REPETITIVE DNA OF MICROTUS AGRESTIS

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Summary: DNA from the European field vole Microtus agrestis was sheared to fragments approximately 500 nucleotides in length in a French pressure cell and fractionated according to repetitiveness on hydroxyapatite. Four fractions, amounting to 26% of the total DNA, were obtained at Cot values between $2x10^{-4}$ and $8x10^{-1}$. Analysis of the fractions by density gradient centrifugation in CsCl and determination of base composition and melting profiles, revealed the presence of 3 main components: two minor highly repetitive components, representing about 5 and 3% of the total DNA and rich in GC and AT, respectively, and a major component of intermediate repetitiveness with a base composition similar to that of total DNA.

Examination of mammalian DNA for repeated polynucleotide sequences normally shows 3 main fractions: 1) a unique minor fraction of satellite or highly repetitive DNA ($>10^6$ copies) which represents approximately 5-10% of the genome, 2) a fraction of intermediate repetitiveness (10^3 to 10^5 copies) which has not been investigated to any great detail and may represent as much as 30% of the genome, and 3) a major fraction, comprising the remainder of the genome, which shows little or no repetitiveness (1-6). The recent observation that mammalian satellite DNA is located in constitutive heterochromatin (2-5) and is found by "in situ" hybridization in the heterochromatic regions surrounding the centromere and the nucleolar organizer (7,8), have provided grounds for speculation concerning the role of this sizable portion of the genome which is now believed to be largely non-transcriptional (5,6,9).

A good model for the investigation of the above relationships is the well known European field vole Microtus agrestis in which the bulk of the constitutive heterochromatin is present in the giant sex chromosomes and is

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estimated to comprise some 17% of the total chromatin in the genome. In our laboratory, this animal has recently been the subject of a detailed cytological study (6,10) which showed that the two giant chromosomes remain condensed, in one form or another, in a large number of somatic cells, as well as in cells at all stages of development. The recent reports that DNA from M. agrestis contains little or no detectable satellite DNA (11-13) and that its repeated sequences amount to only 7% of the total DNA and are of the intermediate repetitive type (13), were quite disturbing because of their inconsistency with the above observations in other mammals. In the present investigation, fractionation of sheared DNA from M. agrestis according to repetitiveness on hydroxyapatite (1), followed by density gradient centrifugation in CsCl, revealed that about 26% of the DNA is repetitive and includes two minor components amounting to approximately 5 and 3% of the total DNA and possessing most of the characteristics of satellite DNA.

MATERIALS AND METHODS

DNA from M. agrestis was extracted from liver nuclei isolated in heavy sucrose by an adaptation of Marmur's procedure (14) described in a previous paper (3). Fractionation on hydroxyapatite was performed as described by Britten (1) with some modifications. DNA, at a concentration of 0.5 mg/ml in 0.4M phosphate buffer, pH 6.8, was sheared to fragments about 500 nucleotides in length by two passages through a French pressure cell at 46,000 psi. The solution was dialyzed against 0.01M phosphate buffer, heat denatured at 100°C for 10 minutes and fast cooled in dry ice-ethanol. Reassociation was performed at 65°C in 0.02 or 0.08M phosphate buffer, as shown in Table I. Following the separation of each reassociated fraction on hydroxyapatite, the subsequent fractions of decreasing repetitiveness were isolated by subjecting the previous fractions of non-reassociated DNA to another schedule of dialysis against 0.01M phosphate, heat denaturation (to ensure that non specific duplexes are broken up) and reassociation.

Melting profiles of the DNA fractions were determined in 0.1SSC in seal-

ed Beckman cuvettes (1 ml capacity), as described in a previous paper (5). Upon completion of the dissociation of the DNA strands, the samples were slowly cooled and the profiles monitored further, to detect any fast reassociation.

Base composition of the various DNA fractions was determined on formic acid hydrolyzates, as described in a previous paper (3).

Density gradient centrifugation was performed in 0.01M Tris, pH 8.2, as described by Flamm et al. (15).

RESULTS

The results of the fractionation of the repetitive sequences of M. agrestis DNA on hydroxyapatite are shown in Table I. The various fractions were reassociated under conditions which were much more strigent than those used by

Table I. Estimated Cot values and GC content of hydroxyapatite fractions from the DNA of M. agrestis[a].

Fraction	Phosphate (Molar)	Time of Reassoc.	% DNA Reassoc.[b]	Estimated Cot value[c]	% GC Content[d]
I	0.02	10 min	6.7	2×10^{-4}	42.6
II	0.08	5 min	4.0	2×10^{-3}	39.4
III	0.08	60 min	8.1	5×10^{-2}	39.4
IV	0.08	19 hrs	6.8	8×10^{-1}	39.5
Non repeated	-	-	-	-	39.5
Total DNA	-	-	-	-	39.7

a Approximately 3 mg of sheared DNA were fractionated as described under Methods. The DNA solutions were loaded on a 50x10 mm column of hydroxyapatite at 65°C and the reassociated DNA fractions isolated from the single stranded DNA by elution with 0.4 and 0.12M phosphate buffer, respectively. The concentration of DNA in the eluates was determined by measuring the optical density at 260 mμ in a Beckman spectrophotometer, using cuvettes of 0.2 cm path length.

b Values corrected for change in concentrations of DNA due to hyperchromic effect; this was estimated as the difference between the hyperchromic effect of sheared undissociated DNA (30.2%) and that of the non-repetitive fraction (8.5%) at 260 mμ (See Fig. 1).

c Co = Initial concentration of repeated sequences in the reassociation mixture; t = time of reassociation in seconds. Cot values in 0.02 and 0.08M phosphate were divided by 62 and 2.5, respectively, to compare with the standard rate of reassociation in 0.12M phosphate buffer established by Britten (1).

d Base composition was determined on 1-2 OD_{260} units of DNA. The variation in the per cent composition of the complementary bases was less than 5% in all cases.

Britten (1) - 65°C and 0.02 or 0.08M phosphate instead of 60°C and 0.12M phosphate - for two reasons: First, the lower buffer concentration slowed down the rate of the reaction and allowed the use of relatively higher concentrations of DNA, thus avoiding the need for labelled DNA (which is required for the detection of reassociation at low Cot values) and providing sizable repetitive fractions for further characterization; second, both the lower buffer concentration and higher temperature contributed to the reassociation of the highly ordered sequences only. As seen in the table, four fractions, amounting to 26% of the total DNA were reassociated at Cot values of 2×10^{-4}, 2×10^{-3}, 5×10^{-2} and 8×10^{-1}. The values were extrapolated to those expected in 0.12M phosphate, as indicated in the table legend. The table also includes the GC content of the 4 fractions; Fraction I yielded a value (42.6%) which was significantly higher than that of the other three fractions (39.4, 39.4 and 39.5%) and of non-repetitive (39.5%) and total DNA (39.7%).

An estimate of the faithfulness of duplex renaturation of the four fractions was inferred from the melting profiles shown in Fig. 1 (closed circles). All four fractions showed sigmoid profiles, somewhat comparable to that of total sheared DNA, although upon close examination Fraction I appeared to be biphasic. The overall Tm value for Fraction I was 66.0°C, only slightly lower than that of undissociated sheared total DNA (68.7°C). Fractions II, III and IV yielded Tm values of 59.1, 58.1 and 59.2, respectively, about 10°C lower than that of total DNA. Upon cooling at a fairly rapid rate (1°C/2 minutes), total DNA and Fractions II, III and IV showed little reassociation of strands, while Fraction I showed almost a precise reassociation with a recovery of approximately 60% of the hyperchromicity (Fig. 1; closed circles). In other experiments it was determined that such reassociation occurred even when the temperature was brought down at a much faster rate (from 80°C to 40°C within 10 minutes). Since these profiles were determined in 0.1SSC (containing the sodium ion equivalent of 0.01M phosphate buffer at neutral pH) the rate of reassociation of Fraction I must be much faster than indicated by the Cot reported

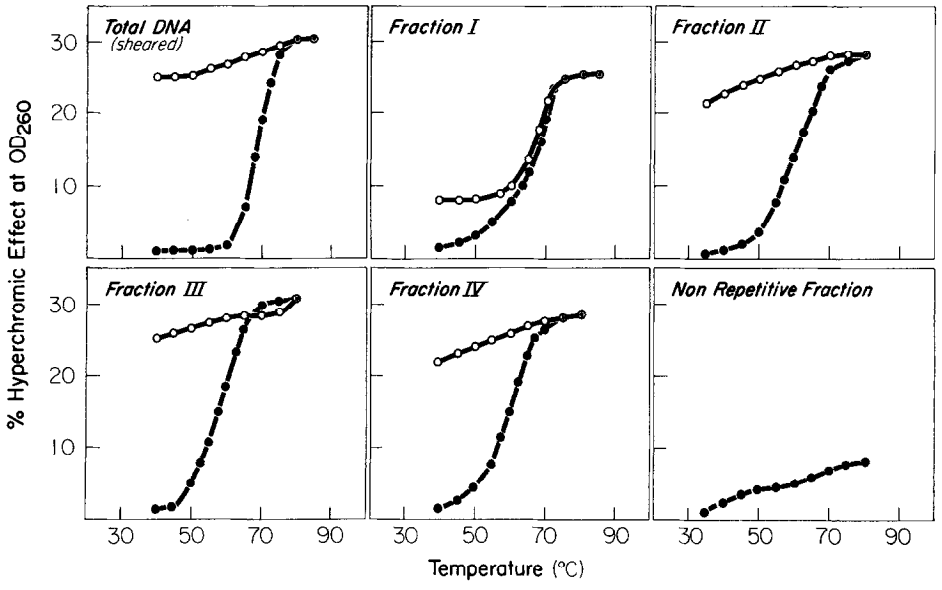

Fig. 1. Thermal denaturation and renaturation profiles of hydroxyapatite fractions from M. agrestis DNA. The profiles were determined on 0.3 OD_{260} unit of DNA in a final volume of 0.8 ml, as described under Methods. Denaturation profiles (closed circles); renaturation profiles (opened circles).

in Table I ($2x10^{-4}$), which was the earliest possible under the experimental conditions used. The hyperchromic effect of the four fractions varied between 25 and 30% and was essentially similar to that of total sheared DNA (30%).

Further characterization of the four fractions was accomplished by density gradient centrifugation in neutral CsCl, as shown in Fig. 2. In spite of the small size of the DNA fragments, three main peaks were observed at densities 1.717, 1.709 and 1.700 gm/cm^3, respectively. Fraction I was composed mainly of the 1.717 gm/cm^3 component (about 70%; or 4.8% of the total DNA), but also contained a significant amount of the 1.700 gm/cm^3 component (about 30%; or 2% of the total DNA). Fractions II and III contained mainly the 1.709 gm/cm^3 component plus minor amounts of the 1.700 gm/cm^3 component (about 15% and 5%, respectively; or 0.6 and 0.4% of the total DNA). Fraction IV was composed primarily of the 1.709 gm/cm^3 component, but also contained some DNA on both sides of this peak, probably representing non-repeated DNA sequences in various stages of reassociation. Upon pooling the tubes representative of each

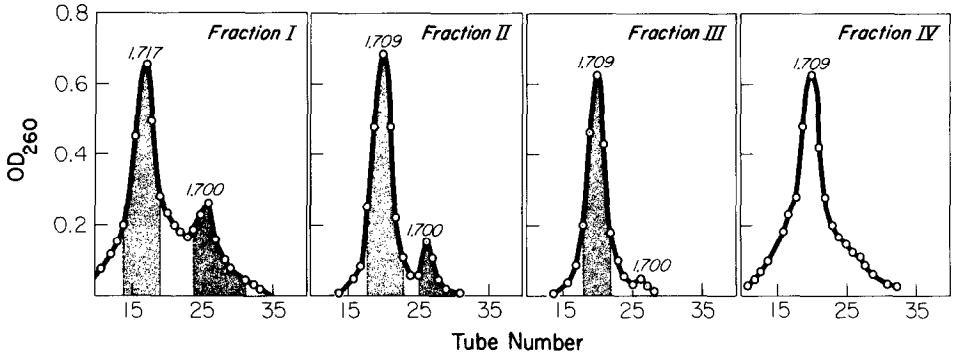

Fig. 2. CsCl sedimentation patterns of hydroxyapatite fractions from M. agrestis DNA. Each gradient contained approximately 1 OD_{260} unit. At the end of the run, 10 drop aliquots (0.1 ml) were collected, diluted with 0.15 ml of water and the optical density at 260 mμ measured in micro cuvettes of 1 cm path length. Aliquots represented by tubes 15, 30 and 45 were used for measuring refractive indices. Peak numbers represent buoyant densities calculated from the refractive indices at 25°C.

peak (as shown by the shaded areas in Fig. 2), dialysis vs. 0.1SSC and determination of melting profiles of the purified components, the 1.717 gm/cm^3 component - or main component of Fraction I - was quite sigmoid and monophasic with a Tm value of 66.8°C. The 1.709 and 1.700 gm/cm^3 components showed profiles essentially similar to those of the other three fractions in Fig. 2, with Tm values of 59.0 and 61.5°C, respectively. Upon subsequent cooling in 0.1SSC the 1.717 gm/cm^3 strands showed virtually complete reassociation while those of the other two components showed almost none, as expected. However, when dialysis was performed against 0.5SSC the 1.700 gm/cm^3 strands reassociated almost completely upon cooling, while those of the 1.709 gm/cm^3 component showed little reassociation. Under this condition, the Tm values were about 12°C higher due to the higher concentration of cations (16). Base analysis further revealed that the GC content of the 1.717 gm/cm^3 component is 49.3% and is 6.7% higher than indicated by the GC content of Fraction I (42.6%; Table I). The GC content of the 1.700 gm/cm^3 component was not determined, due to its presence in small amounts, but is probably low since it caused this decrease in the GC content of Fraction I by virtue of its presence as a minor

component (about 30%; see Fig. 2). The GC content of the 1.709 gm/cm^3 component was similar to that of total DNA (39%).

DISCUSSION

The presence of an appreciable portion of the DNA of M. agrestis as repeated sequences is consistent with similar observations in other mammals (1-6). The isolation of highly repetitive satellite-like DNA's from hydroxyapatite fractions, as shown here for the two minor components of M. agrestis, represents, to our best knowledge, the first successful attempt at such an isolation. The procedures described should aid in the isolation of similar components from other DNA's which are not amenable to the usual fractionation techniques in Cesium salts. Furthermore, such components should be easier to locate within density gradients of undissociated DNA when first isolated in this manner. In the case of M. agrestis, although the satellite DNA's have not yet been isolated in their original double stranded form to establish their identity with the components isolated on hydroxyapatite, the evidence presented suggests that they are quite similar. There are also preliminary indications that the complementary strands of the 1.717 gm/cm^3 component are so well matched that a strand bias can be shown by density gradient centrifugation in alkaline CsCl. This possibility, together with the possibility of isolating the satellite DNA's by zonal density gradient centrifugation, is currently being investigated.

The relation of the repetitive fractions of M. agrestis to the giant sex chromosomes is of special interest. Recently we have described a technique for the localization of repetitive DNA in metaphase chromosome preparations, using a conventional stain (17). The results indicated that the bulk of the giant sex chromosomes and the pericentromeric chromatin are composed of repetitive DNA.

REFERENCES

1. Britten, R.J., in "Problems in Biology: RNA in Development." (E. W. Hanly, ed.), p. 187. University of Utah Press, Salt Lake City, Utah, 1969.
2. Yasmineh, W.G., and Yunis, J.J. Biochem. Biophys. Res. Comm. 35:783 (1969).
3. Yasmineh, W.G., and Yunis, J.J. Exp. Cell Res. 59:69 (1970).
4. Yunis, J.J., and Yasmineh, W.G. Science 168:263 (1970).

5. Yasmineh, W.G., and Yunis, J.J. Exp. Cell Res. 64:41 (1971).
6. Yunis, J.J. and Yasmineh, W.G., in "Advances in Cell and Molecular Biology." (E. J. DuPraw, ed.), Vol II. Academic Press, New York, in press.
7. Jones, K.W. Nature 225:912 (1970).
8. Pardue, M.L., and Gall, J.G. Science 168:1356 (1970).
9. Walker, P.M.B., Flamm, W.G., and McClaren, A., in "Handbook of Molecular Cytology." (A. Lima-de-Faria, ed.), p. 52. American Elsevier Publishing Co., New York, 1969.
10. Lee, J.C., and Yunis, J.J. Chromosoma 32:239 (1971).
11. Schildkraut, C.L., and Maio, J.J. Biochim. Biophys. Acta 161:76 (1968).
12. Arrighi, F.E., Mandel, M., Bergendahl, J., and Hsu, T.C. Biochem. Genetics 4:367 (1970).
13. Hennig, W., and Walker, P.M.B. Nature 225:515 (1970).
14. Marmur, J.J. Mol. Biol. 3:208 (1961).
15. Flamm, W.G., Bond, H.E., and Burr, H.E. Biochim. Biophys. Acta 129:310 (1966).
16. Mandel, M., and Marmur, J., in "Methods in Enzymology." (L. Grossman and K. Moldave, eds.), Vol XII, part B. Academic Press, New York, 1968.
17. Yunis, J.J., Roldan, L., Yasmineh, W.G., and Lee, J.C. Nature, in press.