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Single-Strand-Specific Degradation of DNA during Isolation of Rat Liver Nuclei†

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ABSTRACT: We have investigated structural change in rat liver DNA produced by different isolation procedures and specifically compared the integrity of DNA derived by phenol extraction from isolated and purified nuclei with preparations extracted immediately from a crude liver homogenate containing intact nuclei. As indicated by stepwise elution from benzoylated DEAE-cellulose, most structural change in DNA was evident following nuclei isolation. Damage principally involved generation of single-stranded regions in otherwise double-stranded DNA fragments; totally single-stranded DNA was not detected by hydroxylapatite chromatography. Caffeine gradient elution suggested formation of single-stranded regions extending for up to several kilobases. In neutral sucrose gradients, differences in sedimentation rates of respective DNA samples consequent upon S1 nuclease digestion could be detected after isolation of nuclei, though not in other circumstances. The observed single-strand-specific nuclease digestion of DNA could apparently be reduced if steps were taken to reduce autodigestion during nuclei isolation by reduction of temperature and covalent cation concentration. The results are discussed in terms of the use of exogenous and endogenous nucleases in chromatin fractionation studies involving isolated nuclei and possible artifactual findings that may be generated by single-strand-specific autodigestion.

Digestion of chromatin with DNase I or micrococcal nuclease under carefully defined conditions is considered to permit fractionation of DNA on the basis of its association with transcribed or nontranscribed genes (Garel & Axel, 1976; Bloom & Anderson, 1978; Tata & Baker, 1978; Anderson et al., 1983). Typically, this procedure involves isolation of nuclei prior to digestion after which the respective fractions are separated by centrifugation. During isolation, chromatin may

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be subject to digestion by endogenous nucleases, a reaction which provided the basis for understanding of nucleosome structure (Hewish & Burgoyne, 1973; Burgoyne et al., 1974). Study of the mode of degradation of DNA by endogenous nuclease activity, including that which occurs as a consequence of cell death (Ayusawa et al., 1983), has been almost entirely restricted to analysis of strand breakage. For the most part, single-strand breaks have been assessed by sedimentation of DNA in alkaline sucrose while the effect of double-strand breaks has been indicated by electrophoresis.

Repair- and replication-induced structual change in DNA may be monitored by chromatography using benzoylated DEAE-cellulose (BD-cellulose)[1] or benzoylated naphthoylated DEAE-cellulose (BND-cellulose). These media were introduced as a means of fractionating nucleic acids according to secondary structure (Gillam et al., 1967). In respect of DNA, extensive investigations of the chromatographic characteristics of BD-cellulose (Sedat et al., 1969; Caffin & Mackinlay, 1975) and BND-cellulose (Schlegel & Thomas, 1972; Henson, 1978) have been undertaken. Both exhibit the same characteristics: single-stranded DNA or DNA containing single-stranded regions is bound under conditions (elution with 1.0 M NaCl) in which double-stranded DNA is eluted, and bound DNA may be eluted by addition of caffeine (or ethanol or formamide) to the NaCl solution (Strauss, 1981). Thus, in mammalian cell culture, repair-induced thymidine incorporation is distinguished from incorporation due to de novo synthesis, the latter being associated with single-stranded regions of replicating forks (Scudiero et al., 1975). Study of replicative intermediates indicated that the concentration of caffeine required to elute DNA from BD- or BND-cellulose depends on the proportion of single strandedness. For the purpose of sizing short single-stranded regions chromatographically, analytical relationships between polynucleotide size and eluting caffeine concentration for BND-cellulose have been established for strand lengths up to 2000 nucleotides (Iyer & Rupp, 1971; Schlegel et al., 1972). Analogous experiments using BD-cellulose permitted recovery of much longer single-stranded DNA in the range of usable caffeine concentrations. A consistent relationship between eluting caffeine concentration and polynucleotide size for strand lengths up to 50 000 bases has been demonstrated (Haber & Stewart, 1981).

Research in this laboratory has involved structural analysis of DNA by chromatography on BD-cellulose, specifically in reference to carcinogen-induced repair processes (Huang & Stewart, 1977; Stewart, 1981; Stewart & Haski, 1984). Preparatory to assessing such structural change in relation to chromatin structure, we examined the structural integrity of DNA preparations obtained following isolation of rat liver nuclei. A range of analyses is consistent with strand-specific degradation of such DNA on a large scale. The extent of such digestion seems likely to interfere with subsequent chromatin studies.

Materials and Methods

Materials. BD-cellulose was purchased from Boehringer-Mannheim (Mannheim, West Germany), [*methyl*-^{3}H]thymidine (20 Ci/mmol) from the Radiochemical Centre (Amersham, U.K.), hydroxylapatite (DNA grade) from Bio-Rad Laboratories (Richmond, CA), and S1 nuclease (*Aspergillus oryzae*) from P-L Biochemicals (Milwaukee, WI). Micrococcal nuclease was from Boehringer Mannheim, and DNA markers (*Hin*dIII digest of λ phage) were from New England Nuclear (Boston, MA).

Animals. Female Wistar rats were obtained and caged as previously described (Stewart & Haski, 1984). For the purpose of subsequent analysis, hepatic DNA was radiolabeled in vivo by subjecting 120-g animals to partial hepatectomy (Higgins & Anderson, 1931) and administering [^{3}H]thymidine (50 μCi) 21 and 29 h after surgery. Animals were then maintained under normal conditions for a minimum of 2 weeks before being used for experimental purposes. When necessary, rats were killed by a sharp blow to the head followed by jugular vein exsanguination.

Isolation of DNA. Hepatic DNA was isolated by phenol extraction, based on the method described by Kirby & Cook (1967), either from a crude liver homogenate or after prior purification of liver nuclei.

(*A*) *From Liver Homogenate.* Liver (approximately 7 g) was homogenized at 4 °C in 0.15 M NaCl–0.015 M trisodium citrate, pH 7.0 (SSC, 35 mL), with 5 strokes of a Teflon–glass Potter–Elvehjem homogenizer and the suspension centrifuged at 575*g* for 10 min. The crude nuclear pellet was resuspended in the same volume of SSC, centrifuged again under the same conditions, and resuspended in SSC (8 mL). To the preparation were added 2 M NaCl (20 mL) and 10% w/v sodium dodecyl sulfate (4 mL) followed by 30 mL of a mixture containing phenol (500 g), *m*-cresol (70 mL), water (55 mL), and 8-hydroxyquinoline (0.5 g). The mixture was stirred for 30 min at room temperature and centrifuged at 9225*g* for 30 min, and the aqueous layer was reextracted with phenol as before. The aqueous phase was dialyzed overnight at 4 °C against 10 mM Tris-HCl–1 mM EDTA, pH 8.0 (TE buffer).

(*B*) *From Isolated Nuclei.* Nuclei were purified by sedimentation based upon the methods of Blobel & Potter (1966) and Tata & Baker (1978). After perfusion in situ with ice-cold isotonic saline (20 mL), the liver was removed and homogenized with 10 strokes in 5 volumes of buffer containing 50 mM Tris-HCl (pH 7.5), 12.5 mM NaCl, 12.5 mM KCl, 5 mM $MgCl_2$, 2 mM phenylmethanesulfonyl fluoride, and 0.25 M sucrose. After filtration through gauze and centrifugation at 575*g* for 10 min, the crude nuclei pellet was resuspended in 0.25 M sucrose buffer (20 mL). The suspension was added to 2 volumes of the same buffer containing 2.3 M sucrose, mixed, and layered onto a 1.8 M sucrose buffer "cushion" (5 mL) in a SW-27 cellulose nitrate centrifuge tube. Nuclei pelleted through this sucrose scrub at 130000*g* for 40 min were resuspended by brief homogenization in 0.25 M sucrose buffer (30 mL) containing 0.1% Triton X-100. After centrifugation for 10 min at 575*g*, the nuclei were washed in a further 30 mL of 0.25 M sucrose buffer without Triton X-100 before being repelleted under the same conditions. All procedures were performed at 4 °C. The nuclei were then suspended in SSC (8 mL), and DNA was isolated by phenol extraction as described above.

In some experiments, nuclei were isolated at −20 °C according to the method described by Schibler & Weber (1974). Following in situ perfusion as described, the liver was removed and homogenized with 10 strokes in 5 volumes of a solution containing 50% v/v glycerol and 2 mM phenylmethanesulfonyl fluoride either in 10 mM Tris-HCl (pH 7.4 at 0 °C), 10 mM NaCl, and 1.5 mM $MgCl_2$ or in SSC. For this purpose, a jacketed Teflon–glass Potter–Elvehjem homogenizer which had been cooled to approximately −20 °C by using an ice–salt–water slurry was employed. After filtration through gauze

[1] Abbreviations: BD-cellulose, benzoylated DEAE-cellulose; DEAE, diethylaminoethyl; BND-cellulose, benzoylated naphthoylated DEAE-cellulose; EDTA, ethylenediaminetetraacetic acid; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; PPO, 2,5-diphenyloxazole; bp, base pair(s).

and centrifugation at 4000*g* (−20 °C) for 10 min, the pelleted nuclei were resuspended by gentle homogenization in the same buffer containing 0.1% v/v Triton X-100. Following centrifugation as described, the nuclei were washed in detergent-free buffer and finally repelleted. This pellet was resuspended in SSC (8 mL) and DNA isolated by phenol extraction.

Structural Analysis by Chromatography. (A) BD-cellulose. BD-cellulose, stored and treated to maintain reproducible binding characteristics (Haber et al., 1984), was loaded into either a 1-cm-diameter chromatography column or a 5-mL syringe barrel containing glass wool and a layer of fine glass beads (1 mL). Prior to use, columns were equilibrated with 0.3 M NaCl in TE buffer. Samples of DNA (0.38 mg containing at least 10 000 dpm) in 2 mL of equilibration buffer were sheared by six passages through a 25-gauge needle at 4 °C to a modal length of 7000 base pairs (Stewart et al., 1979), diluted to 4 mL, and loaded on columns at a flow rate of 0.5 mL/min. The columns were washed with a further 4 mL of the same buffer, and the flow rate was adjusted to 1.0 mL/min. Wholly double-stranded DNA was eluted with 1.0 M NaCl in TE buffer (10 mL) and the remaining DNA recovered by elution with prewarmed caffeine solution in either a stepwise or a gradient procedure. For stepwise elution, 2% w/v caffeine in 1.0 M NaCl–TE buffer solution (10 mL) was applied to the column. Gradient elutions involved a progressive linear increase in the caffeine concentration of a 1.0 M NaCl–TE buffer solution (Dialagrad pump, ISCO Model 382), initially increasing from 0% to 0.1% (74 mL) and then in a similar fashion to 0.8% (166 mL), the column being finally washed with 2% caffeine (10 mL). Radioactivity in collected fractions (2 mL) was determined by scintillation counting (Instagel, Packard Instruments) at 35% efficiency for 10 min or to 1% σ and expressed as a percentage of the total recovered from the column.

The linear caffeine gradient profile generated by the gradient pump was confirmed by using a solution of amido-Schwarz dye made to approximately 2 A_{615} units. This solution was used in place of the 0.8% w/v caffeine in 1 M NaCl–TE buffer, and an otherwise normal gradient was generated. Absorbances of the resulting fractions were determined at 615 nm.

(B) Hydroxylapatite. A slurry of hydroxylapatite was prepared in 0.05 M sodium phosphate buffer (pH 7.0) by heating for 10 min at 70 °C with gentle stirring. The suspension was washed and decanted several times to remove fines. Hydroxylapatite (2.5–3.0-mL packed volume) was loaded into a 5-mL syringe barrel, modified as described for BD-cellulose chromatography, and washed with 0.05 M sodium phosphate buffer (30 mL). Following overnight dialysis against 0.05 M sodium phosphate buffer, samples containing up to 0.5 mg of DNA were sheared as previously described. Where indicated, DNA was denatured by heating to 100 °C for 15 min and rapidly cooling in ice water. DNA samples were adsorbed to the column prior to the column being washed with 0.05 M sodium phosphate buffer (12 mL). Stepwise elution followed, using 10 mL each of 0.15, 0.20, 0.25, and 0.40 M sodium phosphate buffers. A flow rate of 0.5 mL/min was maintained throughout the chromatography, and radioactivity in collected fractions (2 mL) was determined as described previously.

Structural Analysis by Digestion and Sedimentation. (A) Nuclease S1. Nuclease S1 [1 unit/μg of DNA; 1 unit of activity catalyzes the formation of 1 μg of acid-soluble deoxynucleotides following 30-min incubation with denatured DNA at 37 °C according to Ando (1966)] was added to DNA samples in a solution containing (final concentration) 40 mM sodium acetate (pH 5.0), 2 mM zinc sulfate, and 75 mM NaCl. The reaction was terminated by adding ice-cold trichloroacetic acid to a final concentration of 5% w/v following the addition of calf thymus carrier DNA (50 μg/mL). Radioactivity of acid-precipitable material was determined after collection on Whatman glass fiber filters and addition of 0.4% PPO in toluene. The proportion of DNA solubilized was calculated from the loss of acid-precipitable radioactivity in the course of digestion.

(B) Neutral Sucrose Gradients. DNA-containing fractions, collected during stepwise elution of BD-cellulose with caffeine solution, were pooled and dialyzed overnight against distilled water at 4 °C. The solution was then rotary evaporated to dryness (30–35 °C) and the product dissolved in 0.3 M NaCl–TE buffer. Such DNA samples were divided and either analyzed immediately by sedimentation in neutral sucrose gradients or subjected to digestion by nuclease S1 prior to sedimentation. Nuclease S1 digestion was performed as described above, the reaction being terminated by addition of 4.0 mM EDTA rather than trichloroacetic acid.

Logarithmic sucrose gradients (5–20% w/v in 50 mM Tris–HCl–0.15 M NaCl, pH 7.5; total volume 5 mL) were prepared by using a programmable pump (Dialagrad) and layered on a cushion of 2.3 M sucrose buffer (1 mL) in a 16 × 102 mm cellulose nitrate centrifuge tube. Routinely, gradients were poured at room temperature and cooled to 4 °C prior to use. DNA samples of not more than 0.5 mL containing approximately 50 μg of DNA were layered onto the top of gradients followed by precooled light paraffin oil. In some experiments, DNA markers were used in place of DNA samples. Gradients were centrifuged at 130000*g* (27 000 rpm; SW-27.1 rotor) at 4 °C for 6 h. Fractionation of gradients was achieved by displacement with light paraffin oil at a flow rate of 0.75 mL/min.

Tritiated deoxyribonucleic acid markers were separated on a 1% agarose low-temperature gel (Bio-Rad, Richmond CA). Marker sizes of 6700 and 4300 bp were cut out and extracted by using a NACS minicolumn system (BRL, Gaithersburg, MD) according to the NACS Pre-Pac instruction manual. Prior to neutral sucrose gradient centrifugation, markers were desalted by using Centricon-30 (Amicon Inc.) microconcentrators.

Structural Analysis by Digestion, Gel Electrophoresis, and Rechromatography on BD-cellulose. Stepwise caffeine-eluted DNA samples, concentrated and desalted by using Centricon-30 microconcentrators, were subject to S1 digestion as described, EDTA being used to terminate the reaction. Digestion products were analyzed by 1% agarose gel electrophoresis. Gels were run at 75 V for 2 h and stained with ethidium bromide. An appropriate range of DNA markers was included. In the case of caffeine-eluted DNA derived from nuclei isolated at 4 °C, the effect of S1 nuclease digestion on binding sites was determined by rechromatography on BD-cellulose.

Results

Following BD-cellulose chromatography, greater than 90% of sheared rat liver DNA isolated by phenol extraction is recovered in 1.0 M NaCl solution, indicative of wholly double-stranded DNA. By comparison, chromatography of DNA extracted from isolated nuclei resulted in a larger proportion of DNA being retained during 1.0 M NaCl elution and recovered in 2% caffeine solution, thereby exhibiting single-stranded character. Thus, when nuclei were isolated by using a conventional procedure which involved buffers containing

Table I: Effect of Isolation Procedures and Conditions on Stepwise Elution of DNA from BD-cellulose[a]

source of DNA and conditions of isolation	proportion of caffeine-eluted DNA
crude homogenate (4 °C, no Mg^{2+})	0.07
crude homogenate (4 °C, 5 mM Mg^{2+})	0.25
isolated nuclei (−20 °C, no Mg^{2+})	0.14
isolated nuclei (−20 °C, 1.5 mM Mg^{2+})	0.47
isolated nuclei (4 °C, 5 mM Mg^{2+})	0.60

[a] The manner in which the isolation temperature and divalent cation concentration were varied is described under Materials and Methods.

Table II: S1 Nuclease Digestion of DNA[a]

treatment prior to digestion	extent of digestion (%) prepn 1	prepn 2
unfractionated	ND[b]	ND
unfractionated, denatured	99	98
caffeine eluted	10.5	17
caffeine eluted, denatured	99	97

[a] Preparation 1 was DNA prepared from crude liver homogenate. Preparation 2 consisted of DNA prepared from isolated nuclei. Once isolated, DNA preparations were variously treated by stepwise elution from BD-cellulose and/or by heat denaturation (see Materials and Methods) before nuclease digestion. Proportion of DNA solubilized is expressed as a percentage. [b] ND, not detectable.

5 mM Mg^{2+} prior to phenol extraction, the caffeine-eluted fraction constituted the major fraction after BD-cellulose chromatography (Table I). This initial observation was suggestive of gross structural damage to DNA as a consequence of initial separation of a nuclear fraction from the whole homogenate.

To further characterize structural defects in DNA eluted from BD-cellulose with 2% caffeine, relevant fractions were pooled, dialyzed, and together with DNA samples obtained immediately after isolation, subjected to S1 nuclease digestion. With respect to the latter, that is, in the absence of prior fractionation on BD-cellulose, digestion of DNA by S1 nuclease could not be detected. When caffeine-eluted preparations were used as substrate for S1 nuclease, a small proportion of the DNA was digested. However, the extent of this digestion was markedly greater for caffeine-eluted DNA prepared following nuclei purification (Table II). Upon denaturation of the same samples, there was almost complete digestion with S1 nuclease.

A qualitative difference between caffeine-eluted DNA fractions obtained following either immediate phenol extraction of whole liver homogenate or isolation of purified nuclei prior to extraction could be discerned by sedimentation in neutral sucrose gradients after S1 digestion. After treatment with S1 nuclease, sedimentation of respective samples in neutral sucrose gradients revealed little change attributable to the single-strand-specific nuclease in the "whole homogenate" sample (Figure 1). In contrast, similar comparison of samples derived from caffeine-eluted fractions of DNA from isolated nuclei revealed a distinct decrease in the extent of sedimentation after reaction with S1 nuclease (Figure 2). This change in behavior attributable to S1 digestion could not be demonstrated by using agarose gels. Examination of the same samples by gel electrophoresis before and after S1 digestion did not reveal any difference in the rate of migration in agarose.

Regardless of the isolation procedure employed, only a minor fraction of caffeine-eluted DNA was S1 nuclease sensitive. Results of hydroxylapatite chromatography indicate that preparations of caffeine-eluted DNA, regardless of the isolation

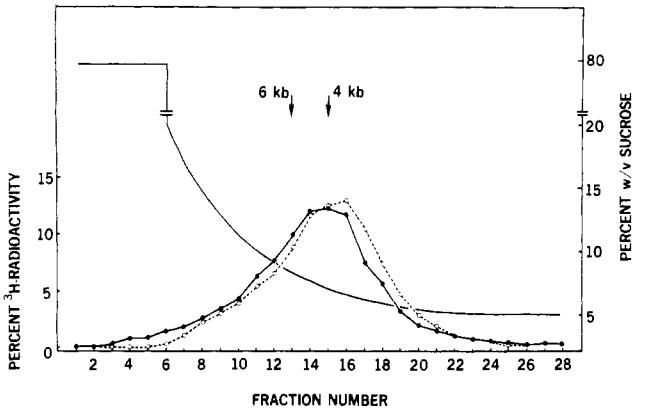

FIGURE 1: Sedimentation of caffeine-eluted DNA obtained by stepwise BD-cellulose chromatography of phenol-isolated whole liver homogenate DNA in neutral sucrose gradients before (●) and after (○) S1 nuclease digestion. 5–20% sucrose gradients were supported on a 2.3 M sucrose cushion. Positions of the DNA markers are shown (see Materials and Methods). Sedimentation is from right to left.

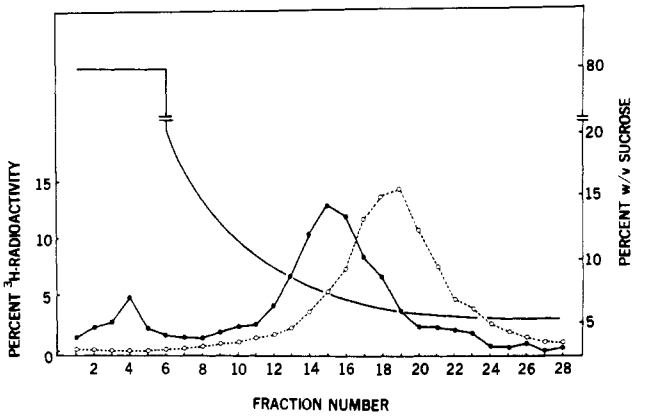

FIGURE 2: Sedimentation of caffeine-eluted DNA obtained by stepwise BD-cellulose chromatography of phenol-isolated DNA from isolated nuclei, 4 °C, in neutral sucrose gradients before (●) and after (○) S1 nuclease digestion. See Figure 1 and Materials and Methods.

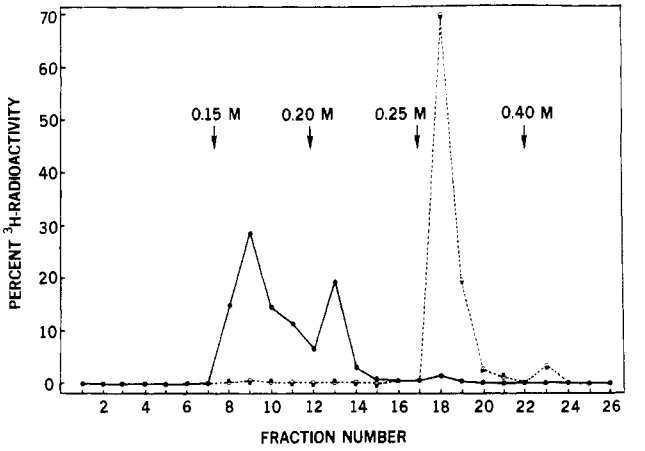

FIGURE 3: Hydroxylapatite chromatograms of various preparations of rat liver DNA obtained by a stepwise increase in eluting phosphate concentration as shown. DNA extracted from isolated nuclei and recovered after caffeine elution of BD-cellulose (○) cochromatographed with similar preparations of DNA not subjected to prior BD-cellulose chromatography (■). No difference in the chromatograms was observed by using DNA prepared by immediate phenol extraction of a crude homogenate (data not shown). Heat denaturation of rat liver DNA (●) resulted in its anticipated recovery at low phosphate concentration.

procedure, cochromatographed with unfractionated DNA and were eluted at phosphate concentrations indicative of double-stranded DNA; no evidence of single-stranded character

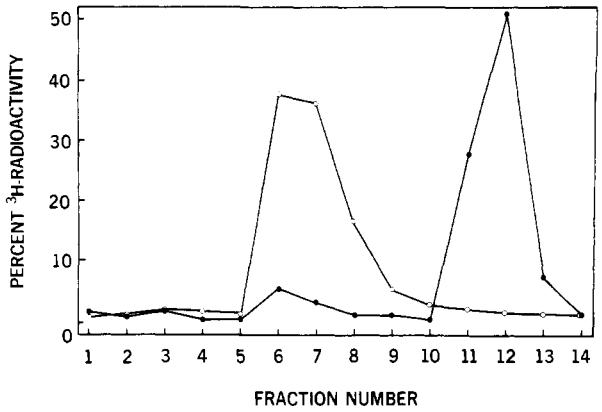

FIGURE 4: Effect of S1 nuclease digestion on the binding of DNA to BD-cellulose. DNA prepared by phenol extraction of isolated nuclei at 4 °C was subjected to stepwise elution from BD-cellulose. The caffeine-eluted fraction was subject to rechromatography on BD-cellulose either before (●) or after (○) digestion with S1 nuclease as described under Materials and Methods. After the DNA was loaded in 0.3 M NaCl solution (fractions 1–4), chromatograms were generated by stepwise elution of BD-cellulose with 1.0 M NaCl (fractions 5–9) to which was added 2% caffeine (fractions 10–14).

could be detected. For control purposes, it was established that heat denaturation of any of these samples resulted, as anticipated, in recovery of DNA early in the course of stepwise increase in phosphate concentration (Figure 3). The implication that structural lesions responsible for binding of DNA to BD-cellulose may be imperfections in otherwise double-stranded DNA was further strengthened by the results of rechromatography of caffeine-eluted DNA after S1 nuclease digestion. In the absence of S1 treatment, only minimal contamination of the caffeine-eluted fraction, of DNA preparations derived from isolated nuclei (4 °C), by double-stranded DNA was evident. However, following S1 nuclease digestion, greater than 90% was eluted as double-stranded DNA from BD-cellulose (Figure 4).

Data presented thus far are consistent with endogenous nuclease activity causing structural changes in DNA during nuclei isolation. Increased nuclease activity may readily be attributed to the extended interval between death of the animal and phenol extraction which occurs when nuclei are purified, by comparison with immediate phenol extraction. Moreover, nuclei were purified in sucrose buffer containing Mg^{2+} cations in order to maintain chromatin structure for subsequent enzymatic fractionations (Tata & Baker, 1978; Vanderbilt et al., 1982). To confirm the role of endogenous nuclease activity and its dependence upon Mg^{2+} concentration, nuclei were prepared in buffers containing 50% glycerol. In addition, the concentration of $MgCl_2$ was reduced from 5 to 1.5 mM, or it was omitted entirely (Schibler & Weber, 1974). The nuclei isolation could thus be conducted at −20 °C as described under Materials and Methods. Under such conditions of reduced Mg^{2+} concentration, the proportion of caffeine-eluted DNA from isolated nuclei was reduced by 20% (Table I). A 75% reduction in this proportion could be achieved when Mg^{2+}-free SSC was used. Further, when Mg^{2+}-free SSC used in the whole liver preparation was replaced with the same sucrose buffer (containing 5 mM Mg^{2+}) used in isolation of nuclei, there was a 3-fold increase in the proportion of caffeine-eluted DNA in whole liver preparations (Table I).

The extent of single-stranded regions in DNA, presumably a consequence of endogenous nuclease activity during nuclei purification, was assessed by caffeine gradient elution from BD-cellulose. BD-cellulose chromatograms of DNA from whole liver and from nuclei isolated at 0 and −20 °C were

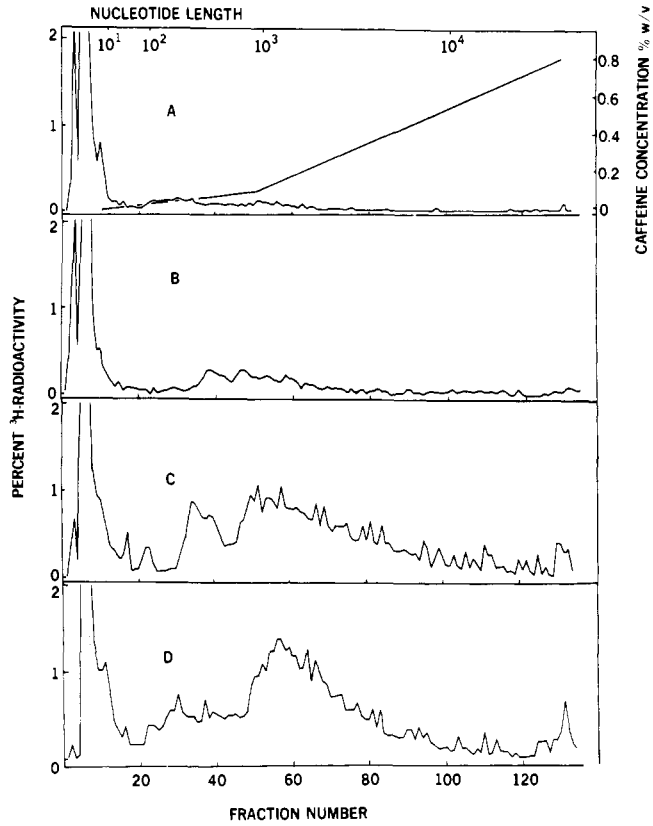

FIGURE 5: Differences in structural characteristics of the various DNA preparations as indicated by caffeine-gradient elution from BD-cellulose. DNA was prepared by phenol extraction of a crude homogenate (A) or of rat liver nuclei isolated at −20 °C in the absence of Mg^{2+} (B) or in the presence of 1.5 mM Mg^{2+} (C) or isolated at 4 °C in the presence of 5 mM Mg^{2+} (D). The biphasic caffeine gradient (same for all chromatograms) is indicated in the top panel, and details are given under Materials and Methods. The affinity of single-stranded DNA for BD-cellulose under the present conditions (Haber & Stewart, 1981) is indicated at the top of the figure. Radioactivity per chromatogram was (2–5) × 10000 dpm.

obtained by using an extended 0–0.8% caffeine gradient prior to a stepwise increase to 2% caffeine. The marked difference between the total proportion of caffeine-eluted DNA is extended by gradient analysis. Thus, not only is the total amount of caffeine-eluted DNA increased by nuclei purification but also its distribution within the caffeine gradient is completely different from what may be regarded as the control preparation. The latter was characterized by a low-level recovery of DNA early in the caffeine gradient (fractions 20–60) with peak recovery in the region of fraction 30 (Figure 5A). After isolation of nuclei, an increase in the amount of caffeine-eluted DNA by almost an order of magnitude is associated with a qualitatively distinct chromatogram. Radioactivity was recovered throughout the gradient and most significantly between fractions 50 and 100, with a peak at fraction 55 (Figure 5D). The chromatograms of DNA from purified nuclei isolated at −20 °C in the absence and presence of Mg^{2+} are clearly intermediate in the transition from the "immediate extraction" result and "purified nuclei" at 0 °C. The recovery of DNA at progressively higher caffeine concentrations is indicative of longer single-stranded regions. After immediate extraction, the small amount of DNA bound to BD-cellulose during NaCl elution was recovered at low caffeine concentration, maximum recovery (fraction 30) corresponding to 200 nucleotides (Haber & Stewart, 1981). In contrast, most DNA prepared after isolation of nuclei was bound to BD-cellulose in the presence of NaCl. Of this, approximately 25% was

contained in fractions 25–50 of the caffeine gradient where single-stranded DNA of 100–1000 nucleotides would be recovered. The major peak, covering fractions 50–70, is correlated with recovery of single-stranded DNA 1–4 kilobases in length (Haber & Stewart, 1981).

Discussion

Retention of DNA by BD-cellulose, despite elution with 1 M NaCl, implies structural lesions in the DNA which may range from short (20 nucleotides) single-stranded regions to totally single-stranded DNA (Stewart et al., 1979). In the course of the present investigation, caffeine-eluted DNA, regardless of the method of isolation, cochromatographed on hydoxylapatite with double-stranded DNA (Figure 3). Having been eluted with caffeine from BD-cellulose under conditions required to recover single-stranded DNA, such a preparation may thus be characterized as double-stranded DNA with single-stranded regions. There was no evidence of a totally single-stranded component even within the most extensively degraded DNA preparation examined, in contrast, for example, with generation of single-stranded DNA following reaction of nucleosomes with acetic anhydride (Jordano et al., 1984).

Preparations of DNA retained by BD-cellulose were only partially digested by S1 nuclease under conditions in which wholly single-stranded DNA was totally degraded (Table II). However, the relatively small fraction of DNA solubilized by S1 nuclease was conspicuously larger when the "purified nuclei" subfraction of DNA was used rather than that obtained following BD-cellulose chromatography of DNA immediately extracted from a crude homogenate (Table II). Regardless of the method of preparation (and prior to S1 nuclease digestion), the respective caffeine-eluted fractions sedimented similarly in neutral sucrose gradients (Figures 1 and 2). Both caffeine-eluted fractions exhibited a molecular weight slightly less than the modal length of approximately 7000 base pairs generated by the shearing procedure to which the whole preparations were subjected before BD-cellulose chromatography (Stewart et al., 1979). S1 digestion failed to alter the sedimentation characteristics of caffeine-eluted DNA obtained by chromatography of DNA extracted from whole liver homogenate (Figure 1). S1 nuclease digestion of the corresponding "isolated nuclei" DNA subfraction clearly affected sedimentation in neutral sucrose (Figure 2). The result is suggestive of double-stranded DNA derived from isolated nuclei having internal single-stranded sites, in contrast to the apparent absence of such sites in DNA immediately isolated from a crude homogenate. This latter preparation may have contained a smaller number of single-stranded sites which were subject to hydrodynamic shear and subsequently located terminally. Rechromatography of caffeine-eluted DNA derived from isolated nuclei indicated that BD-cellulose binding sites were stable. However, binding to BD-cellulose did not occur when the caffeine-eluted DNA was subjected to S1 digestion prior to rechromatography (Figure 4), an identical result being observed when replicating DNA was digested and rechromatographed (E. J. Ward and M. D. Norris, unpublished results). These data indicated that single-stranded regions responsible for binding to BD-cellulose are the same sites which are attacked by S1 nuclease.

Gradient elution of DNA from BD-cellulose permits quantitation of the proportion of DNA having single-stranded regions and the extent of this degradation. In terms of both these parameters, there is clear evidence of a transition from the DNA sample immediately extracted from a crude homogenate through the preparation obtained from "−20 °C" nuclei to that extracted from nuclei isolated at 0 °C. Consistent with the stepwise elution data (Table I), the total proportion of DNA retained by BD-cellulose in the presence of 1 M NaCl was increased when nuclei were purified. Moreover, this transition was associated with recovery of bound DNA at higher caffeine concentrations, indicating that longer single-stranded regions were responsible for binding to BD-cellulose. For totally single-stranded DNA chromatographed on BD-cellulose under the present experimental conditions, eluting caffeine concentration may be used to characterize strand length (Haber & Stewart, 1981). More recent investigations in this laboratory with molecules of known single- and double-stranded length suggest that the strength of binding to BD-cellulose is indicative only of the number of unpaired nucleotides (M. D. Norris, unpublished results). That is, for DNA molecules sheared as in the present study, eluting caffeine concentration during BD-cellulose chromatography is determined by the length of single-stranded DNA irrespective of the length of double-stranded DNA within each molecule. Accordingly, it may be surmised that in the most degraded preparation (Figure 5D) single-stranded regions totalling between 900 and 3600 nucleotides per molecule (fractions 50–70) are characteristic, the peak fraction (57) corresponding to approximately 1600 nucleotides.

The extent of binding of the various DNA preparations (Table I) to BD-cellulose depended upon parameters known to affect nuclease activity: duration of potential exposure (i.e., interval after death until phenol extraction), temperature, and divalent cation concentration of solutions used in the preparation. Reduction in BD-cellulose binding, achieved when isolation procedures were conducted at −20 °C and reduced Mg^{2+} concentration, is completely consistent with nuclease activity being involved. On the other hand, addition of $MgCl_2$ to the medium in which liver was homogenized prior to immediate extraction markedly increased the proportion of caffeine-eluted DNA. Mammalian nucleases are generally activated by cations [for a review, see Sierakowska & Shugar (1977) and Burgoyne & Hewish (1978)]. Most data, however, concern endonucleases (Vinter et al., 1977; Fischman et al., 1979; Machray & Bonner, 1981). Reference to single-stranded exonucleolytic activity is restricted to its anticipated role in DNA repair (Cleaver, 1983). Nuclease activity of the type inferred by our data has been noted by Ishida et al. (1974), who examined degradation of *Escherichia coli* DNA by extracts of rat liver nuclei. They observed that formation of acid-soluble products involved an exonucleolytic enzyme which was not precipitated by 40% ammonium sulfate.

Endogenous nucleases are capable of solubilizing euchromatic DNA from nuclei in the absence of exogenous nuclease (Paul & Duerkson, 1976; Sargan & Butterworth, 1982; Vanderbilt et al., 1982). Prentice & Gurley (1983) have reported that changing isolation parameters have a direct effect upon subsequent nuclei digestion kinetics with DNase I, presumably via chromatin substructural alterations. Thus, the role of Mg^{2+} in determining nucleolytic degradation of DNA must be considered not only in relation to enzyme activation but also with reference to chromatin structure. Vanderbilt et al. (1982) suggested that high cation concentrations inhibited autodigestion of chromatin as a consequence of the inability of endogenous nucleases to attack charge-neutralized chromatin. When the concentration of cations was reduced from physiological (10–80 mM Mg^{2+}) to that used in the present isolation solution (5 mM), autodigestion was facilitated.

Exposure of isolated mammalian nuclei to nucleases is widely used as a means for investigating chromatin structure

(Cartwright et al., 1982; Igo-Kemenes et al., 1982; Mathis et al., 1980). However, Reeves (1984) has noted that when the literature on any particular aspect of chromatin digestion sensitivity is considered in detail, conflicting results or interpretations often emerge. Such differences are attributable to the diversity of salt cation concentrations used in different laboratories, thereby affecting nucleolytic attack on chromatin. Within chromatin, "hypersensitive sites" (Wu et al., 1979) are characterized by localized denaturability and are specifically sensitive to S1 nuclease digestion. Weintraub (1983) has concluded that his and other data indicate that DNase I hypersensitive sites are actually regions of DNA 200–400 nucleotides in length and having certain sequence characteristics. In view of the significance given to the results of nuclease digestion of chromatin preparations and inferences made from structural analysis of DNA, the present findings are cause for concern. Generation of structural defects in DNA during routine isolation procedures could give rise to artifactual findings for digestion studies. Since DNase I hypersensitive sites are nonradom (Elgin, 1981), the single-stranded sites presently under investigation may have specific locations and/or be influenced by the configuration of chromatin. It is possible, for example, that DNase I sensitive sites are the product of endogenous digestion in the course of nuclei isolation.

Single-strand-specific autodigestion of DNA or chromatin does not appear to have previously received close attention. Such degradation is not conspicuous in terms of a range of analytical procedures often used to verify the integrity of DNA. In consequence, it would appear that use of BD- or BND-cellulose chromatography could be particularly useful.

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