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Physical Analysis of a (dG+dC)-rich Fraction of DNA Obtained from the Ovine Genome

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Abstract

Using the organomercuric compound 3,6-bis(acetatomercurimethyl)dioxane in conjunction with Cs_2SO_4 density gradient equilibrium centrifugation a (dG+dC)-rich DNA fraction constituting 10% of the ovine genome was separated from the remainder. Further fractionation allowed four distinct classes of DNA to be identified with buoyant densities in neutral CsCl of 1·714, 1·717, 1·725 and 1·716 g/cm^3. Computerized curve resolving of the data indicated the presence of several additional DNA classes.

Data obtained using restriction endonuclease digestions indicated that the 1·714 g/cm^3 satellite consists principally of an 820-base pair (bp) tandemly repeating unit. The 820-bp DNA is also present in the 1·717 and 1·725 g/cm^3 satellites but as a minor component. The principal components of the 1·717 and 1·725 g/cm^3 fractions are 125-, 176- and 235-bp fragments. In addition, these fractions contain 705-bp tandemly repeated material. Two or possibly three species of 22-bp tandem repeats were found only in the 1·725 g/cm^3 DNA.

Introduction

In contrast to a considerable body of work on the bovine genome few studies have been reported on ovine DNA (Curtain *et al.* 1973; Votavova and Sponar 1975; Maio *et al.* 1977; Forstova *et al.* 1979; Reisner and Bucholtz 1980). In previous work two (dG+dC)-rich satellite DNAs have been identified with certainty in the ovine genome. They exhibited buoyant densities in CsCl of 1·712 and 1·723 g/cm^3 (Forstova *et al.* 1979). A third satellite with a buoyant density of 1·705 g/cm^3 has been reported by them, but they suggest that as yet it has been obtained in relatively impure form.

In previous work netropsin–CsCl centrifuged density gradients and kieselguhr–methylated serum albumin (MAK) columns were used. In the studies reported below, we have chosen to use the organomercuric compound 3,6-bis(acetatomercurimethyl)-dioxane (BAMD) (Bünemann and Dattagupta 1973; see also Cortadas *et al.* 1977). It is reported to show an affinity for (dA+T)-rich regions of DNA, resulting in an increased buoyant density, and to exhibit a degree of sequence selectivity (Cortadas *et al.* 1977; Macaya *et al.* 1978; Streeck and Zachau 1978).

Materials and Methods

BAMD was purchased as AR grade from Koch–Light Laboratories (Colnbrook, Bucks, England), and recrystallized once from hot water. Cs_2SO_4 was Merck Suprapur (Darmstadt, Germany), and CsCl was Sequanal grade (Pierce Chemical Co., Rockford, Ill., U.S.A.). SSC is 0·15 M NaCl, 0·015 M sodium citrate.

Ovine DNA was prepared from fresh thymus obtained from a local abattoir (Kay *et al.* 1952). The modal sedimentation coefficient ($S_{20,w}$) and molecular weight were 21 S and 8×10^6 respectively (Studier 1965; Reisner 1980).

Analytical centrifugations were performed in a Beckman model E ultracentrifuge equipped with mirror optics, monochromator and photoelectric scanner. It was interfaced with a Digital Equipment Corporation (DEC) PDP-11/10 computer (Reisner 1980) for automated data acquisition and subsequent analysis. Density gradient sedimentation equilibrium analyses were performed at 44 770 rpm (165 000 *g*) and 25°C for 20 h (Cortadas *et al.* 1977). Neutral CsCl analyses contained *Micrococcus lysodeikticus* DNA (1·731 g/cm^3) as a reference. Zonal sedimentation velocity runs were made at 29 500 rpm (71 000 *g*) at about 20°C (Studier 1965; Prunell and Bernardi 1973).

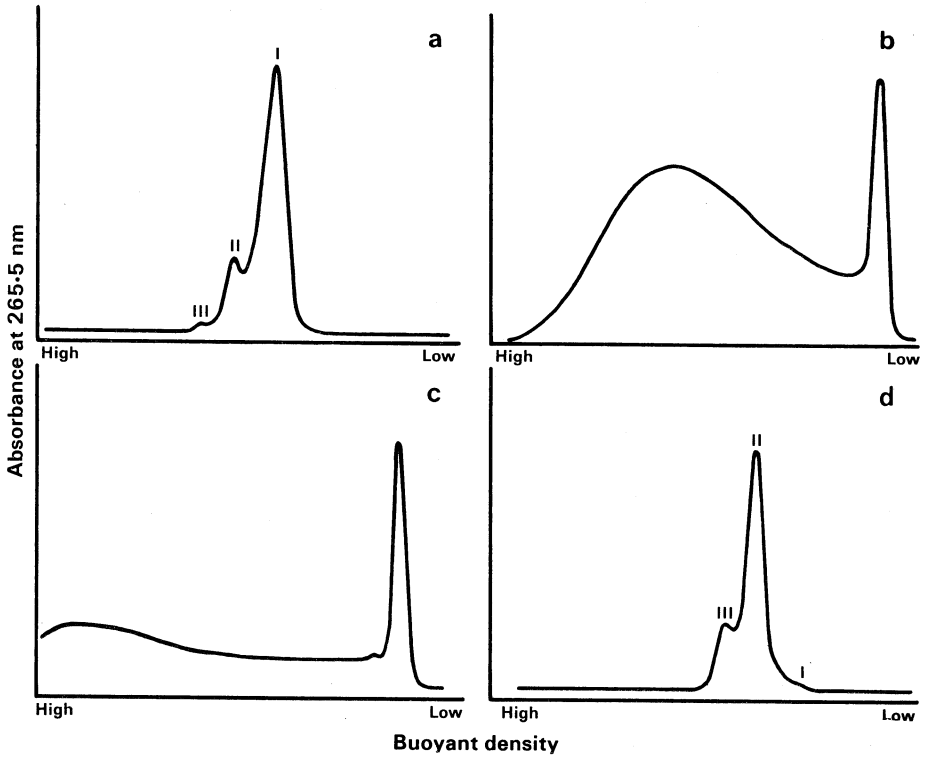

Fig. 1. (*a*) Analysis of unfractionated ovine DNA in neutral CsCl. Reference DNA removed by processing according to computer program. Buoyant densities (g/cm^3): I, 1·699; II, 1·713; III, 1·723. (*b*) Cs_2SO_4 analysis of BAMD–unfractionated ovine DNA complex, $r_f = 0{\cdot}08$. (*c*) Cs_2SO_4 analysis of BAMD–unfractionated ovine DNA complex, $r_f = 0{\cdot}18$. (*d*) Analysis of the (dG+dC)-rich fraction from ovine DNA in neutral CsCl. Reference DNA removed by processing according to computer program. Buoyant densities (g/cm^3): I, 1·702; II, 1·714; III, 1·724. Data were obtained through computer processing of data logged from buoyant density sedimentation equilibrium analyses in an analytical ultracentrifuge run at 44 770 rpm for 20 h at 25°C.

Preparative ultracentrifugations of Cs_2SO_4 gradients were performed at 38 000 rpm (120 000 *g*) and 25°C for 60 h in a Beckman type 60-Ti rotor. Otherwise, conditions for preparing, centrifuging and sampling the DNA–BAMD complex were as described by Cortadas *et al.* (1977).

For restriction endonuclease analyses DNA fractions were obtained using first a ratio of BAMD to DNA phosphate (r_f) of 0·18 to precipitate the bulk of the DNA upon equilibrium centrifugation, but leaving the (dG+dC)-rich material as a sharply defined zone (Fig. 1*c*). After removing the BAMD by dialysis, the solution was adjusted to an r_f of 0·13 to obtain the 1·714 g/cm^3 material or an r_f of 0·19, which precipitated the 1·714 g/cm^3 DNA while cleanly separating the 1·717 g/cm^3 fraction from the 1·725 g/cm^3 fraction in the Cs_2SO_4 gradient.

Thermal-denaturation data were obtained in a specially adapted Gilford instrument similar to the system described by Vizard and Ansevin (1976) and using similar conditions. The solvent was 0·001 M Tris–0·0001 M EDTA (disodium salt), pH 8·0. Measurements were carried out at 250 nm so that the contribution to hyperchromicity of breaking (dA-T) or (dG-dC) pairs was nearly equal.

Restriction endonucleases were obtained from New England Biolabs Inc. Beverly, MA 0195, USA and digestions were performed as recommended by the suppliers. The plasmid pBR322 was obtained from the Plasmid Reference Center, Stanford University, Stanford, CA 94305, USA. It was propagated in *Escherichia coli* RR1 and amplified and purified as described by Sleigh *et al.* (1979). DNA markers of suitable sizes were obtained by appropriate restriction endonuclease digestion of pBR322 (0·1 μg). Fragment sizes are based on Sutcliffe's values (1978). ^{32}P-labelling of DNA fragments was done at their 3′ ends as described by Sleigh *et al.* (1979).

Polyacrylamide gel electrophoresis was performed in 4% (w/v) acrylamide–0·21% (w/v) *N,N*-methylene bisacrylamide vertical gels and run as described by Sleigh *et al.* (1979) but at about 700 V (20 mA constant current) for 1–1·5 h. Thin vertical 1·5% (w/v) agarose gels (Marine Colloids Inc., HGT agarose, 0·5 by 200 by 400 mm) were run using the same buffer system under the same conditions. If denaturing conditions were required the 4% (w/v) polyacrylamide gels were prepared containing 7 M urea and were run at 35 mA. For application to the gel, dried samples were redissolved in 95% (v/v) formamide, 0·02 M EDTA (disodium salt) (10 μl).

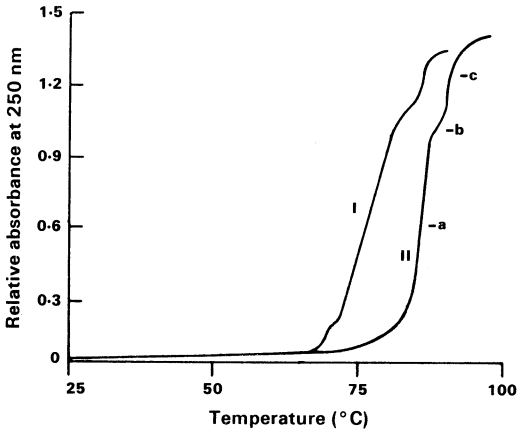

Fig. 2. Thermal-denaturation curves for unfractionated ovine DNA (I); and the (dG+dC)-rich fraction (II). $T_{m(a)}$ = 81·5°C, $T_{m(b)}$ = 83·0°C, $T_{m(c)}$ = 85·0°C. For further details see text.

Results

The appearance of unfractionated ovine DNA at buoyant equilibrium in a neutral CsCl density gradient is shown in Fig. 1*a*. The introduction of BAMD to yield a BAMD to DNA phosphate molar ratio (r_f) of 0·08 (Cortadas *et al.* 1977) resulted in the equilibrium profile in Cs_2SO_4 shown in Fig. 1*b*, while increasing the r_f to 0·18 caused the precipitation of over 70% of DNA (Fig. 1*c*). The sharply defined (dG+dC)-rich region seen in Fig. 1*c* was also obtained in a preparative run done under comparable conditions. This (dG+dC)-rich material (about 10% of the DNA) was dialysed against 2 M NaCl to remove BAMD. It gave the buoyant-equilibrium profile in CsCl (Fig. 1*d*) which shows the separation of 1·714 and 1·724 g/cm^3 material from the rest of the DNA. The thermal-denaturation profile of this material compared with unfractionated ovine DNA is shown in Fig. 2. In the buffer used, T_m for the unfractionated material was 74·5°C and that for the (dG+dC)-rich fraction was 82·0°C. After removal of Cl^- from the solvent system by dialysis against borate–sulfate buffer (Cortadas *et al.* 1977) BAMD was reintroduced to give an r_f of 0·13. Flow analysis of the preparative sedimentation equilibrium run yielded the tetramodal profile shown in Fig. 3*a*. Twenty-five samples each constituting about

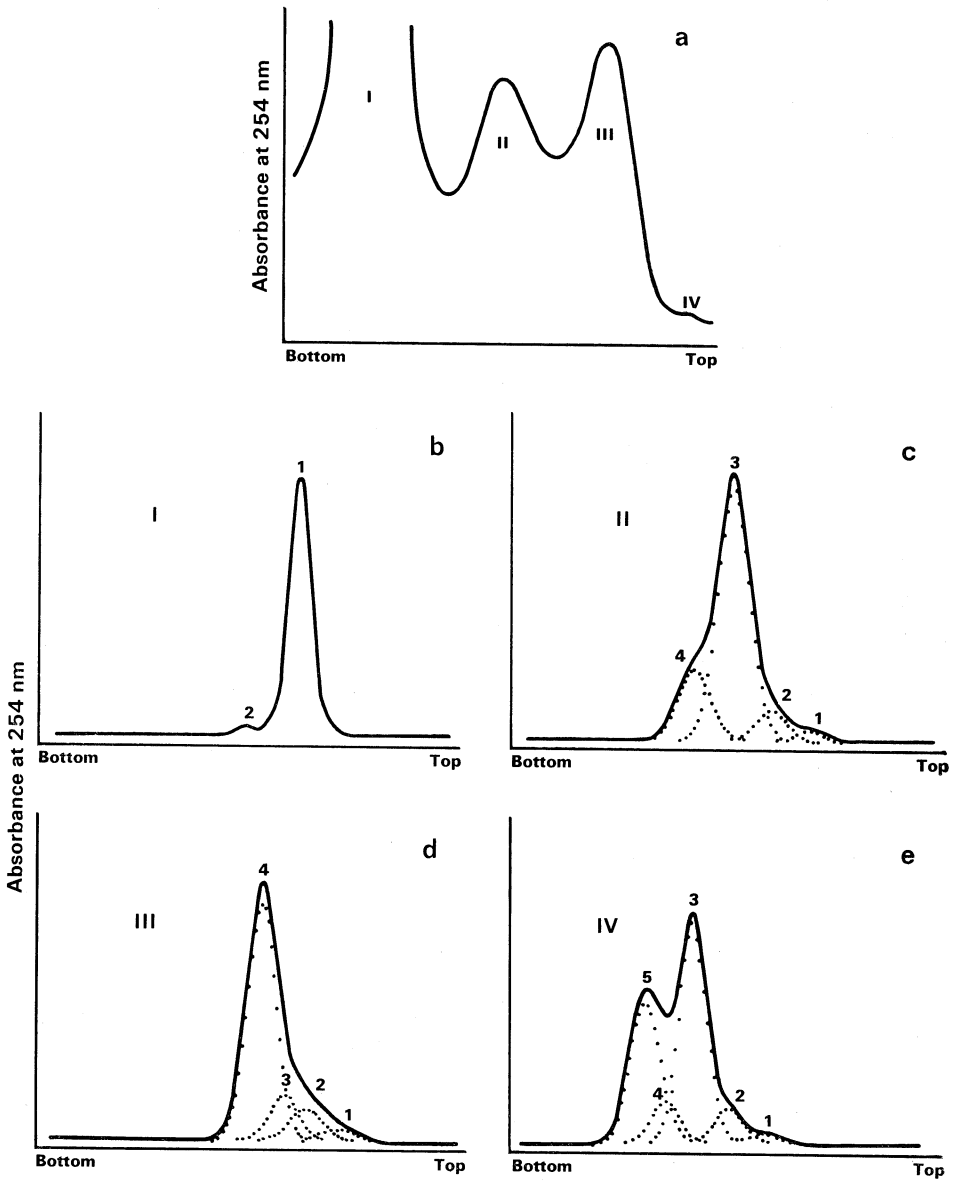

Fig. 3. (*a*) Flow-analysis profile of preparative ultracentrifuge run—see Materials and Methods for details. Zones I–IV are referred to in the text and below. Reference DNA removed by processing according to computer program. (*b*) CsCl analysis of zone I. Buoyant densities (g/cm^3) and relative areas: *1*, 1·714, 87·4%; *2*, 1·723, 2·6%. (*c*) CsCl analysis of zone II. Buoyant densities (g/cm^3) and relative areas of fitted curves: *1*, 1·704, 2·9%; *2*, 1·710, 7·9%; *3*, 1·717, 70·0%; *4*, 1·723, 19·2%. (*d*) CsCl analysis of zone III. Buoyant densities (g/cm^3) and relative areas of fitted curves: *1*, 1·713, 4·4%; *2*, 1·718, 10·6%; *3*, 1·721, 13·2%; *4*, 1·725, 71·8%. (*e*) $CsCl_2$ analysis of zone IV. Buoyant densities (g/cm^3) and relative areas of fitted curves: *1*, 1·703, 1·6%; *2*, 1·709, 6·8%; *3*, 1·716, 48·9%; *4*, 1·721, 8·8%; *5*, 1·725, 34·0%.

4% of the gradient's volume were taken, dialysed against 2 M NaCl and then SSC diluted 1 in 10 before analytical centrifugation in neutral CsCl. The results of the

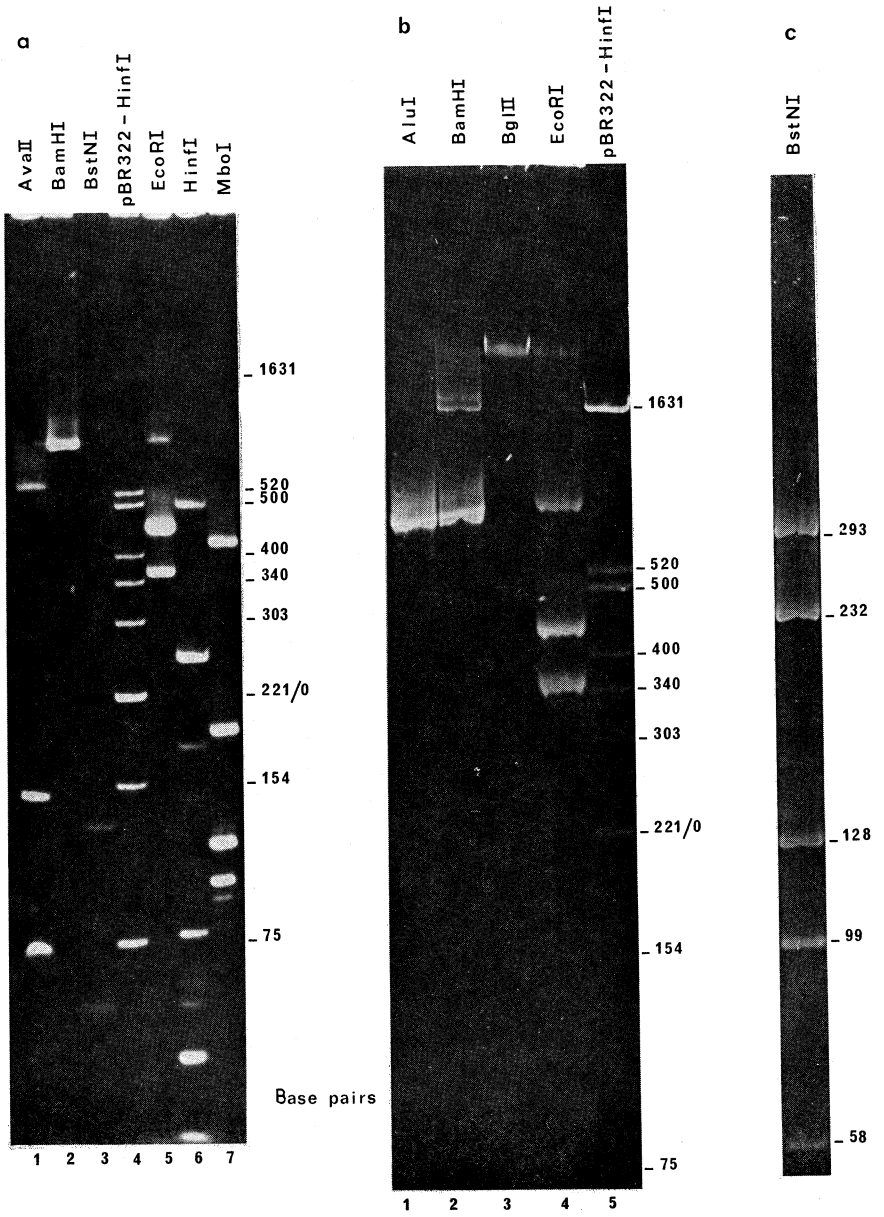

Fig. 4. Electrophoretograms of ovine 1·714 g/cm³ DNA in 4% (w/v) polyacrylamide gels. (*a*) Autoradiograph of ^{32}P-labelled material. (*b*) and (*c*) Electrophoretogram stained with 1% ethidium bromide.

analyses of mid-peak fractions taken from the preparative run are shown in Figs 3*b*–3*e* and indicate that the (dG+dC)-rich fraction contains distinct populations, with buoyant densities of 1·714 g/cm³, Fig. 3*b*; 1·717 g/cm³, Fig. 3*c*; 1·725 g/cm³,

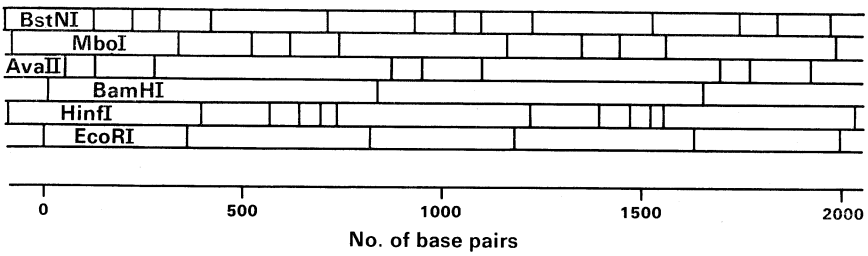

Fig. 5. Restriction endonuclease map of the 820-base pair repeated unit found in ovine 1·714 g/cm³ satellite DNA.

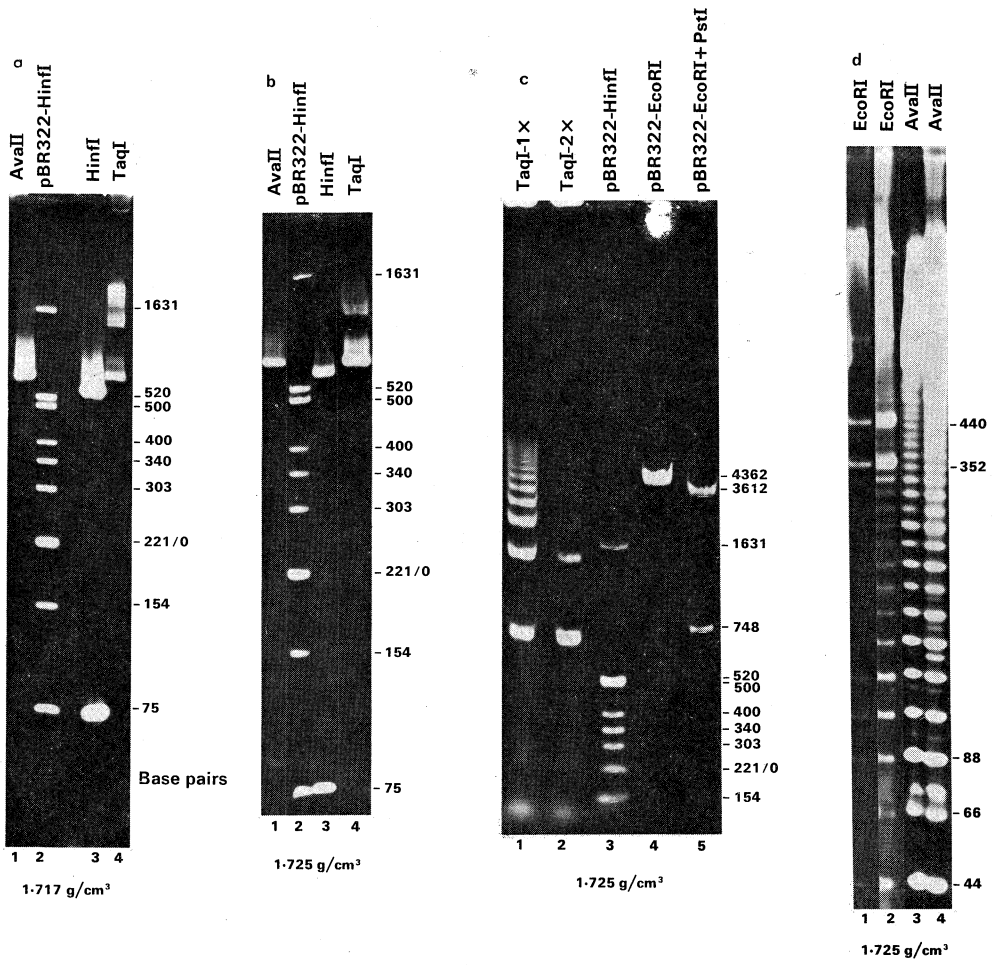

Fig. 6. Electrophoretograms of ovine 1·717 and 1·725 g/cm³ DNA. Autoradiographs of ^{32}P-labelled material in 4% (w/v) polyacrylamide (*a*, *b*, and *d*) and in 1·5% (w/v) agarose (*c*). (*a*) Relatively long exposure of film; 22-base pair repeating unit is absent. (*b*) Relatively short exposure showing presence of 705-base pair material. (*c*) Effect of two concentrations of TaqI on the 705-base pair DNA. (*d*) Short and long exposure of film to illustrate presence of the 465-, 355- and 820-base pair repeating unit (lanes 1 and 2) and different products of the AvaII digest (lanes 3 and 4).

Fig. 3*d*; and 1·716 g/cm^3, Fig. 3*e*. In addition, DNA with a buoyant density in CsCl of about 1·723 g/cm^3 was noted as a minor component in all samples (e.g. Fig. 3*b*), while curve resolution suggests the presence of a 1·721 g/cm^3 component as well as several other possible species.

Restriction endonuclease digestion of 1·714, 1·717 and 1·725 g/cm^3 DNAs are illustrated in Figs 4 and 6. No attempt was made to analyse the small 1.716 g/cm^3 fraction. The 1·714 g/cm^3 material (Fig. 4) consisted predominantly of an 820-base pair (bp) repeating unit (Fig. 4*a*, lane 2). Its restriction map, shown in Fig. 5, is consistent with data obtained from double digestions; however, the relative locations of some of the smaller fragments are not certain. Most, but not all, of the material contained an internal EcoRI site which allowed the cutting of the 820-bp unit into 365- and 455-bp pieces (Fig. 4*a*, lane 5). In addition, small amounts of higher-order oligomers (up to 2500 bp) were not removed by further digestion (Fig. 4*b*, lanes 2, 3 and 4). Fig. 4*c* shows the five fragments obtained with BstNI (isoschizomeric with EcoRII).

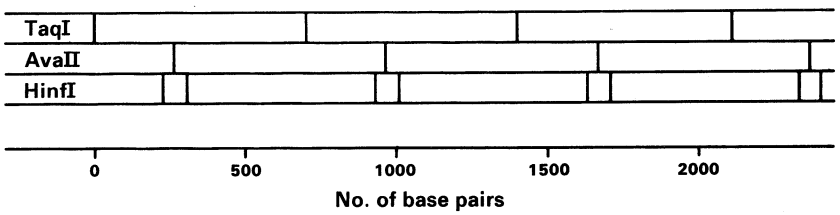

Fig. 7. Restriction endonuclease map of the 705-base pair repeated unit found in ovine 1·717 and 1·725 g/cm^3 satellite DNA.

Small amounts of 820-bp material were also found in the 1·725 g/cm^3 satellite (Fig. 6*d*, lane 1) and the 1·717 g/cm^3 satellite (not shown). In addition, AvaII and EcoRI digests of 1·725 g/cm^3 DNA produced oligomeric series having an increment of 22 bp (Fig. 6*d*, lanes 2, 3 and 4). Neither double digestions nor excision of the 44-, 66- or 88-bp fragments followed by redigestion with the second endonuclease produced smaller fragments. Also noted with AvaII digestion was a series from 77 through 187 bp, modulo 22 (Fig. 6*d*, lane 4) which was not detected with EcoRI digestion.

HaeIII digests of both 1·717 and 1·725 g/cm^3 DNA produced fragments of 235, 176 and 125 bp as reported previously for the 1·723 g/cm^3 satellite by Maio *et al.* (1977) and Forstova *et al.* (1979), the latter using the isoschizomer BspRI. In addition, however, significant populations of fragments 80 and 135 bp in size were found as well as a number of minor populations of fragments. We were able to characterize one of these minor populations in some detail. AvaII and TaqI produced fragments 705 bp long from both 1·725 and 1·717 g/cm^3 preparations when analysed in denaturing gels but which behaved like 640 bp fragments in non-denaturing gels. Digests of pBR322 were used as standards (Fig. 6*b*, lanes 1 and 4). The tandem repetitive nature of the material is illustrated by a partial TaqI digest (Fig. 6*c*, lane 1). In non-denaturing gels HinfI produced two fragments of 74 and 610 bp (Fig. 6, lane 3). The restriction maps of this DNA, based on double digestions, is shown in Fig. 7.

Discussion

In previous work MAK columns together with netropsin–CsCl centrifuged density gradients have been used to purify two (dG+dC)-rich components from sheep DNA with reported buoyant densities of 1·712 and 1·723 g/cm^3 (Forstova *et al.* 1979). A third component with a buoyant density of 1·705 g/cm^3 has also been isolated.

The use of BAMD–Cs_2SO_4 centrifuged density gradients has allowed us to separate remarkably cleanly a (dG+dC)-rich component which comprises 10% of ovine DNA (Fig. 1*c*) and which corresponds to the 1·714–1·725 g/cm^3 region of the ovine genome (cf. Figs 1*a* and 1*d*). (Note: the buoyant density values obtained in the present study are consistently 2 mg/cm^3 higher than those reported by Forstova *et al.* 1979.) The thermal-denaturation analysis (Fig. 2) shows that the material obtained from the first BAMD fractionation corresponds to the high-temperature thermolite seen in the unfractionated material. The pattern appears consistent with the profile obtained for the further fractionation in the ultracentrifuge (Fig. 3*a*) of the (dG+dC)-rich material; i.e. there are three thermolites with T_m's of 81·5, 83·0 and 85·0°C, which probably correspond to fractions I, II and III respectively.

This fractionation (Fig. 3*a*) has allowed the identification of four distinct (dG+dC)-rich components: the 1·714 g/cm^3 material (Fig. 3*b*), which appears to approach homogeneity (about 97·5% of the sample) and the remainder of the (dG+dC)-rich fraction which has been separated into three components (Fig. 3*a*, II–IV). The buoyant densities in CsCl of these three fractions are 1·717 (Fig. 3*c*), 1·725 (Fig. 3*d*) and 1·716 g/cm^3 (Fig. 3*e*, curve 3) respectively. The 1·716 g/cm^3 fraction constitutes about 0·1% of the genome but as shown in Fig. 3*a*, IV is clearly separated from the rest of the (dG+dC)-rich fraction. Significant differences in base sequence may be responsible for this. The computerized resolution of the density gradient profiles into normal distributions (Reisner 1980) suggests the presence of other (dG+dC)-rich components [e.g. one with a buoyant density of 1·721 g/cm^3 (Figs 3*d* and 3*e*), others with buoyant densities of 1·710 and 1·703 g/cm^3].

Forstova *et al.* (1979) reported a 760-bp fundamental unit together with its oligomers in their 1·712 g/cm^3 satellite using EcoRI, BamHI, AluI and BglII. Most of the 760-bp units contained an EcoRI internal recognition site which produced 420- and 340-bp fragments. Although we find that the fundamental unit in the purified 1·714 g/cm^3 fraction is 820 bp in length, it contains an internal EcoRI recognition site producing fragments of 365 and 455 bp and has a single BamHI site close to the main EcoRI site as Forstova *et al.* (1979) found. It appears likely that despite the sizable difference in estimation of length, this is the same material. This difference of 60 bp may be a strain difference reflecting a sizable insertion or deletion.

BAMD appears to have resolved the 1·723 g/cm^3 satellite reported by earlier workers into 1·717 and 1·725 g/cm^3 DNA. Restriction endonuclease digestion with HaeIII produced similar patterns with both fractions upon gel electrophoresis. There were major components of 235, 176 and 125 bp as reported previously for the 1·723 g/cm^3 material (Maio *et al.* 1977; Forstova *et al.* 1979). A number of additional components have been observed as well. Fragments of 135 and 80 bp are obtained using HaeIII together with a number of smaller species and 705-bp material was detected by AvaII, TaqI and HinfI. Therefore, the most striking difference between the two fractions is the presence of the 22-bp material in the

1·725 g/cm^3 DNA (Fig. 6*d*). Forstova *et al.* (1979) found no 20–25-bp material in ovine DNA but did in goat. The separation of the 1·717 g/cm^3 DNA from the 1·725 g/cm^3 DNA using BAMD, together with the use of (α-^{32}P)-labelling rather than ethidium bromide staining, are probably the reasons that we were able to detect the 22-bp material in the 1·725 g/cm^3 DNA. The situation is complex for our data are consistent with there being two, possibly three, different populations of 22-bp repetitive material in which successive internal restriction sites have been altered to produce the oligomeric series detected in Fig. 6*d*. Thus the 22-bp repetitive material visualized with EcoRI is not attacked by AvaII and *vice versa*. In addition, AvaII visualizes two DNA populations (22 bp and 22+11 bp). Not until appropriate material is cloned and sequenced will it be possible to understand the inter-relationships.

Finally, we have no evidence for a population of 1·705 g/cm^3 material in this (dG+dC)-rich material. However, analysis of the remaining 90% of the DNA (Reisner and Bucholtz 1980) has revealed a hypersharp zone in CsCl density gradients with a buoyant density of 1·707 g/cm^3.

Acknowledgments

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References

Bünemann, H., and Dattagupta, N. (1973). On the binding and specificity of 3,6-bis-(acetatomercurimethyl)dioxane to DNAs of different base composition. *Biochim. Biophys. Acta* **331**, 341–8.

Cortadas, J., Macaya, G., and Bernardi, G. (1977). An analysis of the bovine genome by density gradient centrifugation: fractionation in Cs_2SO_4/3,6-bis(acetatomercurimethyl)dioxane density gradient. *Eur. J. Biochem.* **76**, 13–19.

Curtain, G. C., Pascoe, G., and Hayman, R. (1973). Satellite DNA in the sheep and goat. *Biochem. Genet.* **10**, 253–62.

Forstova, J., Votavova, H., Guttmann, T., Pivec, L., and Doskocil, J. (1979). Components of sheep and goat DNA. *Nucleic Acids Res.* **6**, 57–70.

Kay, E. R. M., Simmons, N. S., and Dounce, A. L. (1952). An improved preparation of sodium-deoxyribonucleate. *J. Am. Chem. Soc.* **74**, 1724–6.

Macaya, G., Cortadas, J., and Bernardi, G. (1978). An analysis of the bovine genome by density gradient centrifugation: preparation of dG+dC-rich DNA components. *Eur. J. Biochem.* **84**, 179–88.

Macaya, G., Thiery, J-P., and Bernardi, G. (1976). An approach to the organisation of eukaryotic genomes at a macromolecular level. *J. Mol. Biol.* **108**, 237–54.

Maio, J. J., Brown, F. L., and Musich, P. R. (1977). Subunit structure of chromatin and the organization of eukaryotic highly repetitive DNA: recurrent periodicities and models for the evolutionary origins of repetitive DNA. *J. Mol. Biol.* **117**, 637–55.

Prunell, A., and Bernardi, G. (1973). Fractionation of native and denatured deoxyribonucleic acid on agarose columns. *J. Biol. Chem.* **248**, 3433–40.

Reisner, A. H. (1980). Analyses of DNA by automated logging and processing of data from the analytical ultracentrifuge. *Anal. Biochem.* **105**, 24–31.

Reisner, A. H., and Bucholtz, C. A. (1980). A physical study of the ovine genome. *Biochim. Biophys. Acta* **609**, 97–106.

Sleigh, M. J., Both, G. W., and Brownlee, G. G. (1979). A new method for the size estimation of the RNA genome segments of influenza virus. *Nucleic Acids Res.* **6**, 1309–21.

Streeck, R. E., and Zachau, H. G. (1978). A long-range and two short-range periodicities are superimposed in the 1·706 g/cm^3 satellite DNA. *Eur. J. Biochem.* **89**, 267–79.

Studier, F. W. (1965). Sedimentation studies of the size and shape of DNA. *J. Mol. Biol.* **11**, 373–90.

Sutcliffe, J. G. (1978). pBR322 restriction map derived from the DNA sequence: accurate DNA size markers up to 4361 nucleotide pairs long. *Nucleic Acids Res.* **5**, 2721–8.

Vizard, D. L., and Ansevin, A. T. (1976). High resolution thermal denaturation of DNA: thermolites of bacteriophage DNA. *Biochemistry* **15**, 741–50.

Votavova, H., and Sponar, J. (1975). Identification and separation of components of calf thymus DNA using a CsCl–netropsin density gradient. *Nucleic Acids Res.* **2**, 431–46.

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