calf thymus DNA were fractionated at r_f = 0.23 (Fig. 6). The new peak corresponded to the 1.723-g/cm³ satellite. No enrichment was observed on the light side of the 1.715-g/cm³ peak for the 1.720-g/cm³ satellite, as could be expected on the basis of the redistribution of the 1.715-g/cm³ and 1.723-g/cm³ components. The peak of the 1.711-g/cm³ satellite is shifted to higher densities than at r_f = 0.18. The minor peak present in fractions 13−15 is associated with a 1.719-g/cm³ minor component.

DISCUSSION

It is of interest to compare the relative amounts of the bovine DNA components, as estimated in the present work, with our previous results from Cs₂SO₄/

Ag⁺ and CsCl investigations (Table 1). As far as the major components are concerned, the amount of the heaviest one (1.709 g/cm³) is in excellent agreement with previous data; the components of density 1.704 g/cm³ and lower were not separated in this work, but the sum of the amounts of unfractionated material pelleted at the bottom of the tube (60%) and of the 1.704-g/cm³ component still left in solution (8%) are close to those previously found. Owing to the larger amount of material submitted to fractionation, and to the higher specificity of BAMD interaction, much more information about the minor components could be obtained in this work compared to the previous ones; first of all, six minor components could be identified as opposed to the three or four previously seen; furthermore, precise assessments of both densities and amounts could be obtained. Finally, a number of other points raised by this work have been clarified by the Cs₂SO₄/Ag⁺ analysis of the fractions (to be published later).

The choice of the bovine genome for this analysis was largely due to the fact that this genome is particularly rich in heavy components, which lend themselves particularly well to fractionation in Cs₂SO₄/BAMD; another reason was our previous experience with this genome. It should be stressed, however, that the general usefulness of the present approach has already been confirmed in our laboratory with other eukaryotic genomes.

Finally, we would like to make three comments concerning the methods used here. The first is that the

Cs_2SO_4/BAMD fractionation of the high-density components of the bovine genome really is a large-scale fractionation allowing, for instance, milligram amounts of the major satellite to be obtained in only two preparative centrifugations. The second is that, even if the general order of components in the gradient is a function of their $dA+dT$ content, the $(dA+dT)$-rich ones being the heaviest, an indication of a sequence specificity is given by the exceptions found: for instance, the 1.706-g/cm³ satellite (Fig. 3, fractions $9-13$) is lighter than the 1.709-g/cm³ major component (fractions $5-9$). Thirdly, the large amount of bovine DNA that can be processed by Cs_2SO_4/BAMD preparative centrifugation (2500 A_{260} units in a rotor 30) allows in subsequent Cs_2SO_4/Ag^+ gradients the detection of components present in extremely minute relative amounts.

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Note Added in Proof. A combined Cs_2SO_4/Ag^- and restriction enzyme analysis (to be published) has revealed that the minor component 1.7115 g/cm³ (Fig. 4, fractions $10-12$) is in fact a satellite different from the 1.711 g/cm³ satellite present in fractions $13-16$ of Fig. 4.