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Analysis of eucaryotic DNAs with a restriction endonuclease from H. influenzae: isolation of "hidden" satellite DNAs

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ABSTRACT

The restriction enzymes from H. influenzae have been used to study various eucaryotic DNAs. Fractions resistant to the action of these enzymes have been isolated from rat, mouse and calf DNAs. The method revealed the existence in rat of several components having the properties of satellite DNAs. Mouse DNA was shown to contain a new satellite of buoyant density 1.704. Therefore, this appears to be a powerfull method for the isolation of "hidden" satellite DNAs.

Calf satellite DNAs ρ = 1.705 and ρ = 1.723 were those resistant to the action of the restriction enzymes whilst satellite DNAs ρ = 1.714 and ρ = 1.710 gave fragments of discrete lengths, suggesting internal repeat units of 1 500 and 5 000 base pairs respectively.

INTRODUCTION

In some organisms fractions of the repeated sequences can be separated from the bulk of the DNA by equilibrium density centrifugation in either CsCl or Cs_2SO_4-Ag^+ gradients. These "satellite" DNAs separate when their GC content is very different from the bulk of the DNA and when their proportion in the genome is great enough to give detectable peaks (1, 2).

The proportions and the number of the satellite DNAs are variable among species. Mouse has a light satellite DNA which makes up about 10 % of the genome. The calf genome contains at least four satellite DNAs which make up 15 % of the total DNA (3). In some species, the proportion is even higher ; for example, kangaroo rat DNA contains fractions

separable on CsCl gradients which make up more than 50 % of the total DNA (4). In some other species, by contrast, no satellite DNA is detectable, neither in CsCl nor in Cs_2SO_4-Ag^+ gradients. Rat DNA gives a single heterogenous peak in neutral CsCl as well as in Cs_2SO_4-Ag^+ gradients. However, rat DNA does contain repetitive sequences, as shown by its reassociation kinetics (5). Among them, are very fast reassociating sequences which renature at a rate comparable to most satellite DNAs.

As shown elsewhere (6) many satellite DNA fractions in eucaryotes have the property to be relatively resistant to restriction endonucleases because of their low complexity. These enzymes hydrolyse generally a short sequence of 4 to 6 base pairs long in double stranded DNA. In a random sequence, such a site should occur from about one in 250 to one in 4 000 base pairs. It is expected then that a restriction sequence would generally occur with a low probability for sequences of 100 base pairs long or so. This prediction has been verified in most of the examples of satellite DNAs treated with the H. influenzae restriction enzymes Hin_d II plus Hin_d III (Southern E. M. & Roizès G., in preparation).

One obvious consequence of this property is that it will be possible to separate satellite like DNAs from the rest of the DNA as high molecular weight fractions after degradation with restriction endonucleases, thus allowing the isolation of fractions with the same properties as satellite DNAs which normally cannot be separated in density gradients.

Moreover, if the satellite DNAs have been formed by the duplication of very simple sequences in stages as postulated by Southern (7), one should find examples where the sequence recognized by a restriction endonuclease has been taken for such a multiplication. The consequence of this would be that these satellite DNAs will give a single fragment length after degradation with such an enzyme.

In this paper, we examine the high molecular weight fractions after treatment with the endonucleases from H. influenzae of rat, mouse and calf DNAs. We show, with these examples, that it is possible to isolate "hidden" satellite DNAs. Some of the properties of these fractions

are examined. Furthermore, we show examples of calf satellite DNAs where discrete lengths are obtained after degradation with restriction endonucleases.

MATERIAL AND METHODS

DNA Preparation :

DNAs from calf thymus and rat liver were extracted from nuclei prepared by the method of Frenster et al. (8). Mouse DNA labelled with ^{32}P was prepared from L cells grown in calcium-free F-12 medium containing one tenth of the usual amount of phosphate, with isotonicity and pH maintained with borate buffer. ^{32}P orthophosphate was added at 10mCi/l and the culture harvested 3 days later for extraction of DNA (9). The cells were washed and suspended in a hypotonic buffer containing 10^{-3} M EDTA. The suspension was then treated with a Dounce homogeniser and the nuclei were pelleted and washed at 2 000 rpm for 2 min, several times till they were clean under the microscope.

The different DNAs were then extracted from the purified nuclei, using the Sevag procedure with successive chloroform extractions, ethanol precipitation, RNase treatment, further chloroform extractions and isopropanol precipitation. DNA samples were finally dissolved and dialysed against the appropriate buffers and sedimentation coefficients of between 20 to 25 S were determined.

DNA fractionation on Cs gradients :

Preparative centrifugation to equilibrium in Cs_2SO_4-Ag^+ and CsCl were used in order to either fractionate or analyse some DNA samples.

Calculated quantities of DNA solution in 0.1M Na_2SO_4, of borate buffer (0.1M $Na_2B_4O_7$, pH 9.2), of 10^{-3}M $AgNO_3$ and 0.5 ml of Cs_2SO_4 (suprapur, Merck) saturated solution in 0.1M Na_2SO_4 per ml of final volume were mixed to obtain a final borate concentration of 0.005M and Ag^+/DNA-P molar ratio, R_f, as indicated in legends of figures.

DNA solutions in CsCl were obtained by adding the right amount of solid CsCl to the DNA solution.

The densities of the solutions were adjusted approximately to 1.5 g/cm^3 and 1.7 g/cm^3 for Cs_2SO_4 and CsCl respectively, as determi-

ned from the refractive index at 25°C. The solutions obtained were then centrifuged in conditions given in the legends of figures. At equilibrium, fractions were collected through a hole pierced on the bottom of the tube. The fractions were analysed by measuring A_{260} for cold DNA and the radioactivity in scintillator for the labelled ones.

Analytical CsCl centrifugation :

DNA samples to be analysed at equilibrium in neutral CsCl were dissolved at a concentration of 2-3 mg/ml in CsCl. The density of the solution was adjusted to 1.7 g/cm^3, as determined from the refractive index at 25°C. Micrococcus lysodeikticus DNA (buoyant density ρ = 1.731) was added as marker. The centrifugation was performed at 44 700 rpm, 25°C in a MSE analytical centrifuge. At equilibrium (24 hours), scanning was performed and the buoyant densities calculated.

Melting curves :

The melting curves of DNA were measured on the Gilford 2400 apparatus. The temperature was raised at 0.5°C/min. The samples were previously flushed for 5 min with helium to avoid the formation of air bubbles during heating.

DNA reassociation experiments :

DNA denaturation was carried out by heating DNA solutions in low salt (0.02 M NaCl, 10^{-4}EDTA) at 100°C for 10 min. DNA was then rapidly cooled to 0°C. The DNA concentration was 10-15 µg/ml for cold DNAs and less than a µg/ml for the radioactive samples.

The reassociation was performed at 60°C in 0.12M phosphate buffer (equimolar in Na_2HPO_4 and NaH_2PO_4). In order to avoid the size effect in the reassociation reaction, all DNA samples had previously been sonicated. The reassociation proceeded till the C_0t (nucleotide concentration in moles/l x time in sec) indicated in the legends and table were reached. The change in absorbance at 260 nm (hypochromicity) was the measure of the percentages of duplexes reformed in the experiments with cold DNA samples. In experiments with labelled DNAs, when the chosen C_0t was reached DNA samples were immediately added to hydroxypatite in 0.12 M phosphate at 60°C. The slurry was mixed and briefly centrifuged

at the same temperature. The same elution procedure was applied at 0.15 and 0.3M phosphate buffer concentrations. The supernatants and washings were pooled together and counted by Cerenkov radiation. Percentages of reannealed duplexes were given by the eluted fractions at 0.3M phosphate.

Enzyme preparation :

The enzyme was extracted from H. influenzae Rd 123 which was provided by S. Glover. The enzyme was purified by the procedure described by Smith & Wilkox (10). It was stored in the cold room in 0.2M NaCl, 0.02M Tris (pH 7.4), 0.3 % bovine serum albumin at a final activity of 8 Units/ml. No loss of activity was detectable over a period of one year. One preparation was entirely deficient in Hin_dII and was, therefore, used as pure Hin_dIII. It has been used to establish the sequence splitted by Hin_dIII (R. Old, K. Murray & G. Roizès, in preparation).

Degradation of DNA by the restriction endonucleases from H. influenzae :

DNA to be degraded was dialysed against Tris-HCl 10^{-3}M, pH 7.4. It was made 50 mM NaCl, 6.6 mM Tris-HCl pH 7.4, 6.6 mM $MgCl_2$ and 6.6 mM mercaptoethanol. An appropriate amount of enzyme was added and the solution was incubated at 37°C for at least 20 hours. λ DNA was incubated in the same conditions to make sure the degradation has gone to completion. In this case, approximately 25 bands were obtained when a mixture of the two enzymes were used, while only 6 were visible in an agarose gel electrophoresis when using only Hin_dIII. The enzyme reaction was stopped by adding EDTA to a final concentration of 0.01M.

DNA fractionation by sucrose gradient centrifugation :

After degradation by the restriction endonuclease, the DNA samples were fractionated by centrifugation on sucrose gradients. Conditions were, if not otherwise noticed in the legends of figures : 6-20 % sucrose ; 0.01M Tris (pH 7.4) ; 0.1M EDTA ; 0.04 % sodium lauryl sulphate (SLS). The sucrose gradients were run in the SW 25 rotor of a Spinco preparative centrifuge at 25 000 rpm, 20°C for 8 hours. At the end of the run, fractions were collected from the bottom of the tubes and further analysed.

Preparation of DNA samples for electron microscopy :

Samples of DNA to be examined in the electron microscope were spread on a drop of water as described by Inman & Schnös (11), at pH 7.5. They were then examined in a Jeol electron microscope 100 B, photographs were taken, the final magnification factor being 50 000. The molecules were measured on an apparatus connected to a computer devised by F. Cuzin in the Institut Pasteur (Paris). Histograms were made from a large number of molecules as indicated in the legends of the figures.

Agarose slab gel electrophoresis :

Gel electrophoresis was performed in a slab apparatus very similar to that described by Studier (12) ; 1 and 2 % agarose (Sigma for use in electrophoresis) gels were used. The gels were made by boiling agarose in the buffer in which the electrophoresis was performed (36 mM Tris, pH 7.5 ; 30 mM NaH_2PO_4 ; 1 mM $EDTANa_2$). After cooling at 50°C and addition of ethidium bromide at a concentration of 0.5 μg/ml, the agarose solution was poured in the slab apparatus. The samples were applied in the slots of the gel in 5 % sucrose. A voltage of 50 volts was applied for 30 min in order to prevent heating and mixing of the sample with the buffer. It was then raised to the desired value ; usually, 100 volts for 10 hours in 2 % and 200 volts for 4 hours in 1 % gels were the conditions used. The DNA was visualised by the fluorescence in UV of ethidium bromide bound to it. Photographs were taken through an objective set with two filters (one anti-UV and one orange) on FP4 Panchromatic films from Ilford. Microdensitometer tracings were made from them with a Joyce-Loebl apparatus.

RESULTS

Size distribution of the fragments obtained from the restricted DNAs.

When analysed by centrifugation in sucrose gradients or by electrophoresis in agarose gels, the restricted DNAs from rat, mouse and calf showed similar profiles. Most of the fragments had a length of a few hundred to a few thousands of base pairs (Fig. 1).

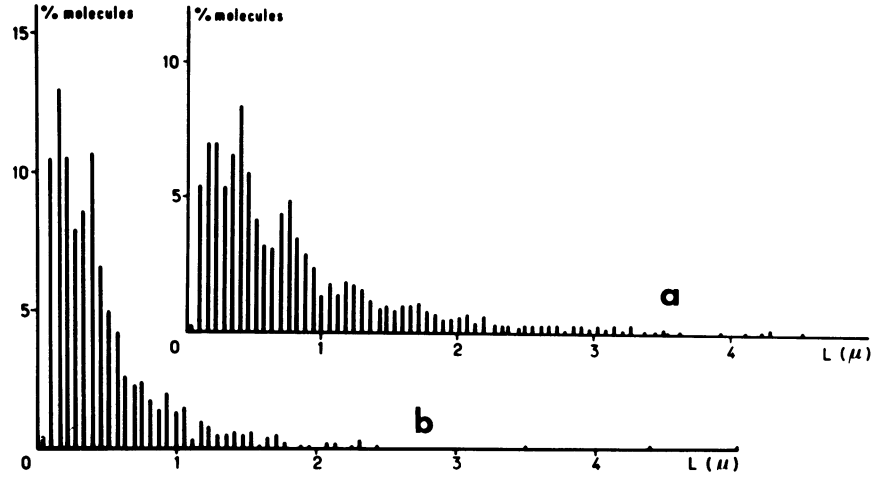

Figure 1 : Lengths distribution of total rat and calf DNAs after restriction by H. influenzae endonucleases. The restricted fragments of rat and calf DNAs were spread, as indicated in Material and Methods and examined in the electron microscope. The histograms were plotted by taking the fragments at intervals of 0.06 μ; 475 and 1025 DNA fragments were measured for rat (a) and calf (b) DNA respectively.

No band was visible in the gel electrophoresis microdensitometer tracing of mouse DNA degraded by the mixture of Hin_dII plus Hin_dIII (Hin) or by Hin_dIII alone whilst several bands were visible in both calf and rat DNAs (Fig. 2).

The number of bands detectable was five in the case of rat DNA degraded by the two restriction endonucleases of H. influenzae. Their lengths, calculated as the number of base pairs, were approximately 4 000 ; 2 500 ; 700 ; 540 and 400. They were determined by comparison with those obtained by degradation of λ DNA by the mixture of the two enzymes. When Hin_dIII alone was used, only three bands were still visible ; they were identical to those of lengths 4 000, 700 and 400 base pairs.

Calf DNA gave a more complex pattern of detectable bands which were visible on a smear of unresolved fragments. With the mixture of the

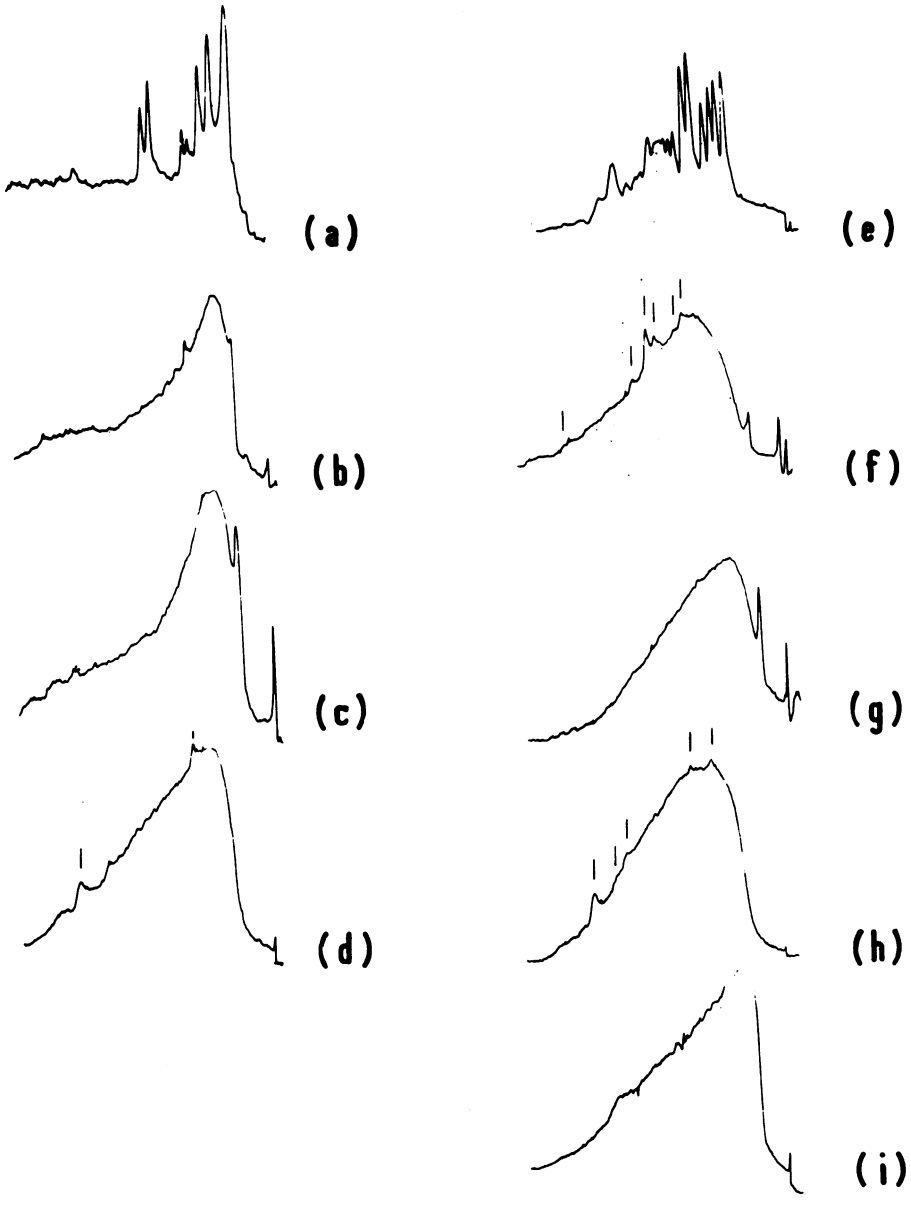

Figure 2 : Agarose slab gel electrophoresis of restricted DNAs : DNA samples after restriction by R Hin_dIII (a, b, c, d) or Hin (e, f, g, h, i) were applied on a 1 % slab agarose gel as described in Material and Methods. Microdensitometer tracings were made from the films taken at the end of the electrophoresis. The bands are indicated by arrows. (a, e) DNA ; (b, f) calf DNA ; (c, g) mouse DNA ; d, h) rat DNA and (i) Micrococcus lysodeikticus DNA.

two enzymes, at least six were visible, - but, it was difficult to establish, because of the too low intensity of some of them, if they all resulted from a complete digestion of the DNA ; the main ones had lengths which were 2 500 and 1 200 in base pairs. As it was not possible to analyse all of them in a digest of the total DNA, calf DNA was fractionated in caesium gradients in order to get homogenous fractions which are analysed in a separate paragraph. When total calf DNA was degraded by R Hin_dIII alone, only one band was detectable. These results confirm previous findings described in earlier papers by Southern & Roizès (6) and by Botchan et al. (13).

After degradation using restriction endonucleases, only a small proportion of the genome of rat, calf and mouse was of high molecular weight. Their proportions are not quantitatively comparable since they depend on the molecular weight of the starting material which has not been measured systematically in this study.

The analysis of the size distribution in the electron microscope show, for both rat and calf DNAs, that they are plurimodal and can be divided in several classes of different mean lengths.

Analysis of the restricted rat DNA fragments :

The fractions in the sucrose gradient profile of rat DNA (Fig. 3) were analysed more thoroughly since we chose this organism to show the presence of "hidden" satellite DNAs. The main classes of DNA molecules were pooled together, high molecular weight (R 1), moderately high molecular weight (R 2) and low molecular weight (R 3). In the experiment shown, the proportions were : R 1 = 5.2 % ; R 2 = 13.3 % ; R 3 = 81.5 % . It appears that these fractions are very different from each other on analytical equilibrium centrifugation in neutral CsCl (Fig. 4 , a). Several satellite DNAs are apparent, particularly in R 1, the main one having a buoyant density of 1.704, two minor peaks being detected at ρ = 1.712 and ρ = 1.719. A shoulder having the density of total rat DNA appears also in the light side of the peak. R2 has the same components, but increases its proportion of total like DNA ; while R 3 gives a profile identical to total low molecular weight rat DNA.

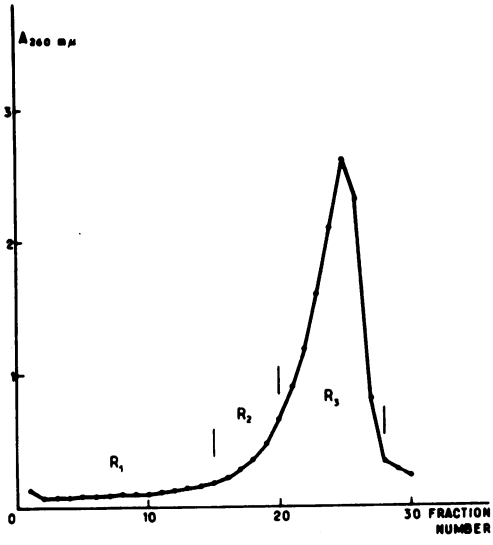

Figure 3 : Sucrose gradient centrifugation of restricted rat DNA : Total rat DNA was restricted by Hin and layered on top of a 6-20 % sucrose gradient. At the end of the run, 1 ml fractions were collected and their OD_{260mu} measured. The fractions were pooled in three main classes of molecules, dialysed and analysed.

The melting profiles of the three fractions were also different : the T_m values increased with the buoyant density of the fraction. R1 exhibited a multiple steps melting profile (Fig. 5) which again shows the heterogeneity of this fraction.

Measurements of the reassociation rates of the three fractions confirmed that repeated were enriched in the high molecular weight fractions. In order to eliminate the effect of size in the rates of reassociation, the fragments were sonicated to the same size.

A high proportion (52 %) of R1 renatured almost instantaneously ($C_0t < 10^{-3}$), this proportion of rapidly renaturing DNA was much lower (16 %) in R2 and was 5 % in R3. By $C_0t = 2$, R1 was reassociated to 62 %, R2 to 25 %, while R3 had reassociated to an extent (7 %) comparable with total rat DNA in similar conditions.

The reassociated fractions (to $C_0t = 2$) were analysed in neutral CsCl gradients (Fig4, b). The three peaks in R1 were 2-3 mg/ml more dense in the reassociated than in the native DNA and this is consistent with what

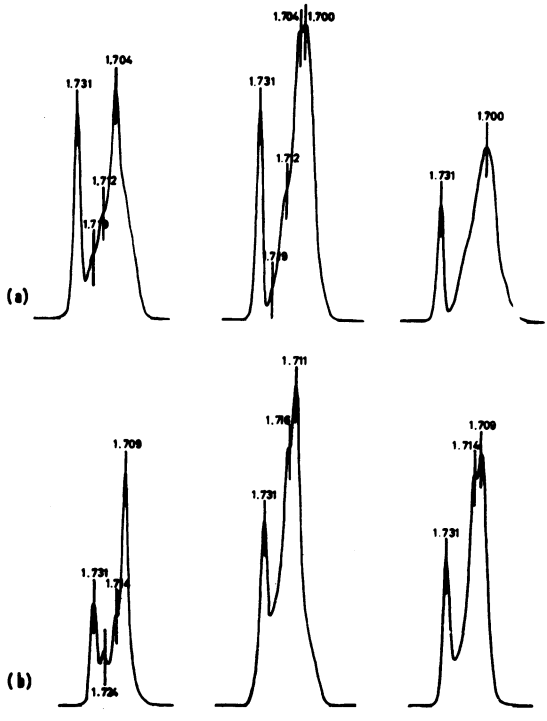

Figure 4 : Analytical centrifugation in caesium chloride of rat DNA fractions.

(a) R1, R2 and R3 analysed with Micrococcus lysodeikticus DNA (ρ = 1.731) as marker (left to right)

(b) R1, R2 and R3 reassociated at C_0t = 2

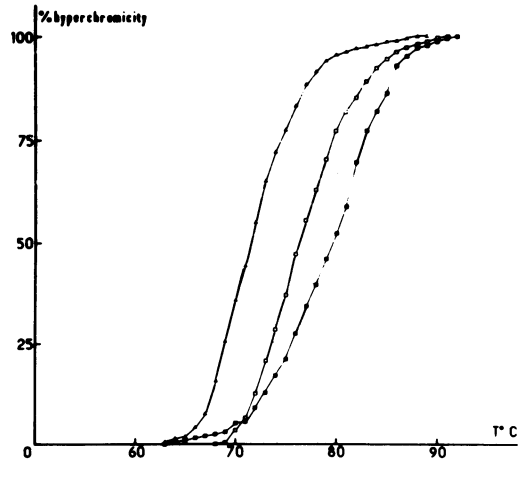

Figure 5 : Melting curves of rat DNA fractions.

R1, R2, R3

has been found with other satellite DNAs. The density increase in other fractions (10-15 mg/ml) is also consistent with that found with partially reassociated DNA from other eucaryotes. From these experiments, it is clear that the homogeneous density fractions contained in R1 and R2 have the properties associated with so-called satellite DNAs.

Analysis of the restricted mouse DNA fragments :

In spite of the many studies of mouse DNA, no density satellite DNA has been found other than the light satellite first described by Kit. Using restriction enzymes other "cryptic"satellites are revealed.

^{32}P labelled mouse DNA was fractionated in a CsCl gradient and the satellite DNA was refractionated in a Cs_2SO_4-Ag^+ gradient ; one could get, thus, a fraction originating from the light side of the main band (LSMB) which was almost free of the light satellite DNA.

This fraction was treated with Hin and the fragments separated on a sucrose gradient. Most of the DNA was broken to small fragments but there was a tail of faster sedimenting DNA. Fractions of the sucrose gradient were sonicated, denatured and reassociated to $C_0t = 10^{-2}$ and $C_0t = 10$. The extent of reassociation was measured by chromatography on HAP. As was found for rat DNA, the longer fragments contain the most rapidly reassociating sequences (Fig. 6). Two fractions from the sucrose gradient of the Hin fragments of light side of the main band DNA were analysed in silver-caesium sulphate gradient together with an excess of non radioactive total DNA. A high molecular weight fraction (fraction 10 from the sucrose gradient) contained a high proportion of satellite DNA, whereas a low molecular weight fraction (fraction 26) contained only main band DNA (Fig. 7). This is in agreement with earlier results which showed that mouse satellite DNA is comparatively resistant to Hin.

Main band from which the light fractions has been removed by two centrifugations in silver caesium sulphate was analysed in a similar manner : the sucrose gradient (Fig. 8) was divided into three fractions. M1 contained the largest and M3 the smallest fragments. Reassociation to $C_0t = 10^{-2}$ and $C_0t = 1$ (Table I) showed that the longer fragments were greatly enriched for repeated sequences. The three fraction were also analysed in preparative caesium chloride gradients with ^3H labelled total

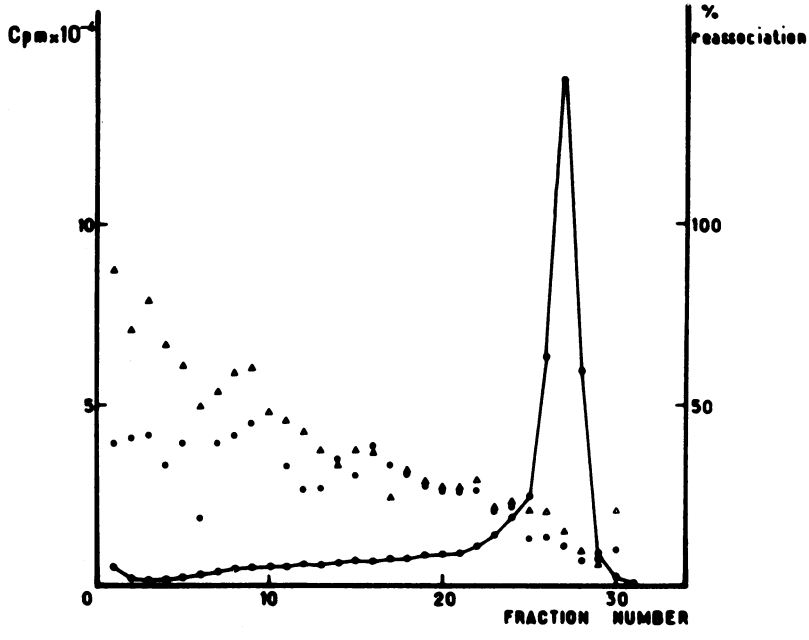

Figure 6 : Sucrose gradient centrifugation of mouse LSMB DNA after restriction by Hin :

The light side of the main band (LSMB) of mouse DNA was purified from the light satellite DNA which had been previously separated by one caesium chloride centrifugation at equilibrium in the 16 ml tubes of a 8 x 40 Ti MSE rotor at 26 000 rpm, 25°C for three days. The light satellite plus LSMB mouse DNAs were then run in silver-caesium sulphate (R_f = 0.35) in 10 ml tubes at 40 000 rpm, 25°C for 24 hours. LSMB DNA was then, after dialysis and concentration by sedimentation restricted by Hin in conditions described in Material and Methods. The restricted DNA was then layered on top of a 6-20 % sucrose gradient in the 6 x 15 ml MSE rotor and centrifuged for 6 hours at 25 000 rpm, 20°C. 0.5 ml fractions were collected from the bottom of the tube. The fractions were counted by the Cerenkov' radiation, dialysed and sonicated. They were then heat denatured and rapidly cooled. The concentrations in DNA (μg/ml) of the fractions had been adjusted and the times of incubation calculated to reach $C_0t = 10^{-2}$ and $C_0t = 10$ for each of the fractions. The reassociated duplexes were separated from single stranded DNA on HAP as described previously.

o—o Cerenkov ^{32}P counts of the restricted LSMB on the sucrose gradient.
●—● Percentage of reassociated duplexes at $C_0t = 10^{-2}$
△—△ " " " $C_0t = 10$.

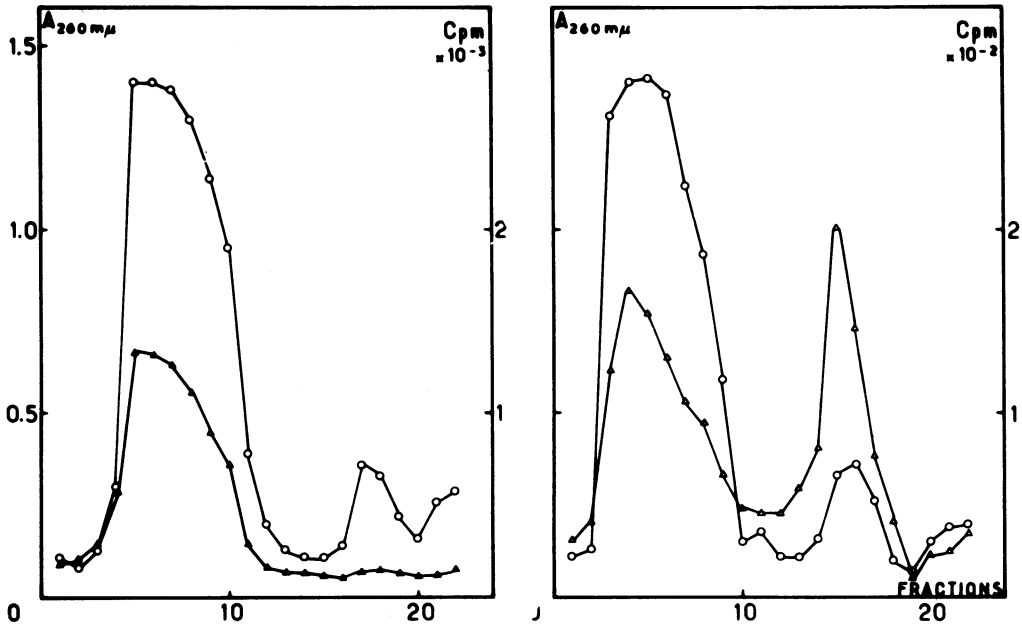

Figure 7 : Cs_2SO_4-Ag^+ gradient centrifugation of # 10 and 26 of the sucrose gradient centrifugation of restricted LSMB :

Aliquots of fractions 10 and 26 from the sucrose gradient of Fig. 6 were dialysed thoroughly against 0.1M Na_2SO_4. They were then prepared for analysis by centrifugation at equilibrium in Cs_2SO_4-Ag^+ gradients. Cold total mouse DNA (25 μg/ml) was added as a carrier. The R_f was 0.35. The run was performed in 10 ml tubes adapted to a 30 x 40 Ti MSE rotor for 24 hours at 40 000 rpm, 25°C. 0.2 ml fractions were collected from the bottom of the tubes. $A_{260\,m}$ was measured for each fraction and the radioactivity of aliquots counted in scintillator.

o—o $A_{260\,m}$ of cold carrier DNA

Δ—Δ ^{32}P of # 10 (a) and # 26 (b).

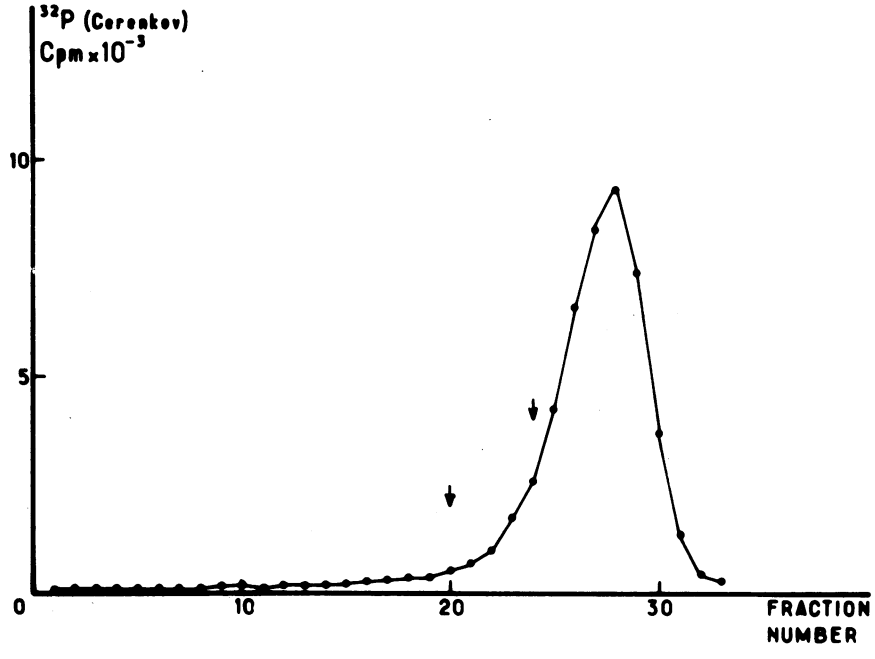

Figure 8 : Sucrose gradient centrifugation of restricted main band mouse DNA. Mouse main band DNA was purified through two centrifugations in caesium sulphate. It was then restricted by Hin and centrifuged in 6-20 % sucrose gradient in a SW 25 rotor, 25 000 rpm, 20°C for 7 hours. 0.5 ml fractions were collected from the bottom, counted by Cerenkov' method and pooled in three main fractions, M_1, M_2 and M_3.

Mouse DNA fraction	Percentage of duplexes formed	
	$C_0t = 10^{-2}$	$C_0t = 1$
M_1	18.7	34.9
M_2	2.5	10.9
M_3	0.5	1
"main band"	5.8	7.1

Table I : The fractions pooled in M_1, M_2 and M_3 were dialysed and concentrated by sedimentation. Aliquots, as well as non fractionated main band mouse DNA were sonicated, heat denatured and rapidly cooled. The concentrations in DNA (μg/ml) of the fractions had been adjusted and the times of incubation adjusted to reach $C_0t = 10^{-2}$ and $C_0t = 1$ for each of the fractions. The reassociation was followed as described previously.

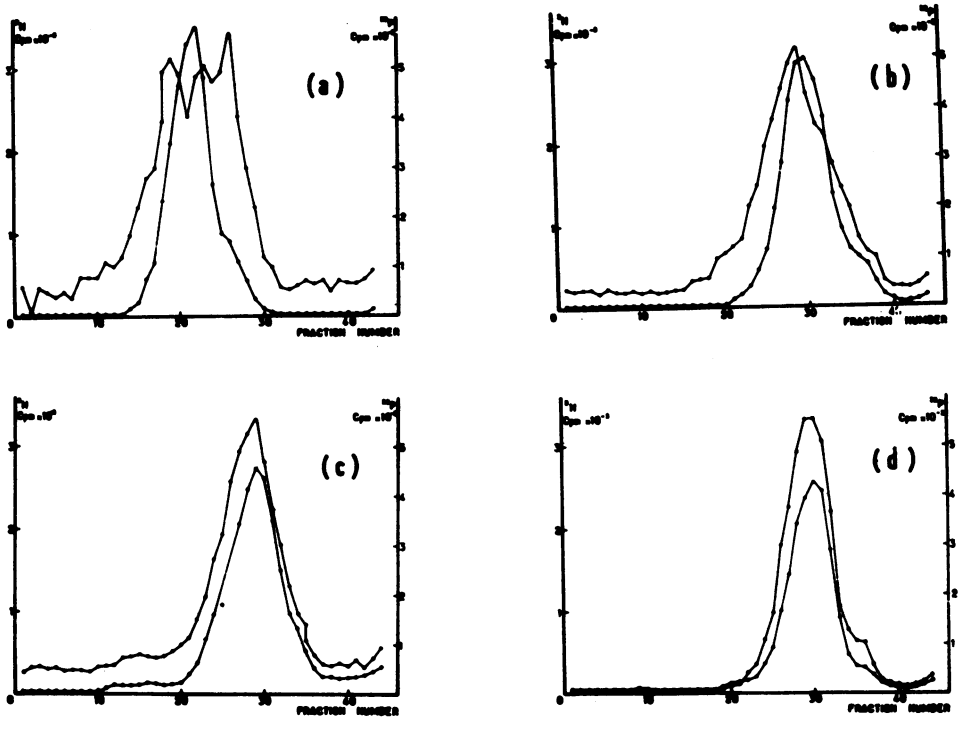

Figure 9 : Preparative CsCl centrifugation of restricted fractions of mouse main band DNA :

Fractions (a) M_1, (b) M_2 and (c) M_3 of fig. 8 were dialysed and concentrated by centrifugation. Aliquots were mixed with ^3H mouse total DNA as marker in a 4.5 ml CsCl solution. The run was performed for 48 hours in a Ti 50 Spinco rotor at 35 000 rpm, 25°C. 0.1ml fractions were collected from the bottom of the tubes and aliquots were counted in scintillator. A control (d) of total ^{32}P mouse DNA was made.

o—o ^{32}P mouse DNA fractions

●—● ^3H total mouse DNA.

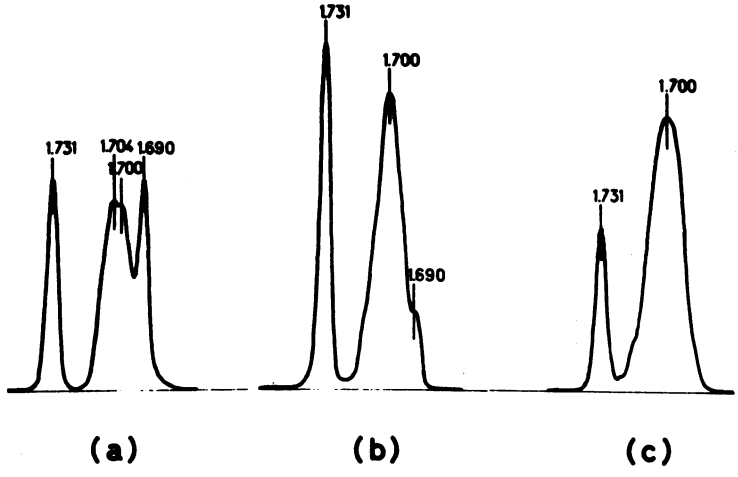

Figure 10 : Analytical centrifugation in caesium chloride of mouse DNA fractions :
M_1(a), M_2(b) and M_3(c) with Micrococcus lysodeikticus (ρ = 1.731) as marker.

DNA (Fig.9). The largest fragments gave three peaks in the density gradient. The densities, measured in the analytical ultracentrifuge were 1.690, 1.700 and 1.704. (Fig. 10). No satellite like DNAs were found in the smaller fragments either in the preparative gradients or in the analytical centrifuge.

Analysis of the restricted calf DNA fragments :

After treatment with Hin calf DNA shows a number of bands by agarose gel electrophoresis (Fig. 2). Filipski et al (3) have shown that calf DNA contains at least four density satellites which can be separated in silver-caesium sulphate gradients. It was therefore of interest to see if any of these satellite DNAs gave rise to the bands. It was found that all the bands were produced from satellite DNAs, and that the "main band" DNA gave a smear in the gel with no visible bands.

Fragments produced by Hin from satellite ρ = 1.714 were measured in the electron microscope (Fig. 11). Equal numbers of fragments were found : a long fragment of 0.4 μ or 1200 base pairs (743 molecules) and a shorter fragment of 0.1 μ or 300 base pairs (716 molecules). When incomplete digests are analysed by agarose gel electrophoresis, a band is seen with a molecular weight corresponding to the sum of the weights of one

short and one long fragment, showing in agreement with Botchan (in press), that the two fragments are linked together in the satellite DNA.

When satellite DNA ρ = 1.710 (largely contaminated with main band DNA) was degraded with R Hin_dIII (Fig. 12), it gave one distinct band which

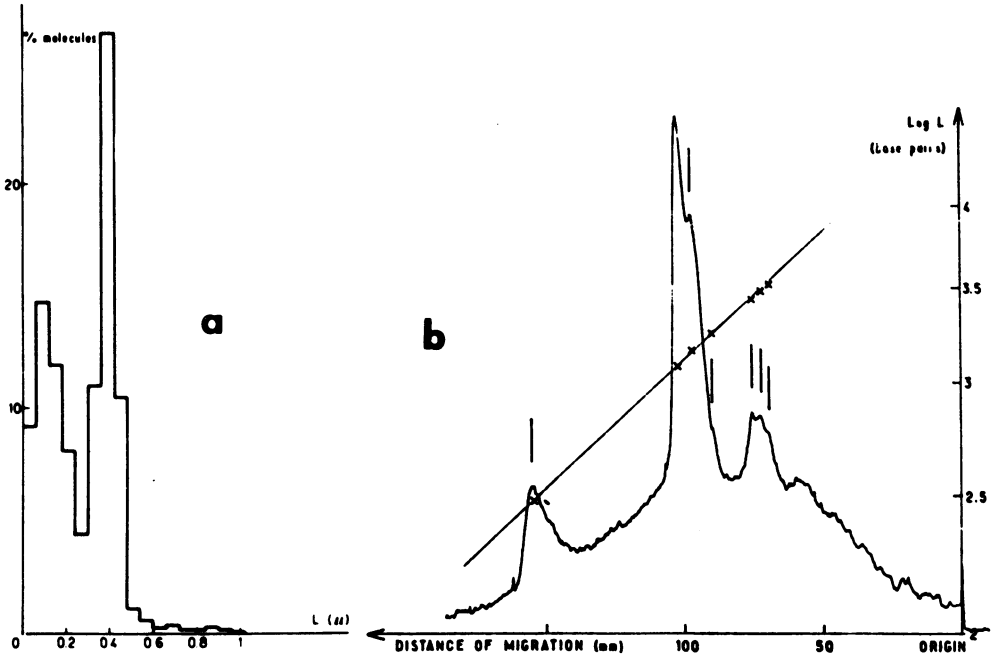

Figure 11 : Analysis by electron microscope of calf satellite DNA ρ = 1.714 restricted by Hin :

Calf satellite DNA ρ = 1.714 was purified from the heavy side of a preparative silver-caesium sulphate centrifugation. This was performed by recycling it three times in preparative neutral caesium chloride gradient centrifugation. In the same time, main band calf DNA was purified of any distinguishable satellite DNA in the analytical ultracentrifuge. Satellite DNA ρ = 1.714 was then restricted by Hin and the DNA fragments spread for electron microscope measurements (a). A partial digestion was in parallel analysed by gel slab electrophoresis in 1 % agarose. On the microdensitometer tracing made from a photograph of the gel (b), all the fragment sizes possible for a partial digestion of a repeat unit of 1500 base pairs tandemly arranged were present: 300, 1200, 1500, 1800, 2700, 3000, 3300 ... were present ; they falled in a straight line when their distances of migration were plotted against their lengths in base pairs of nucleotides.

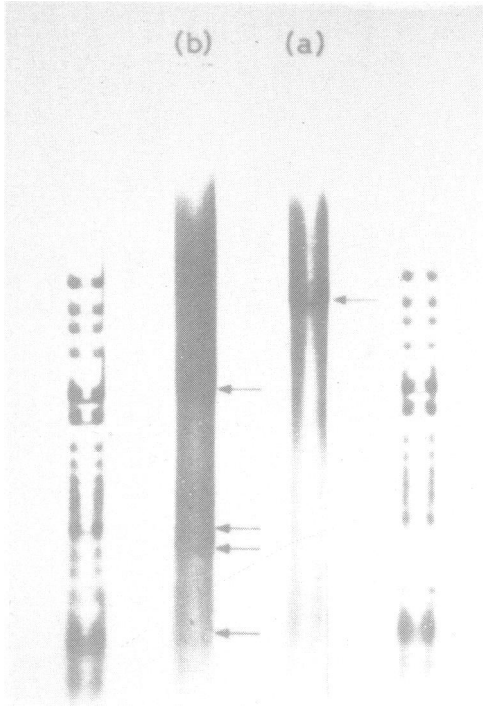

Figure 12 : Calf satellite DNA ρ = 1.710 was prepared according to Filipski et al (3). A large amount of main band DNA was still present in the preparation. However, as main band DNA did not give bands in agarose gel electrophoresis, the fraction was analysed without further purification. Restriction was carried out using Hin_dIII (a) and Hin (b) and gel electrophorised together with two samples of λ DNA degraded by Hin. The bands unambiguously visible are indicated by an arrow.

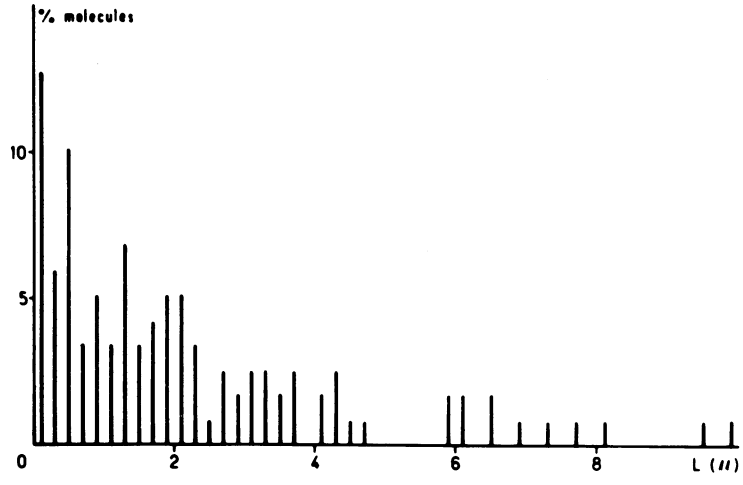

Figure 13 : Analysis by electron microscope of calf satellite ρ = 1.705 restricted by Hin : Calf satellite ρ = 1.705 was extracted as other fractions according to Filipski et al (3). The fraction was pure in the analytical centrifuge. It was restricted by Hin and was spread as described previously. 118 DNA fragments were measured. The histogram was plotted by taking the fragments at 0.2 u intervals.

was estimated by indirect measurement, as being approximately 5 000 base pairs long. It was not obvious if the restriction sequence was present once or twice at distances of 4 700 and 500-600 base pairs. This result, in any case, suggested a repeat unit of approximately 5 000 base pairs long. When the same fraction was degraded by Hin , several bands were obtained. They were approximately 500-600, 900, 1150 and 2500 base pairs long.

Analysis of the fragments produced by Hin from satellite DNA ρ = 1.705 showed that it is relatively resistant to the action of the two nucleases : 63 % of the fragments were longer than 1 μ as against only 13 % of the fragments produced by the restricted main band calf DNA. Expressed as percentage by weight of DNA, the figures were 93 % and 10 % (Fig. 13).

The mixture of two fractions of buoyant densities ρ = 1.710 and ρ = 1.723 gave a complex pattern in which bands were apparent and were similar to those obtained with satellite DNA ρ = 1.710 in a partial digest. However, a large fraction was relatively resistant to the restriction endonucleases. Therefore it could be satellite ρ = 1.723.

Restricted total calf DNA was divided into size classes on a sucrose gradient (Fig. 14). The profiles of these fractions in analytical caesium chloride were clearly different. The long fragments contained satellite DNA ρ = 1.723 and ρ = 1.705 together with fractions of intermediate buoyant density, but with no detectable "main band" DNA. On the other hand the smallest DNA fragments contained only main band DNA.

DISCUSSION

The use of restriction enzymes to isolate repeated sequences from eucaryotic DNAs is likely to be of more general application than isopycnic centrifugation since it depends on a structural feature which is common to all such sequences rather than on the trivial property of base composition. As has been argued elsewhere (E. Southern & G. Roizès, in preparation), tandemly linked repeated sequences remain high molecular weight after treatment with restriction endonucleases, provided the restriction site is not present in the sequence. There is a high probability of the sequence not being present in sequences with the structure of satellite DNAs. The

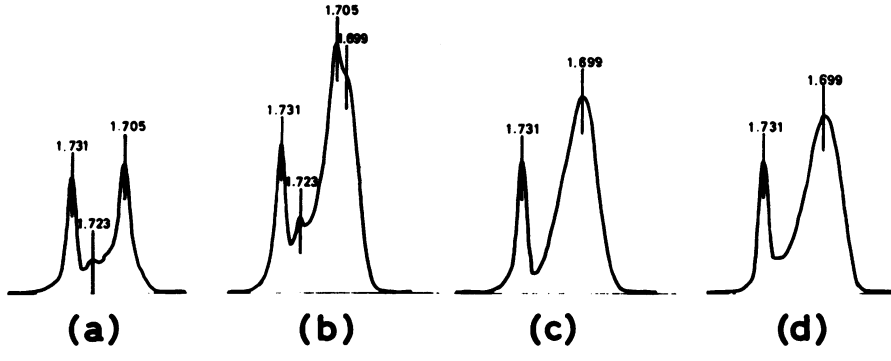

Figure 14 : Analysis of different size classes of calf DNA fragments restricted by Hin :

Total calf DNA was restricted by Hin and fractionated as described previously on a 6-20 % sucrose gradient. Four fractions of decreasing sizes were made. Analytical centrifugations in caesium chloride were performed with Micrococcus lysodeikticus DNA as a marker. The sizes of the DNA fragments are decreasing from (a) to (d).

method was used here to isolate cryptic satellites from rat and mouse DNAs. These cryptic satellites present in rat and mouse have not been isolated previously in high molecular weight native form, though they almost certainly make up part of the fast reassociating DNA of these organisms.

It was found in this work that a high proportion of mouse light satellite DNA is released by treatment with restriction enzyme and sucrose gradient sedimentation of the main band DNA already purified through two cycles of silver-caesium sulphate centrifugation. It could consist mainly of light satellite DNA sequences covalently linked to other sequences. It would be of interest to study the linkage points between the satellite and other sequences.

In the analysis of calf DNA, two satellite fractions are shown to behave in a similar manner to the fractions of mouse and rat DNA described above. In addition examples are given of satellite DNAs which contain the restriction site within the repeating sequence and, as has been shown for other sequences, these are broken to fragments of uniform length by the enzyme, giving a measure of the length of the sequence.

These two types of breakage pattern are consistent with the sequences being linked in tandem. They do not show the behavior expected of "interspersed" repeated sequences of the type described by Davidson et al. for Xenopus laevis DNA (14), Kram et al. and Wu et al. for Drosophila DNA (15, 16) or Cech et al. for mouse DNA (17). It will now be of interest to study the reassociation kinetics of restriction fragments of various size classes to see where the "intermediate" sequences occur. This should help to distinguish between the various models being considered for the organisation of the eucaryotic genome.

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