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Isolation and Analysis of Low Molecular Weight DNA Fraction by Electrophoretic and Chromatographic Techniques (4953)

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After enzymatic digestion of nuclei the chromatin structure is characterized by repeating subunits of DNA fragments as shown by electrophoretic analysis (1–5). Exposure of the chromatin from rat hepatocytes to Ca–Mg endonuclease yielded DNA fragments the smallest single stranded class of which had a molecular weight of 45,000–65,000 (2). DNA fragments of the same length have also been found in native DNA extracted from undigested nuclei and have been separated using analytical and micropreparative electrophoresis not only from DNA of rat hepatic nuclei (6, 7) but also from DNA of calf thymus (7). The latter does not contain any Ca–Mg endonuclease (1). Therefore, it is suggested that these DNA units are produced not only by the digestion of chromatin but are also formed *in vivo*. Hence they may be either a particular type of DNA, such as repetitive DNA or, alternatively, a transitory step during DNA synthesis and/or degradation.

Experiments were performed to rule out the possibility that this low molecular weight DNA fraction could be the result of enzymatic or chemical damage. The findings confirm the presence of these DNA fragments in native nuclear DNA extracted from rat hepatocytes and show that this low molecular weight DNA can be further fractionated in two parts, one formed by double stranded DNA, with an $S^{\circ}_{20,w}$ of 3.88 S and another, by single stranded DNA with an $S^{\circ}_{20,w}$ of 0.96 S, that is one-fourth that of the double helix.

Materials and methods. Thirty day-old Sprague-Dawley rats fed *ad libitum* with pellets (Morini, S. Polo d'Enza, Italy) were used. Hepatocyte nuclei were isolated from the livers using sucrose 2.2 *M* containing 3.3 m*M* $CaCl_2$, according to the method described by Bresnick *et al.* (8). In some experiments the $CaCl_2$ was omitted and 2 m*M* EDTA (Ethylene-diaminetetraacetic acid disodium salt) + 20 m*M* Ethylene glycol-*bis*-(2-amino ethyl ether)-*N*,*N*′-tetraacetic acid were added to the sucrose solution used in the nuclear preparation (1). The nuclear pellet after isolation in sucrose was washed with physiological solution (Barnes *et al.*, 9) or with 1 × SSC (0.15 *M* NaCl, 0.015 *M* Na citrate pH 7). The DNA was then extracted according to the method of Sato *et al.* (10) with the following modifications. RNase dissolved in 0.15 M NaCl, pH 5, was preincubated for 10 minutes at 90° and added to the nuclei dispersed in 1% SDS (sodium dodecylsulfate) + 1 m*M* EDTA solution (1–2 ml/g wet tissue) at the final concentration of 200 μg/ml. After 1 hr of incubation at 37°, pronase (300 μg/ml final concentration) was added and the incubation was prolonged for another 2 hr. Then the DNA solution was brought to 1 *M* NaCl and centrifuged at 10,000*g* for 30 min. With this procedure a large amount of RNA and protein was discarded in the sediment. The supernatant was brought to 1 *M* NaCl O_4 and treated four times with iso-amyl alcohol and chloroform (1:5 v/v). The DNA was precipitated with 2.5 vol of cold ethanol and recovered by centrifuging at 27,000*g* for 30 min. This method yield a recovery of 95% (1) whereas with Marmur (11) and Kirby (12) methods the recovery is only 45–50% (6, 7). The RNA content was 0.65% (13) as determined by the orcinol method (14). The protein content was about 1.5%, as determined by the method of Lowry (15). The DNA was then studied by electrophoretic and chromatographic analyses.

Gels were prepared with polyacrylamide at a concentration of 2.5–5% of that of the acrylamide. Two buffers were used: (i) 0.04 *M* Tris, 2 m*M* EDTA, 0.02 *M* Na acetate pH 7.8 and (ii) 0.025 *M* Tris + 0.033 *M* glycine pH 8.4–8.6 (16). The DNA (10–100 μg) was dissolved either in 0.04, or in 1 *M* phosphate buffer containing 10% sucrose and was layered over the gel. The electrophoresis was carried out at room temperature (25°), for different times and with different voltages

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depending upon the gel concentration and the buffer used. Bromophenol blue was used as the marker in each experiment. Staining of the gels was made with ethidium bromide (1) or Coomassie brilliant blue (17). In some experiments the unstained gel was divided in two parts containing DNA at low migration rate and DNA at high migration rate respectively. The two fractions were eluted with a micropreparative apparatus and were used for determination of DNA and RNA content (7). The chromatographic analysis was performed first on Bio-Gel P 300 for a rough separation of DNA according to molecular weight; then the eluted fractions were analyzed on hydroxyapatite for the separation of single stranded from double stranded DNA (18). The $S^{\circ}_{20,w}$ of the DNA fractions were determined by alkaline sucrose gradients (19, 20), and the Tm with spectrophotometric technique using Gilford 2400 equipped with Colora bath.

Results. Three fluorescent bands were evident on the 5% gels stained with ethidium bromide (Fig. 1a, b). The first, broader band had an Rf of 28.6%, when the DNA was dissolved in 0.04 *M* phosphate buffer and of 10% when 1 *M* phosphate buffer was used; it was formed by "low migrating" DNA and constituted the bulk of DNA, about 75–78% of the total. The other two bands ("high migrating" DNA) had an Rf of 98.6 and 100% respectively when calculated from DNA dissolved in 0.04 *M* phosphate buffer. Their separation was improved when DNA was dissolved in high molarity buffer (Rf 98 and 100%; Fig. 1a, b). The "low migrating" DNA and the "high migrating" DNA with an Rf of 100% were formed by protein-free DNA. On the other hand in the band with an Rf of 98%, proteins linked to DNA were present as shown by Coomassie brilliant blue (17) staining (Fig. 1c). The RNA evaluated as % of DNA was less than 1% and was equally distributed between low and high migrating DNA. Treatment with purified RNase (type A, Worthington Biochemical Co., Freehold, NJ) of high migrating DNA eluted with micropreparative system (7) did not affect at all migration rate and appearance of the bands when the high migrating fraction was further analyzed with electrophoresis.

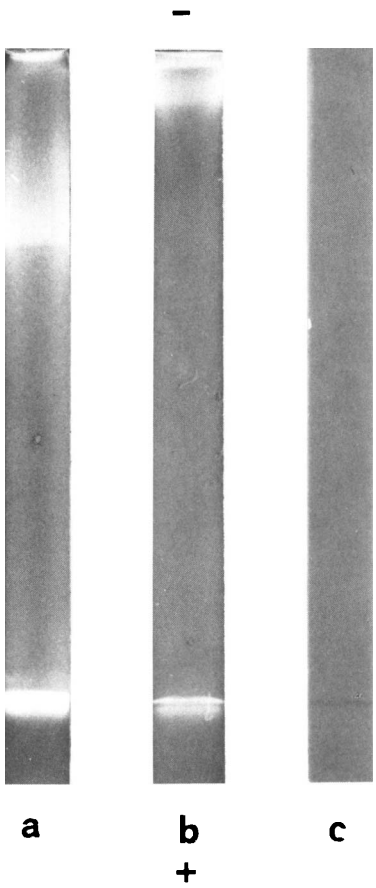

FIG. 1. Polyacrylamide gel electrophoresis of DNA isolated from purified hepatocyte nuclei. (a) DNA dissolved in 0.04 *M* phosphate buffer. The migration pattern shows three bands: the first is broader and has an Rf of 28.6%, "low migrating" DNA. The other two bands, formed by DNA which migrate faster, have an Rf of 98.6 and 100%, respectively. The gel was stained with ethidium bromide. Running condition: 5 mA/tube, 45 min. (b) DNA dissolved in 1 *M* phosphate buffer. The migration pattern is equal to that observed in *a*, but the Rfs of the three bands are 10, 98 and 100% respectively. The gel was stained with ethidium bromide. Running condition: 10 mA/tube, 75 min. (c) DNA, dissolved in 1 M phosphate buffer, has migrated as in *b*, and the gel was stained with Coomassie brilliant blue which is specific for proteins. Only the band with an Rf of 98% remains visible. Running condition: 10 mA/tube, 75 min. The bisacrylamide concentration of the gel was 5% of that of the acrylamide. Bromophenol blue was used as the marker and the Rfs are calculated with respect to it.

The electrophoretic pattern did not depend on the method of extraction since, as shown previously (6), DNA obtained from hepatocyte nuclei with the Marmur (11) and Kirby

(12) methods behaved in the same way on 5% gels. DNA extracted from hepatocyte nucleic isolated in the presence of the chelating agents EGTA and EDTA showed the same electrophoretic pattern (Fig. 2a). Since the nuclei after separation in sucrose were washed with physiological solution containing Mg^{2+} and Ca^{2+}, control experiments were performed on DNA, obtained from nuclei washed with 1 × SSC. The electrophoretic pattern of the DNA extracted from nuclei washed in 1 × SSC was similar to that obtained from nuclei washed with physiological solution (Fig. 2b). This finding was unchanged after adding DNase (SIGMA type I) to the 1 × SSC solution at a final concentration of 500 μg/ml (Fig. 2c).

Different results were obtained by changing the buffer during the electrophoretic run. When Tris acetate buffer was used the DNA did not migrate and remained at the origin, even if the polyacrylamide concentration was 2.5% of that acrylamide. This finding was in agreement with the results reported by Hewish and Burgoyne (1).

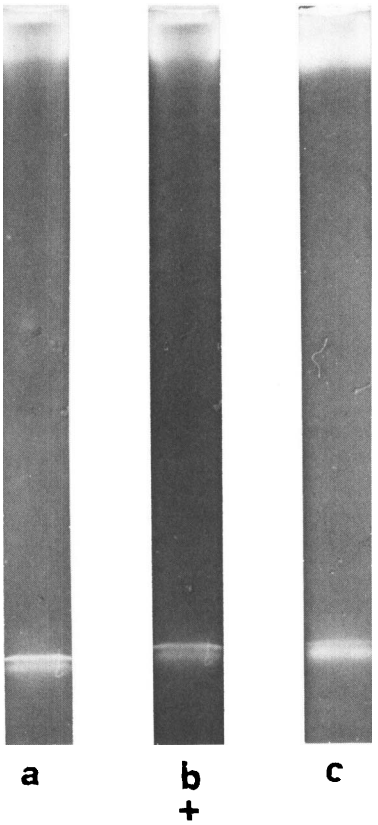

FIG. 2. Effect of endonuclease and DNase inhibitors on the pattern of the disc electrophoretic migration of DNA. (a) DNA extracted from nuclei isolated with sucrose containing 2 m*M* EDTA and 20 m*M* EGTA which inhibit the Ca–Mg endonuclease action (1). (b) DNA isolated from nuclei washed and suspended in 1 × SSC. (c) DNA isolated from nuclei washed and suspended in 1 × SSC containing DNase at the concentration of 500 μg/ml. The gels were prepared as in Fig. 1. The DNA was dissolved in 1 *M* phosphate buffer. Ethidium bromide staining was used. Running condition: 10 mA/tube, 75 min.

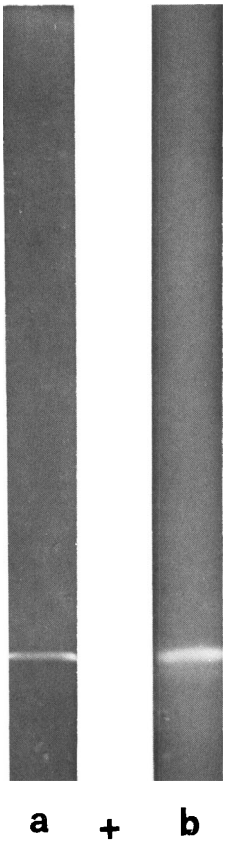

FIG. 3. Polyacrylamide gel electrophoresis of the DNA with a molecular weight less than 4×10^5 as determined by Bio-Gel P 300, after chromatography on hydroxyapatite. (a) Fraction of DNA eluted with 0.12 *M* phosphate buffer (Rf 98%). (b) Fraction of DNA eluted with 0.5 *M* phosphate buffer (Rf 100%). The gels were prepared as in Fig. 1. The DNA was dissolved in 1 *M* phosphate buffer. Ethidium bromide staining was used. Running condition: 10 mA/tube, 75 min.

Chromatography of native DNA on Bio-Gel P 300 allowed the separation of 2 fractions: a larger one which was excluded and a small fraction (10% of the total DNA) which was included, i.e. has a molecular weight of less than 4×10^5 daltons. Chromatography on hydroxyapatite of this DNA showed that it comprised two fractions, one (fraction *a*) which was eluted with 0.12 *M* phosphate buffer and a second fraction (b), which was eluted with 0.5 *M* phosphate buffer. The two fractions migrated with an Rf of 98% (fraction *a;* Fig. 3a) and with an Rf of 100% (fraction *b;* Fig. 3b) respectively. The $S^{\circ}_{20,w}$ obtained with alkaline sucrose gradients, was 0.96 S for the fraction *a* and 3.88 S for the fraction *b* (Fig. 4a). The large peak which was excluded from Bio-Gel P 300 was eluted with 0.5 *M* phosphate buffer from hydroxyapatite. It had a high molecular weight, 12.5 S (Fig. 4b) and migrated on gel electrophoresis mainly as "low migrating" DNA. However a small amount (fraction *c*) migrated with an Rf of 98% together with fraction *a* of the

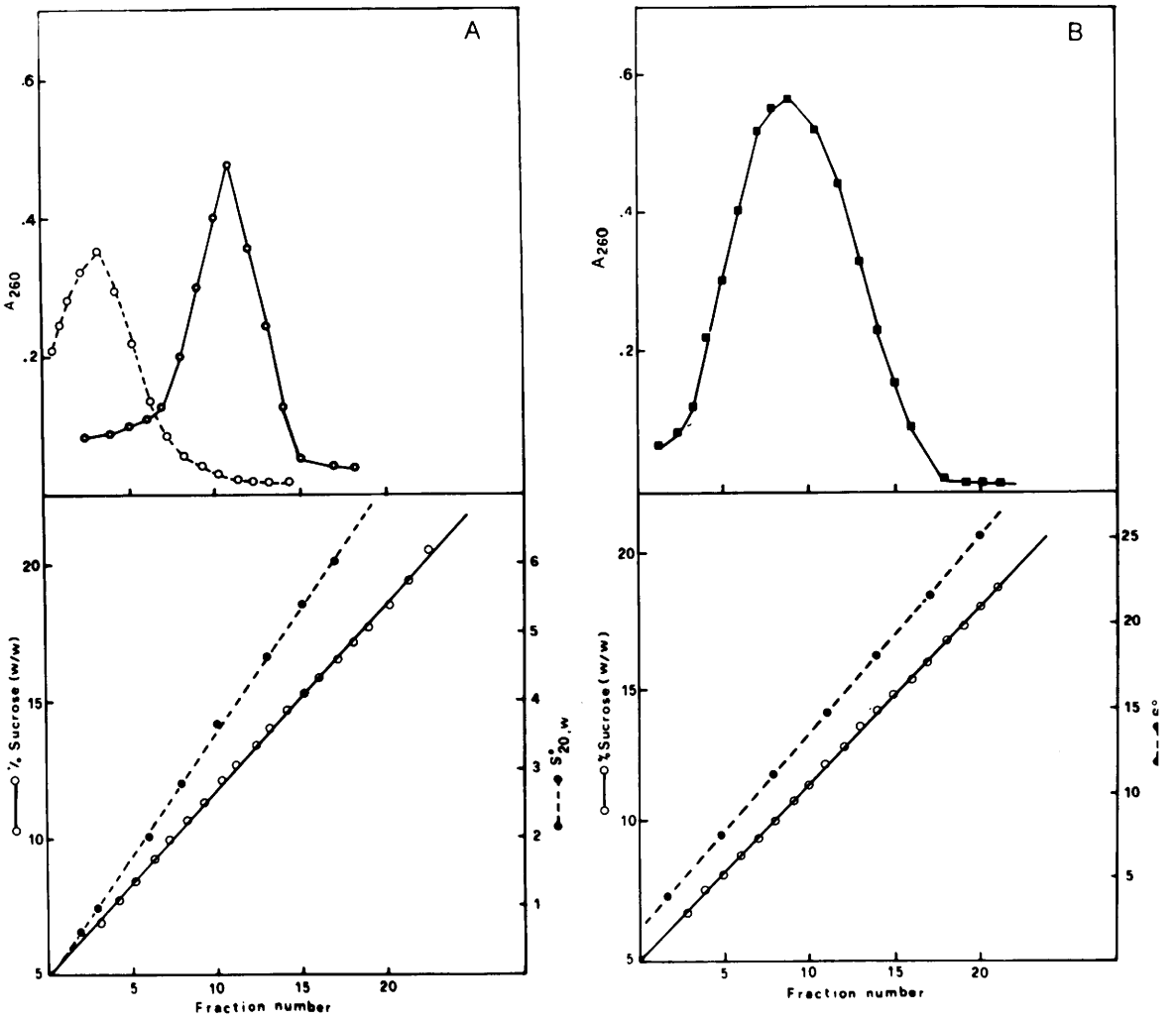

FIG. 4. Alkaline sucrose gradient of DNA. (a) DNA with a molecular weight less than 4×10^5 as determined by Bio-Gel P 300, chromatographed on hydroxyapatite. Top panel: distribution of DNA eluted with 0.5 *M* (O–O–O) and with 0.12 M (O--O--O) phosphate buffer. Bottom panel: sucrose gradients and S value. (b) DNA excluded from Bio-Gel P 300. Top panel: DNA distribution. Bottom panel: as in *a*. The refractive index and the absorbance of each fraction at 260 nm were measured. The sediment coefficients ($S^{\circ}_{20,w}$) of the DNA fragments were calculated from McEwen's table (19).

DNA included in Bio-Gel P 300. The DNA of the fraction *c* was contaminated by proteins. The Tm of the fraction *b* is 70.4° equal to that found for the DNA excluded from Bio-Gel P 300 (70.4°), the hyperchromicity after denaturation was 33%, whereas for the fraction *a* the hyperchromicity was only 6% as expected for single stranded DNA (Fig. 5).

Discussion. A small fraction of DNA was separated from the bulk of native DNA either with chromatography on Bio-Gel P 300 or with polyacrylamide gel electrophoresis. This fraction was characterized by low molecular weight and by high migration rate, suggesting that it was formed by small DNA fragments. The possibility that this fraction was the result of enzymatic or chemical damage was ruled out by the following experiments. Treatment of nuclei with chelants which specifically inhibited the action of the Ca–Mg endonuclease present in the hepatocytes, such as EGTA and EDTA (1), or with sodium citrate, which caused complete inhibition of DNase, had no effect on the results; neither did the DNA extraction technique influence the electrophoretic pattern (7). Therefore, it appears that this fraction was not an artefact, but rather a normal component of native eukaryotic DNA.

The analysis on hydroxyapatite showed that this DNA fraction was not homogeneous but that it could be fractionated further into two aliquots: one consisting of single stranded DNA, as shown by both elution with 0.12 *M* phosphate buffer and Tm; the other consisting of a double stranded DNA. These aliquots differed in their respective molecular weights and in their migration rates.

The double stranded DNA fraction (fraction *b*) migrated with an Rf of 100% and had an $S^{\circ}_{20,w}$ of 3.88 S corresponding to a molecular weight of 45,000–65,000, values similar to those obtained for the chromatin subunits isolated by Burgoyne *et al.* (2) after endogenous nuclease digestion. The DNA eluted from hydroxyapatite with 0.12 *M* phosphate buffer (fraction *a*) had an Rf of 98% (calculated from DNA dissolved in 1 *M* phosphate

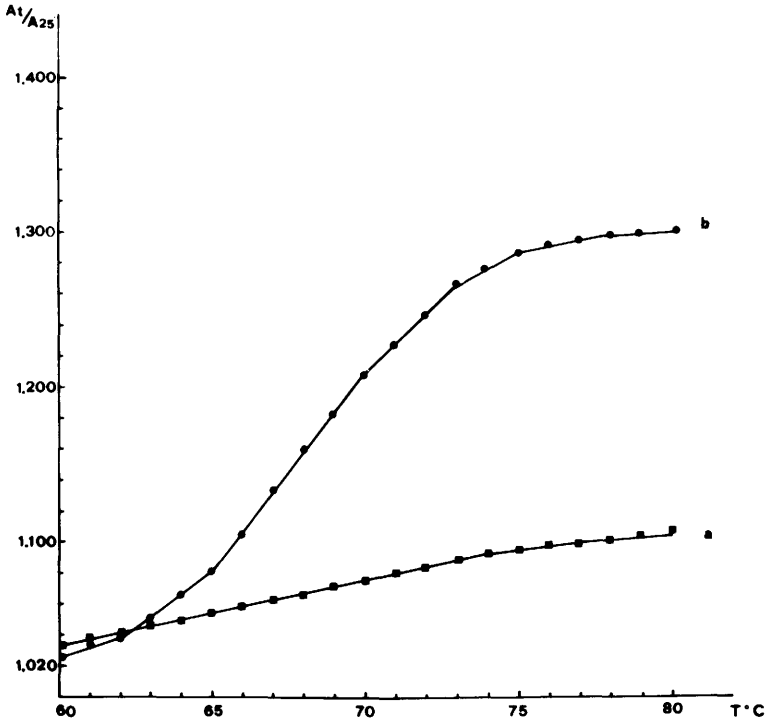

FIG. 5. Thermal denaturation curves of fractions *a* and *b*. The DNAs (20 μg/ml) were dissolved in 5 m*M* Na_2HPO_4 + 10 m*M* Na H_2PO_4 pH 6.8. Relative absorbance (corrected for thermal expansion) at each temperature, divided by the initial absorbance at 25° (At/A25) is plotted *versus* the temperature of the solution.

buffer). This Rf was lower than that of the double stranded DNA (fraction *b*) and was in agreement with the observations of Zeiger *et al.* (21). They showed that denatured DNA had a lower mobility than did native DNA. Since the $S^{\circ}_{20,w}$ of this single stranded fraction was 0.96 S, i.e. one-fourth that of 3.88 S DNA, it seems reasonable to suppose that these DNA fragments may represent the basic unit from which double stranded segments are formed.

To date no evidence has been presented on the nature of this low molecular weight DNA. Its physiological rôle is unknown and further structural studies should be undertaken.

Summary. A small fraction of DNA characterized by low molecular weight was isolated from the nuclear DNA of rat hepatocytes using electrophoretic and chromatographic techniques. Control experiments excluded the likelihood that this result could be due to enzymatic or chemical damage during nuclear separation and DNA extraction. Using hydroxyapatite chromatography this fraction was separated into two aliquots, one eluted by 0.12 *M* phosphate buffer and the other with 0.5 M phosphate buffer. The $S^{\circ}_{20,w}$, as determined by alkaline sucrose gradients, was 0.96 S for the first aliquot and 3.88 S for the second. The single stranded nature of DNA eluted with 0.12 *M* phosphate buffer was confirmed by the denaturation curve.

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