Isolation and Characterization of Mouse and Guinea Pig Satellite Deoxyribonucleic Acids*

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ABSTRACT: Mouse and guinea pig satellite deoxyribonucleic acids have been isolated using Ag^+–Cs_2SO_4 density gradient centrifugation. The complementary strands have been separated in preparative alkaline CsCl density gradients, or in neutral CsCl, after heat denaturation in the presence of formaldehyde. The satellite deoxyribonucleic acids and their complementary strands have been investigated in some of their physical properties (buoyant densities, melting temperatures, and denaturation–renaturation properties), and in their base composition. In contrast with the main deoxyribonucleic acids, the satellite deoxyribonucleic acids show buoyant densities and melting temperatures which are quite different from those expected on the basis of analytical data. The separated complementary strands of satellite deoxyribonucleic acids show base compositions which are very different from each other, particularly in the case of guinea pig satellite.

The presence of satellite DNAs has been shown in several animal species (Kit, 1961; Schildkraut *et al.*, 1962; Polli *et al.*, 1966; Corneo *et al.*, 1967). Studies of their properties have been carried out after isolating them from the main DNA by chromatography on MAK[1] columns (Cheng and Sueoka, 1963) or by CsCl density gradient centrifugation (Polli *et al.*, 1966; Flamm *et al.*, 1966).

Mouse and guinea pig DNAs contain satellite components displaying buoyant densities in CsCl gradients of 1.690 and 1.705 g/cm^3, respectively (Kit, 1961), while the densities of the main DNA of both species is 1.700 g/cm^3. Mouse satellite DNA has been isolated by CsCl density gradient centrifugation and some of its physical properties have been studied (Waring and Britten, 1966; Bond *et al.*, 1967). The base analysis of this satellite DNA and of its complementary strands, as obtained by alkaline CsCl density gradient centrifugation, has been reported (Flamm *et al.*, 1967).

The present paper concerns the isolation of mouse and guinea pig satellite DNAs by centrifugation of the total DNAs in Ag^+–Cs_2SO_4 density gradient and the separation of their complementary strands in alkaline CsCl, or in neutral CsCl, after heat denaturation in the presence of formaldehyde. The satellite DNAs and their complementary strands so obtained have been investigated in some of their physical properties and in their base composition.

Materials and Methods

Isolation of Satellite DNAs. Total, high molecular weight DNA has been isolated from mouse and guinea pig livers according to Marmur (1961). Satellite DNA was separated from the main DNA by centrifugation in Ag^+–Cs_2SO_4 density gradient (Jensen and Davidson, 1966). The solution to be centrifuged contained DNA at an A_{260} of 0.6, $AgClO_4$ to give an Ag^+/DNA-P molar ratio equal to 0.27, 0.1 M borate buffer (pH 9.2) to give a final concentration of 0.005 M, and Cs_2SO_4 (Suprapure, Merck, Darmstadt, Germany) to obtain a final density close to 1.5 g/cm^3, as determined from refractive index measurements (Vinograd and Hearst, 1962). The preparative centrifugation was carried out in a Spinco Model L 2 centrifuge. Volumes of 20 ml were spun in each tube of a Spinco No. 30 fixed-angle rotor for 96 hr at 30,000 rpm; 0.25-ml fractions were collected and their absorbance at 260 mμ was determined after a fourfold dilution. Fractions corresponding to the satellite DNA were pooled, dialyzed exhaustively against 5 M NaCl–0.01 M Tris-HCl (pH 7), then against 0.1× SSC, and concentrated in dialysis tubing by slow evaporation under a gentle stream of air.

The separation of the complementary strands of isolated satellite DNA was carried out by centrifugation in an alkaline CsCl density gradient, prepared as follows. NaOH (0.1 ml of 1 N) was added to 3.5 ml of 0.01 M Tris-HCl buffer (pH 8.5) containing approximately 50 μg of DNA and 500 μg of sodium lauryl sulfate (Flamm *et al.*, 1967); the density of this solution was adjusted to approximately 1.750 g/cm^3 by adding solid CsCl; the final pH was about 12.5.

CsCl Density Gradient Centrifugation. This was performed in a Spinco Model E centrifuge as described by Schildkraut *et al.* (1962). Ultraviolet absorption photo-

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[1] Abbreviations used that are not listed in *Biochemistry 5*, 1445 (1966), are: MAK, methylated serum albumin kieselguhr; SSC, 0.15 M NaCl–0.015 M sodium citrate (pH 7.0).

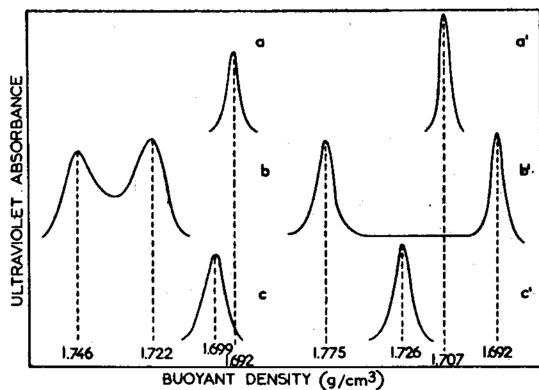

FIGURE 4: Microdensitometer tracings, of mouse (a to c) and guinea pig (a′ to c′) satellite DNA in neutral (a and a′), alkaline (b and b′), and neutralized (c and c′) CsCl.

and digested, in succession, by three enzymes: (a) spleen acid DNase (Bernardi *et al.*, 1966), (b) spleen exonuclease (the preparation used in the present work had been obtained according to a modification of the method of Bernardi and Bernardi (1966) and contained adenosine deaminase at a trace level), and (c) spleen acid phosphomonoesterase II (G. Bernardi, A. Chersi, and A. Bernardi, paper to be published). Digests were loaded on 0.9 × 15 cm columns of DEAE-cellulose (Serva, Heidelberg, Germany; 0.57 mequiv/g; acetate form) washed with water. Nucleosides were washed through with water and nucleotides were eluted with 1 M ammonium acetate buffer (pH 5.4). The over-all recovery was 97–100%; nucleosides formed 90–98% of the recovered material.

Nucleoside Analysis on Polyacrylamide Gel Columns. This was done as described by Carrara and Bernardi (1968), using Bio-Gel P-2 (Bio-Rad, Richmond, Calif.) columns. The nucleosides obtained from the DEAE-cellulose columns were dried by rotary evaporation at 30°, dissolved in 0.4 ml of 2 × 10^{-3} M ammonium carbonate (pH 10.15), and loaded on to a P-2 column equilibrated with the same buffer. The order of elution from the column was G + T, C, A; after having measured its A_{271}, the unresolved G + T fraction was dried, dissolved in 0.4 ml of 2 × 10^{-4} M sodium phosphate (pH 7.2), and loaded on a P-2 column equilibrated with the same buffer. Nucleoside fractions were collected in 5- or 10-ml volumetric flasks and adjusted to pH 1.0 with 1 N HCl. Ultraviolet spectra were measured in a Zeiss PMQ-II or a Cary 15 spectrophotometer using as a blank the buffer obtained from the column and similarly acidified.

Results

Figure 1 shows a comparison of the banding patterns and the buoyant densities of total mouse and guinea pig DNAs obtained in CsCl and Ag^+–Cs_2SO_4 density gradients, respectively. The distance between the main and satellite DNA bands is considerably increased after complexing with Ag^+. The two light peaks shown by guinea pig DNA in Ag^+–Cs_2SO_4 correspond to material from the light side of the main DNA peak as

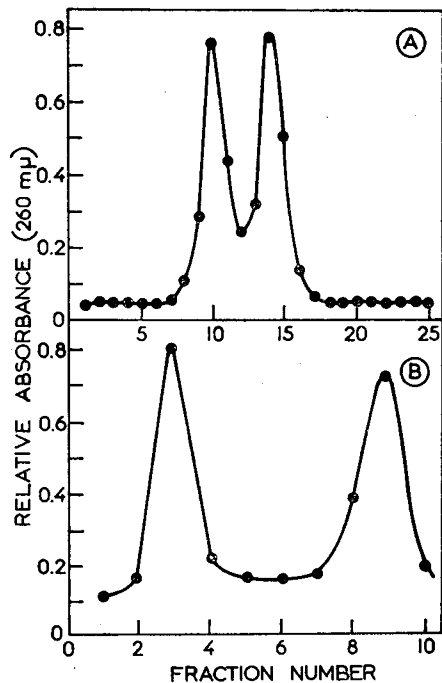

FIGURE 5: Fractionation of the complementary strands of mouse (A) and guinea pig (B) satellite DNA in an alkaline CsCl gradient.

shown by the fact that, when isolated and recentrifuged in CsCl after dialysis, they display a density of 1.695 g/cm³. These light DNA components were not investigated further.

Figure 2 shows the banding patterns of total mouse and guinea pig DNA in a preparative Ag^+–Cs_2SO_4 density gradient. The fractions corresponding to the main peaks and to the satellite peaks, when recentrifuged in CsCl after extensive dialysis to remove Ag^+ from the DNA, displayed densities corresponding to their original densities in CsCl and did not show any contamination from the satellite and main DNA components, respectively.

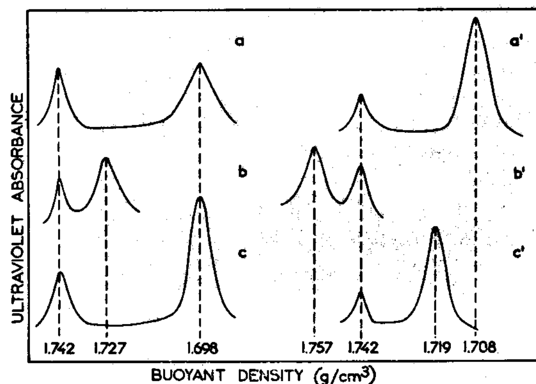

FIGURE 6: Microdensitometer tracings of mouse (a to c) and guinea pig (a′ to c′) satellite DNA centrifuged to equilibrium in neutral CsCl in the analytical ultracentrifuge: light strand (a and a′), heavy strand (b and b′), and an equimolar mixture of the two complementary strands, heated at 65° for 5 hr in 2× SSC (c and c′). In each tracing the peak with a density of 1.742 g/ml corresponds to the reference 2C DNA.

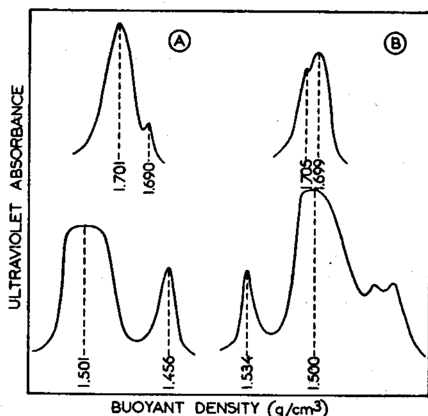

FIGURE 1: Microdensitometer tracings of mouse (A) and guinea pig (B) liver DNA, centrifuged to equilibrium in neutral CsCl (upper tracings) and in Ag^{+}–Cs_2SO_4 (lower tracings) density gradients.

graphs were taken after 20-hr centrifugation at 44,770 rpm at 25° and analyzed with a microdensitometer. Buoyant densities were calculated from the initial density of the solution and the limiting isoconcentration distance according to Vinograd and Hearst (1962), as described by Nandi *et al.* (1965). Alternatively, phage 2C DNA (density = 1.742 g/cm³) was used as a density marker; in this case, densities were calculated according to Schildkraut *et al.* (1962) and were referred to the density of *Escherichia coli* DNA, taken to be 1.710 g/cm³.

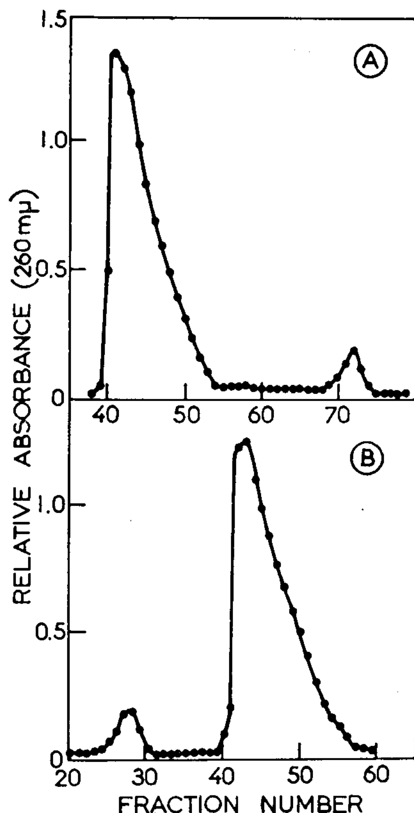

FIGURE 2: Fractionation of mouse (A) and guinea pig (B) liver DNA in a Ag^{+}–Cs_2SO_4 preparative density gradient.

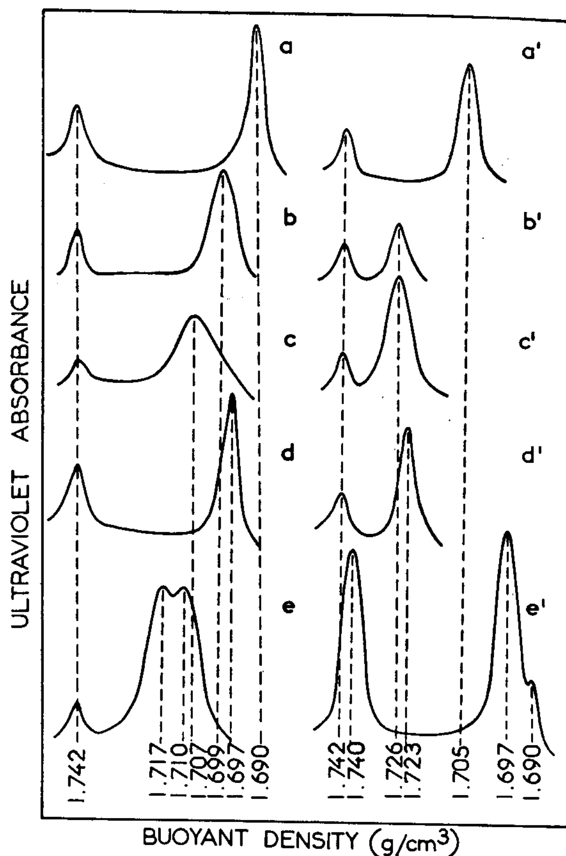

FIGURE 3: Microdensitometer tracings of mouse (a to e) and guinea pig (a′ to e′) satellite DNA, centrifuged to equilibrium in neutral CsCl: native (a and a′), denatured by heating at 100° for 10 min in SSC (b and b′), or in 0.1× SSC (c and c′), heated at 65° for 5 hr in 2× SSC after heat denaturation (d and d′) and denatured by heating at 100° for 3′ in 0.1× SSC containing 2% formaldehyde (e and e′). The peak on the left in each tracing except e′ corresponds to the reference 2C DNA (ρ = 1.742 g/ml). The peak on the right in the tracing e′ corresponds to mouse satellite DNA (ρ = 1.690 g/ml) which was used as a reference in this case.

Zone sedimentation was performed according to Studier (1965). Sedimentation constants were found to range between 20 and 30 S, for different preparations.

Denaturation and Renaturation of Satellite DNAs. DNA denaturation was carried out by heating DNA solutions (20 μg/ml in 0.1× SSC) at 100° for 10 minutes, followed by fast cooling, or by adding 0.1 volume of 1 M NaOH, followed 5 min later by 0.1 volume of 1 M KH_2PO_4, to a DNA solution in 0.1× SSC (20 μg/ml). DNA was also denatured by heating it to 100° for 3 min in 0.01 M phosphate buffer (pH 7.8) containing 2% freshly neutralized formaldehyde. DNA renaturation in the absence of formaldehyde was carried out at a concentration of 10 μg/ml in 2× SSC at 65° for 5 hr.

The absorbance–temperature profiles were determined by heating DNA solutions in glass-stoppered quartz cuvets in a specially adapted Beckman DU spectrophotometer (Marmur and Doty, 1962).

Enzymatic Digestion of DNA Samples to Nucleosides. DNA samples were exhaustively dialyzed against 0.05 M ammonium acetate buffer–0.001 M EDTA (pH 5.4)

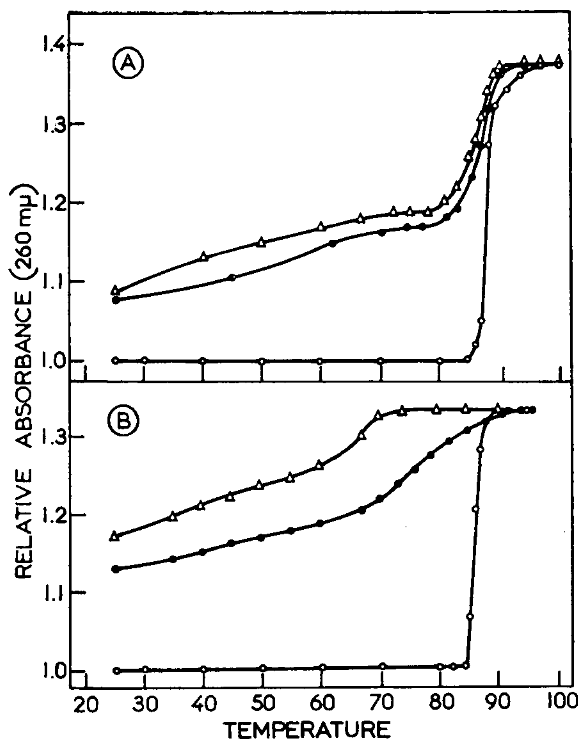

FIGURE 7: Ultraviolet absorbance–temperature curves of mouse (A) and guinea pig (B) satellite DNAs: native DNA (circles), denatured DNA (triangles), renatured DNA (points). The DNA solvent in all experiments was SSC.

When heated in SSC and fast cooled, isolated mouse satellite DNA increased in buoyant density from 1.690 (Figure 3a) to 1.699 g/cm³ (Figure 3b); a larger increase in density to 1.707 g/cm³ was obtained by heat denaturation in 0.1× SSC (Figure 3c). After renaturation, the

TABLE I: Buoyant Densities of Satellite DNAs from Mouse and Guinea Pig Livers and Human Placenta.

	Mouse Liver	Guinea Pig Liver	Human Placenta[a]
Native	1.691	1.705	1.687
Heat denatured, SSC, fast cooled	1.699	1.726	1.703
Heat denatured, 0.1× SSC, fast cooled	1.707	1.726	1.703
Renatured	1.697	1.723	1.694
Denatured 2% HCHO	1.710 1.717	1.697 1.740	1.703 1.717
Alkaline gradient	1.722 1.746	1.692 1.778	1.707 1.738
Separated strands in neutral CsCl	1.698 1.723	1.708 1.757	1.694 1.712

[a] Corneo *et al.* (1968).

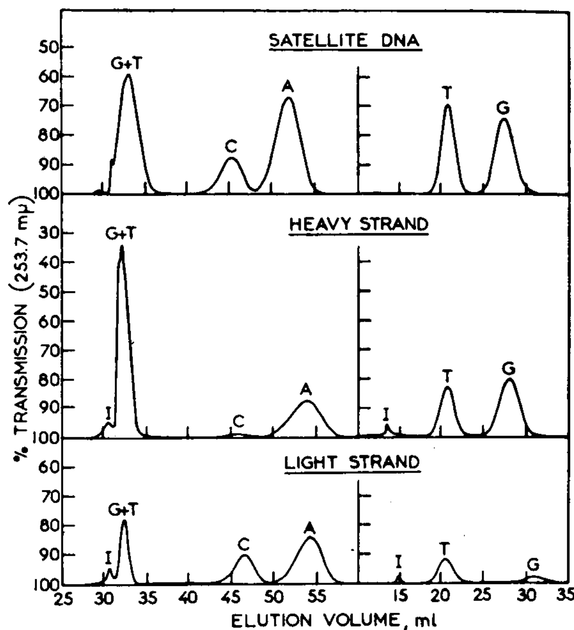

FIGURE 8: Chromatography on Bio-Gel P-2 columns of the deoxyribonucleosides obtained by enzymatic digestion from guinea pig satellite DNA and its complementary strands. Columns (0.78 × 90 and 0.78 × 50 cm) were used for the separations at pH 10.15 and 7.2, respectively. Chromatograms obtained at pH 10.15 are shown on the left-hand side; those obtained at pH 7.2 are shown on the right-hand side. Transmission at 253.7 mμ was recorded using a Uvicord (LKB, Stockholm, Sweden); the cell has an optical path equal to 0.3 cm. Flow rate was 24 ml/hr. Satellite DNA: 4.84 and 2.24 A_{271} units were loaded on the pH 10.15 and 7.2 columns, respectively. Heavy strand: 3.20 and 1.85 A_{271} units were loaded on the pH 10.15 and 7.2 columns, respectively. Light strand: 1.83 and 0.44 A_{271} units were loaded on the pH 10.15 and 7.2 columns, respectively. The small amounts of inosine (peaks indicated by I) present in the digests from the separated strands originated from adenosine by deamination.

satellite DNA displayed a density of 1.697 (Figure 3d). When denatured in the presence of 2% formaldehyde, mouse satellite DNA showed two bands, formed by the complementary strands, which displayed densities of 1.710 and 1.717 g/cm³, respectively (Figure 3e).

When denatured by heat in SSC or 0.1X SSC (Figure 3b′,c′) or alkali, isolated guinea pig satellite DNA increased in density from 1.705 (Figure 3a′) to 1.726 g/cm³. When denatured DNA was exposed to renaturing conditions, it underwent only a very slight density decrease, to 1.723 g/cm³ (Figure 3d′). When denatured by heating at 100° for 3 min in 0.1X SSC containing 2% formaldehyde, guinea pig satellite DNA showed in neutral CsCl density gradient two bands displaying densities close to 1.697 and 1.740 g/cm³, respectively (Figure 3e′).

When centrifuged in an alkaline CsCl density gradient, mouse and guinea pig satellite DNAs showed two bands (Figure 4b,b′) corresponding to the separated complementary strands. The buoyant densities found for the strands of mouse satellite DNA were 1.722 and 1.746, in fair agreement with results by Flamm *et al.* (1967). The densities of the guinea pig satellite strands were found to be equal to 1.692 and 1.778, respectively.

TABLE II: Enzymatic Digestions of Satellite DNAs.[a]

DNA Samples	DNA Concn (A_{260})	Vol (ml)	Enzyme Concn[b] (unit/ml)	Digestion Time (min)	Hyperchromic Shift at 260 mμ (%)	
			DNase Digestion			
Mouse satellite DNA						
Native	1.026	9.0	0.73	80	34.9	
Light strand	0.661	7.2	0.92	130	19.5	
Heavy strand	0.677	7.2	0.92	130	7.4	
Guinea pig satellite DNA						
Native	0.902	4.6	0.72	80	32.6	
Light strand	0.304	7.0	0.94	130	13.2	
Heavy strand	0.329	7.0	0.34	130	10.0	
			Exonuclease Digestion			
Mouse satellite DNA						c
Native	1.384	9.0	0.104	180	24.3	67.6
Light strand	0.790	7.2	0.130	360	28.2	54.0
Heavy strand	0.730	7.2	0.130	360	19.9	29.2
Guinea pig satellite DNA						
Native	1.196	4.6	0.120	180	25.4	66.5
Light strand	0.344	7.0	0.134	300	30.8	48.0
Heavy strand	0.362	7.0	0.134	300	25.4	38.0

[a] All digestions were carried out in 0.05 M ammonium acetate–0.001 M EDTA (pH 5.4). DNase and exonuclease digestions were done at room temperature. Phosphomonoesterase digestion was carried out for 16 hr at 37° using spleen acid phosphomonoesterase II (0.06–0.14 unit/ml of incubation mixture). [b] For the definition of enzyme units see Bernardi *et al.* (1966) for acid DNase, Bernardi and Bernardi (1968) for spleen exonuclease, and Chersi *et al.* (1966) for acid phosphomonoesterase. [c] This column reports the over-all (DNase plus exonuclease) hyperchromic shifts.

After neutralization, just one band was obtained (Figure 4c,c′) displaying densities of 1.699 in the case of mouse DNA and of 1.726 in the case of guinea pig DNA.

The complementary strands from both satellite DNAs were separated in a preparative alkaline CsCl density gradient (Figure 5A,B). The buoyant densities of the separated complementary strands were determined in a neutral CsCl density gradient. Figure 6a,a′,b,b′ shows the banding patterns obtained with the isolated light and heavy strands, respectively. In the case of mouse satellite DNA, the densities of the light and the heavy strands were found to be 1.698 and 1.723, respectively, in good agreement with the data of Flamm *et al.* (1967). In the case of guinea pig satellite DNA the densities of the light and the heavy strands were found to be equal to 1.708 and 1.757, respectively. If equimolar mixtures of the complementary strands from both DNAs were heated at 65° for 5 hr in 2× SSC, they showed, in neutral CsCl density gradients, single bands with densities close to those of satellite DNAs submitted to renaturing conditions (Figure 6c,c′).

Table I summarizes all the buoyant density data obtained under the different experimental conditions mentioned above. Data obtained on human satellite DNA by Corneo *et al.* (1968) are shown for comparison.

Ultraviolet absorbance–temperature curves were investigated in the cases of: (a) native, (b) heat-denatured and fast-cooled, and (c) renatured satellite DNAs from both mouse and guinea pig. The results are shown in Figure 7. The melting temperatures, T_m, were found to be equal to 86° regardless of source.

Table II shows in detail the experimental conditions used in the enzymatic digestions of satellite DNAs and their separated strands. The hyperchromic shifts caused by acid DNase and spleen exonuclease digestions were quite different in the complementary strands, being very high in the light strands, and particularly so in the case of mouse DNA.

The analytical results obtained on the main DNAs, the satellite DNAs, and their complementary strands are presented in Table III. The results of Flamm *et al.* (1967) on mouse satellite DNA are shown for comparison. The chromatographic separations of nucleosides obtained from guinea pig satellite DNA are shown in Figure 8. In all cases, the acid spectra of nucleosides showed absorption maxima and spectral ratios in good agreement with those reported in the literature.

Discussion

When satellite DNAs from mouse and guinea pig are denatured by alkali, or by heating in the presence of

TABLE III: Nucleoside Analysis of Mouse and Guinea Pig Satellite DNAs.

	Mouse Main DNA	Mouse Satellite DNA					
		Native		Light Strand		Heavy Strand	
		a	*b*	*a*	*b*	*a*	*b*
A	30.3	32.4	32.2	44.8	44.2	19.2	20.7
T	29.7	32.5	33.6	20.2	21.6	45.8	44.7
G	20.1	17.8	16.4	22.0	19.9	14.0	12.5
C	19.9	17.4	17.8	13.0	14.3	21.4	22.1
G + C %	40.0	35.2	34.2	35.0	34.2	35.4	34.6
Purine–pyrimidine	1.02	1.01	0.95	2.01	1.79	0.49	0.50

	Guinea Pig Main DNA	Guinea Pig Satellite DNA		
		Native	Light Strand	Heavy Strand
A	30.4	30.8	37.2	24.9
T	30.2	30.7	24.4	36.9
G	19.7	19.2	2.7*	35.4
C	19.7	19.3	36.0	2.7*
G + C %	39.4	38.5	38.7	38.1
Purine–pyrimidine	1.00	1.00	0.66	1.52

[a] Present work. [b] Data of Flamm *et al.* (1967). The results are given in micromoles per cent; they were obtained from duplicate analyses; in all cases the values agreed within 1% (within 2% for the very low values of G and C indicated by the asterisk).

formaldehyde, and are then centrifuged in alkaline or formaldehyde-containing CsCl, respectively, they show two bands of different buoyant densities, corresponding to the separated complementary single strands, which have, indeed, widely different base compositions. When isolated from preparative alkaline CsCl density gradient experiments and rerun separately in neutral CsCl, the complementary strands also show different buoyant densities, as expected.

The separated complementary strands of mouse and guinea pig satellite DNAs easily undergo reassociation[2] when mixed together in neutral solvents at room temperature, as shown by the appearance in CsCl density gradient of a single band. This reassociation explains why satellite DNA solutions heated up to 100°, in the absence of formaldehyde, and fast cooled, show a single band in neutral CsCl density gradient.

The reassociation phenomenon has been explained by the hypothesis (Waring and Britten, 1966) that satellite DNAs contain repetitive nucleotide sequences. Waring and Britten (1966) further suggested, on the basis of their finding that reassociated satellite DNA has virtually the density of the native molecules, that repetitive sequences are strung end to end. In contrast, the results of Flamm *et al.* (1967) and of the present work indicate that reassociated mouse satellite DNA has a density which is significantly higher than that of native DNA. The reassociated DNAs from guinea pig and human placenta, then, have densities which are much higher than those of native materials. Furthermore, heating the denatured (reassociated) satellite DNAs causes variable extents of decrease in buoyant density, but never leads to the recovery of the original density of native deoxyribonucleic acid. All these findings might be best explained by a model where a single repetitive sequence is interspersed among nonrepetitive sequences of various lengths, or where more than one repetitive sequence exists.[3] According to the particular arrangement of repetitive sequences in different satellite DNAs, one might expect different extents of base pairing upon reassociation and, in general, it would be impossible to reach a perfect matching of complementary strands.

The following comments concern the analytical results. As far as mouse satellite DNA is concerned, the present data are in good agreement with those reported by Flamm *et al.* (1967), except for the following minor differences. The G + C contents of unfractionated satellite and of its separated strands were found to be slightly higher than those reported previously; the purine:pyrimidine ratio of the light strand was found to be higher than that reported by Flamm *et al.* (1967) and to match perfectly the ratio obtained for the heavy strand. The analysis of guinea pig satellite DNA also shows very large differences in the base contents of the complementary strands. In this case G and C are present

[2] The word reassociation is used here in the sense given to it by Waring and Britten (1966), namely as indicating the stable pairing of DNA strands by virtue of complementary nucleotide sequences, however imperfect the match.

[3] A special case would be that in which complementary sequences would be present on one strand; in this case, intrastrand reassociation might occur.

TABLE IV: Per Cent GC Content of Mouse and Guinea Pig Satellite DNAs.

	Mouse		Guinea Pig	
	Main	Satellite	Main	Satellite
Nucleoside analysis	40.0	35.2	39.4	38.5
CsCl buoyant density[a]	40.0	30.6	39.7	45.9
Melting temperature[b]	40.0	40.8	39.7	40.7

[a] Calculated according to Schildkraut *et al.* (1962). [b] Calculated according to Marmur and Doty (1962).

at extremely low levels in the light and heavy strands, respectively.

It is very interesting to remark (Table IV) that, in contrast with the main DNAs, the satellite DNAs show buoyant densities and melting temperatures which are significantly different from those predicted on the basis of their composition. The melting temperatures correspond, in both mouse and guinea pig satellite DNAs, to G + C contents which are higher than those found by analysis. In contrast, the buoyant densities lead to estimates of G + C contents which are lower than the analytical ones in the mouse satellite, but higher in the guinea pig satellite. The results of Table IV clearly indicate how dangerous it is to rely on buoyant density or melting data to estimate the base composition of satellite DNA. In this respect, the case of guinea pig DNA is particularly striking since the "heavier" satellite has in fact a slightly lower G + C content than the main DNA. Another anomaly shown by the guinea pig satellite DNA is that it binds more Ag^+ than the main DNA, in spite of the fact that its G + C content is lower.

The discrepancies found between the analytical results and the base composition expected from the physical data might be due to the presence in satellite DNAs of nucleosides, like 5-methyldeoxycytidine or 5-hydroxymethyldeoxyuridine, which cannot be separated (according to unpublished results obtained in Strasbourg) from deoxycytidine and thymidine, respectively, by the method used in the present work. Our finding of correct spectral ratios for the isolated nucleosides and the possible presence of only 3% of 5-methyldeoxycytidine in mouse satellite DNA (Bond *et al.*, 1967) make it very unlikely that the discrepancies found be *only* due to the presence of "rare" nucleosides.

An alternative explanation might be that satellite DNAs are conformationally slightly different from the main DNAs, the differences being related to their peculiar nucleotide sequences. A similar explanation was put forward by Bernardi *et al.* (1968) to account for the different elution molarity from hydroxylapatite columns of mitochondrial DNA, compared with nuclear DNA. In connection with this explanation, it may be recalled that poly d(A-T) and poly (dG)·(dC) show "anomalous" buoyant densities (Schildkraut *et al.*, 1962), melting temperatures (Marmur and Doty, 1962), and optical rotatory dispersions (Samejima and Yang, 1965).

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