Characterization of Rapidly Labeled Detergent-Soluble DNA in Murine Splenocytes†

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ABSTRACT: Freshly prepared spleen cells from concanavalin A stimulated mice incorporate [^{3}H]thymidine into DNA which can be recovered in detergent-soluble (NP40) and detergent-insoluble forms. The presence of detergent-soluble forms occurs despite the fact that the cells are lysed at 4 °C in the presence or absence of 25 mM ethylenediaminetetraacetic acid. After a 2-h pulse with [^{3}H]thymidine, the detergent-soluble fraction contains about 1–3% of the total cellular DNA but 25% of the total labeled high molecular weight material. Since the specific activity of the extensively purified DNA from the detergent-soluble fraction is considerably higher than that of chromosomal DNA, it meets the criteria for being metabolically active. We propose the name "MADS" DNA for metabolically active detergent-soluble DNA. MADS DNA has a density of 1.699 g/mL on cesium chloride gradients and a slightly higher G + C content than chromosomal DNA as determined by high-pressure liquid chromatography. Electrophoresis using native or denaturing agarose gels resolves MADS DNA into discreet sizes between 200 and 4500 base pairs. Nuclease S-1 treatment of native MADS DNA does not alter the size distribution as resolved by means of gel electrophoresis under denaturing conditions. Therefore, MADS DNA is not a collection of single-stranded Okazaki fragments. Southern blot analysis reveals that mitochondrial DNA is a minor component of higher molecular weights above the bulk of the DNA visualized either by staining with ethidium bromide or by incorporation of [^{3}H]thymidine. Inhibitors of ribonucleotide reductase or DNA polymerase α inhibit incorporation of [^{3}H]thymidine into MADS DNA, and hence chromosomal DNA synthesis is required for MADS DNA production. Since Southern blot analysis also reveals homology of larger fragments with the ^{32}P-labeled 200 base pair fragment, the presence of repetitive sequences is suggested.

[^{3}H]Thymidine ([^{3}H]TdR) incorporation is often used as a measure of cellular proliferation. Its utility as an indicator of cell division requires knowledge of how it enters the cell and how it forms the products into which it is incorporated. We have shown that splenocytes from normal unstimulated mice do not take up [^{3}H]TdR by carrier-mediated mechanisms (Strauss et al., 1976). On the other hand, splenocytes stimulated to divide in the animal by intravenous injection of concanavalin A (Con A) (Moatamed et al., 1975) do transport [^{3}H]TdR (Strauss et al., 1977). In vivo stimulation made it possible to avoid the problems commonly faced when murine splenocytes are stimulated in culture. Unless stimulation is performed in vivo, at least 50% of the cells were dead after the first 24 h (Ling & Kay, 1975; Goodman & Weigle, 1977; P. R. Strauss, unpublished results). In the present study, we have exploited earlier findings on TdR transport (Strauss et al., 1976, 1977, 1980; Strauss, 1979) to study the properties of DNA synthesis by Con A stimulated mouse lymphocytes in short-term culture. Over a 2-h period, splenocytes incorporate [^{3}H]TdR into DNA which is found in both detergent-soluble and detergent-insoluble fractions. While DNA in the detergent-soluble fraction contains about 25% of the total incorporation product, it comprises less than 3% of the total cellular DNA, and its specific activity is significantly greater than that of chromosomal DNA. The labeled DNA from the detergent-soluble fraction is nonmitochondrial in origin. We propose to call this DNA MADS DNA, metabolically active detergent-soluble DNA.

Other investigators who have observed metabolically active DNA include Schneider (Schneider & Kuff, 1969; Smith et al., 1975; Schneider & Smith, 1977) and Pelc (Stroun et al., 1967; Pelc, 1968) from mouse liver and muscle and Williamson (1970) from primary cultures of embryonic liver cells. It seems likely that the detergent-soluble DNA from human lymphocytes which labels efficiently with [^{3}H]TdR (Meinke et al., 1973; Meinke & Goldstein, 1974; Rogers, 1976a,b) might be of similar nature. A variety of eukaryotic cells in culture also contain small polydisperse circular (spc) DNA, which is separable from chromosomal DNA by physical means (Delap et al., 1978; Smith & Vingrad, 1972; Stanfield & Helinski, 1976; Bertelson et al., 1982). Since no systematic study of [^{3}H]TdR incorporation has been reported and only circular molecules have been isolated, it is as yet unclear whether spc DNA is related to MADS DNA.

Materials and Methods

Preparation of Cells. Nonadherent spleen cells from male outbred Swiss mice (Sendai virus free CD-1, 4–8-weeks old, from Charles River Breeding Farms, Wilmington, MA) were prepared as described previously (Strauss et al., 1976, 1977). The preparative procedure included Ficoll–Hypaque gradient centrifugation to remove red cells and a 30–60-min interval when the cell suspension was allowed to settle onto plastic petri dishes in order to remove adherent cells. Stimulated mice had been injected intravenously with 250 μg of Con A (Pharmacia Fine Chemicals, Ltd., Piscataway, NJ) 24–48 h prior to sacrifice. Under these conditions, the spleens became enlarged, and numerous mitotic figures were observed (Moatamed et al., 1975). These were the same conditions used in earlier transport and incorporation studies (Strauss et al., 1976, 1977, 1980). The viability of the final cell suspension was 90–95% as judged by trypan blue exclusion.

Chemicals and radiochemicals were obtained from the sources previously listed (Strauss et al., 1976, 1977, 1980). Radiolabeled nucleosides were purified before use by ascending

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chromatography on Whatman 1 paper in one of several systems described by Fink et al. (1963). The strip corresponding to the unlabeled standard was cut out and the radioactive material eluted by descending chromatography.

Incubations. Cells were incubated for 2 h unless indicated otherwise at 37 °C in phosphate-buffered saline containing glucose and bovine serum albumin (PBSGB). In experiments where [^{33}P]phosphate incorporation was measured, incubation was carried out in 10 mM (HEPES) containing 200 μM phosphate, 0.9% NaCl, 5 mM glucose, and 0.1% bovine serum albumin. This concentration of phosphate was chosen after incorporation in a variety of phosphate and HEPES concentrations was examined. Thymidine was present at an external concentration of 60–150 nM, the concentration depending on the specific activity of the radiolabeled isotope (60–90 Ci/mmol). Incubation was terminated by washing 2 times in PBSGB and resuspending in half the original volume. The wash supernates were discarded. To the final suspension the nonionic detergent Nonidet P40 (NP40) (Lerner et al., 1971) and ethylenediaminetetraacetic acid (EDTA) were added to a concentration of 0.5% and 25 mM, respectively. Under these conditions, both cells and nuclei rapidly swelled and became trypan blue permeable, but lysis did not occur. Pellets and supernates were separated from each other immediately after mixing or after a 5-min incubation in one of two ways: in early experiments by centrifugation at 2000 rpm at 4 °C for 10 min in an International PR6000 centrifuge or in later experiments by spinning for 30 s in the Eppendorf microfuge (Model 3200) at 4 °C. Similar results were obtained if lysis and spinning in the Eppendorf were carried out at room temperature in the absence of EDTA or in the presence of 150 mM EDTA. Pellets were taken up by boiling in half the original volume of 2% sodium dodecyl sulfate (SDS) or without boiling in 2% SDS containing 8 M urea. Fractions were stored at −20 °C.

Purification of DNA from Supernates and Pellets. Supernates and pellets were subjected to phenol extraction in the presence of chloroform and isoamyl alcohol, preceded by proteinase K treatment (Maniatis et al., 1982), and repeatedly precipitated with ethanol until all traces of EDTA had been removed. Material contained in the ethanol precipitate was treated with RNase (either 8 μL of RNase T-2 in RNase buffer or heat-treated RNase A) for 2 or 4 h at 37 °C and subjected to cesium chloride centrifugation (Vogt, 1973) at 36 000 rpm for 60–72 h at 15 °C in a Spinco Model L-2 65B ultracentrifuge. After 0.4-mL fractions were collected from the bottom of the tube, those with substantial amounts of radioactivity were pooled, precipitated directly with 70% ethanol (Sclavani & Wechsler, 1981), washed once with 70% ethanol containing 0.1 M NaOAc, dried, and resuspended in 10 mM tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl), pH 8.0. To obtain the recoveries listed in Table I, it was necessary to follow the precautions for single-stranded polynucleotides described by Sclavani & Wechsler (1981). UV spectrophotometry indicated OD_{260}/OD_{280} ratios of 1.7–1.9. The material was completely sensitive to DNase I, and, after heat denaturation, it was completely susceptible to nuclease S-1. It was insensitive to purified Pronase and to repeated treatment with heat-treated RNase A.

In one experiment, [^{3}H]DNA extracted from the NP40 pellet (43.3×10^4 cpm) was mixed with 2.5×10^7 cells (5.0×10^6 cells/mL) immediately prior to detergent lysis. Subsequently, DNA was purified from the NP40 supernate in the usual fashion. Half the initial radiolabeled material (51.5%) was recovered along with DNA from the NP40 supernate. When [^{3}H]DNA extracted from NP40 pellets was treated with 0.5% NP40 in the absence of cells, 48.4% of the radiolabeled DNA was recovered in the supernate. Thus, complete reprecipitation of pellet DNA required the presence of the original chromatin, and the presence of added cellular material was without effect.

Paper and Thin-Layer Chromatography. Metabolites of [^{3}H]TdR found in media, wash solutions, and lysates were resolved in one of several different systems described by Fink et al. (1963) or by Pazur (1962). Alternately, unidirectional thin-layer chromatography was performed by the method of Randerath & Randerath (1967) on 20 × 20 cm poly(ethylenimine)–cellulose (PEI–cellulose) plates (VWR Scientific, Inc., Newton, MA). Appropriate unlabeled standards were included as required. At various times, stock [^{3}H]TdR or stock [^{3}H]TdR mixed with incubation medium was also chromatographed. Combinations of the various chromatography systems were able to resolve thymidine, thymine, TMP, cTMP, 5′-TDP, 3′,5′-TDP, TTP, thymidine diphosphate glucose, and thymidylylthymidine.

The location of the radioactivity after paper chromatography was determined by cutting the paper into 1-cm-wide pieces and counting them in mini vials containing 5.2 mL of scintillation fluid. On some occasions, the pieces were placed in 20-mL vials so that the radiolabeled material was eluted with 0.2 M Tris-HCl containing 0.7 M $MgCl_2$ before the addition of 10 mL of scintillation fluid. This procedure permitted counting efficiencies of up to 38% and complete recovery of the applied starting material. The location of radioactivity after thin-layer chromatography was determined by scraping 0.5-cm bands along the lane of the unknown into scintillation vials and eluting the isotope with Tris–$MgCl_2$ before addition of scintillation fluid for scintillation spectrometry.

Column Chromatography. Column chromatography was performed in PBS at room temperature by using Sephadex G100 (Pharmacia Fine Chemicals, Ltd., Piscataway, NJ) in a 0.9 × 23 cm column. Half-milliliter samples were placed on the column, and 0.4–0.8-mL fractions were collected, depending on the run.

Gel Electrophoresis. Agarose gel electrophoresis (native or denaturing) was performed as described by Maniatis et al. (1982). λ DNA restricted with *Hin*dIII (New England Bio-Labs Inc., Beverly, MA) and φX174RF restricted with *Hae*III (New England Bio-Labs, Inc., Beverly, MA) were used as standards. Electrophoresis was carried out at 100 V for 2–3 h. In order to detect the presence of tritium, the gels were sliced into 2-mm slices with a gel slicer and placed in scintillation vials for melting with 0.5 or 1.0 mL of H_2O at 90 °C before the radioactivity was determined by scintillation spectrometry.

High-Pressure Liquid Chromatography (HPLC). Samples to be analyzed for base ratios were dissolved in 10 mM Tris-HCl, pH 8.0, heat denatured, cooled quickly, treated with 5 μg of snake venom phosphodiesterase (PDE, from Boehringer-Mannheim, GMBH, Indianapolis, IN) for 30–150 min at either 37 or 65 °C, and either analyzed immediately or stored at −20 °C. Samples were injected onto a SAX column (Whatman PXS 10/25 SAX) attached to a Varian Model 5000 high-pressure liquid chromatograph. Nucleotides were eluted at a flow rate of 2.0 mL/min, by means of the following program (Brown, 1975): 5 min in solvent A (7 mM KH_2PO_4–7 mM KCl, pH 4.0), a linear increase to 100% solvent B (25 mM KH_2PO_4–50 mM KCl, pH 5.0) over the next 35 min, followed by 100% solvent B for 10 min. Under these conditions, nucleosides eluted with the solvent front, while mononucleotides elute between 5 and 15 min into the program.

Fractions (1.0 mL) were collected for analysis of radioactivity by scintillation spectrometry. Standards, consisting of mono-, di-, and trinucleotides, deoxynucleosides, and bases, were injected separately and together. Each unknown was run at least twice alone and once with a mixture of the four deoxymononucleotides. In order to quantitate the ratio of peak area to moles of deoxynucleotide, samples of known concentration of each monodeoxynucleotide were injected, and the area under the resultant peak was quantitated by tracing the peak onto chart paper with a no. 6 pencil. The peak was cut out and weighed. The following conversion factors were obtained: dCMP, 131.7 pmol/mg of paper weight; dTMP, 97.0 pmol/mg of paper weight; dAMP, 41.6 pmol/mg of paper weight; dGMP, 35.0 pmol/mg of paper weight.

Nick Translation and Southern Blots. MADS DNA was resolved by means of 1.2% agarose gel electrophoresis and blotted onto nitrocellulose paper (Gene Screen, New England Nuclear Corp., Boston, MA) as described by Maniatis et al. (1982). Whole mouse mitochondrial DNA cloned at a single, *Sph*I site of pBR322 (Bibb et al., 1981) and MADS DNA fragments were labeled with [^{32}P]dCTP by using a New England Nuclear (Boston, MA) nick translation kit in accordance with the manufacturer's recommended procedure. Blots were exposed to radiolabeled probe at 65 °C and washed at the same temperature (Maniatis et al., 1982). They were then autoradiographed and analyzed.

Enzyme Treatments. Hydrolysis with venom phosphodiesterase (Boehringer-Mannheim, GMBH, Indianapolis, IN) was performed in 10 mM Tris-HCl, pH 8.0, as described above. Products were analyzed by high-performance liquid chromatography (see above). Treatment with nuclease S-1 (Boehringer-Mannheim, GMBH, Indianapolis, IN) was carried out in S-1 buffer as described by the manufacturer. Preliminary experiments using trichloroacetic acid (Cl_3CCO-OH) precipitation showed that 10 units of enzyme at 37 °C for 1 h was sufficient to completely degrade denatured MADS DNA or ^{14}C-labeled *Escherichia coli* DNA. The reaction was stopped by the addition of 1 mM EDTA (final concentration).

Restriction endonucleases were obtained from New England Bio-Labs (Beverly, MA). Digestion was carried out as suggested by the manufacturer except that 6.0 mM β-mercaptoethanol was substituted for dithiothreitol and 0.1 mg/mL gelatin was used in place of bovine serum albumin.

Inhibition Experiments. Cells were prepared as described above and incubated for 30 min in the presence of 2 mM hydroxyurea (HU), 5 μg/mL aphidicolin (APHI), or 5 μg/mL ethidium bromide (EB). [^{3}H]TdR was then added, and the incubations were continued for an additional 2 h. Cells were washed and lysed with NP40 as described above. Under these conditions the viabilities of treated and control cells were not different as determined by trypan blue exclusion.

Results

The time course of appearance of [^{3}H]TdR in different subcellular fractions of murine splenocytes is shown in Figure 1. Cells were incubated in the presence of 90 nM [^{3}H]TdR for the indicated period. The NP40 supernatant and NP40 pellet fractions were prepared, and DNA and free TdR + TMP were resolved by gel filtration. During the first 20 min, radioisotope in the NP40 supernate was in the form of TdR and TMP as determined by paper and thin-layer chromatography. After 20 min, progressively greater amounts were found in high molecular weight material. At 2 h, the ^{3}H-labeled incorporation product from the NP40 supernate fraction represented ~25% of all the high molecular weight material (supernate plus pellet) in the cell. A 2-h incubation period

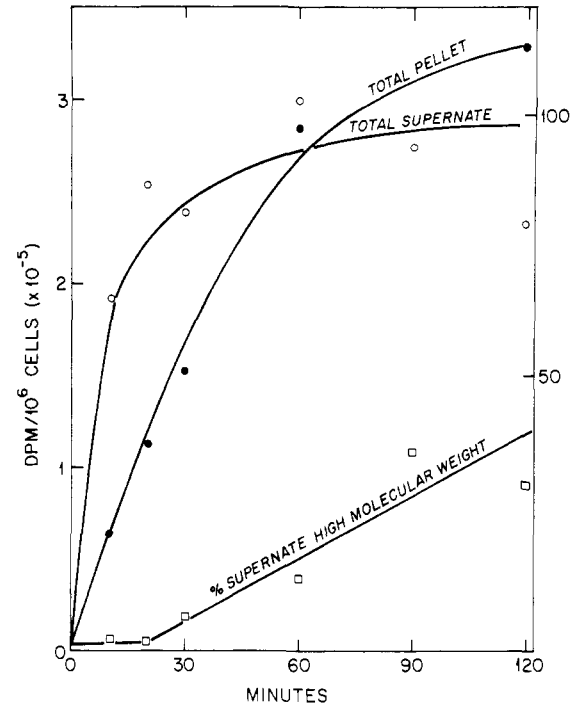

FIGURE 1: Time course of appearance of [^{3}H]TdR in different cell fractions. Splenocytes from Con A stimulated mice (5 × 10^6/mL) were incubated in the presence of [^{3}H]TdR (6 μCi/mL, 90 nM) for the indicated times at 37 °C, washed twice at 4 °C, resuspended in half the original volume, and lysed with 0.5% NP40. Pellet and supernatant fractions were separated rapidly as described under Materials and Methods. Supernates were fractionated further by means of chromatography on Sephadex G100 into high and low molecular weight fractions. Low molecular weight material consisted of thymidine and thymidine nucleotides as shown by paper and thin-layer chromatography. Total radiolabeled material appearing in the NP40 pellet (●) or in the NP40 supernate (○); percentage radiolabeled material from the NP40 supernate appearing in the void volume of the G100 column (□).

was chosen for subsequent experiments.

Isolation and Physical Characteristics of MADS DNA. DNA from NP40 supernatant and NP40 pellet fractions was extensively purified as described under Materials and Methods. Table I lists the amount of DNA from the NP40 supernate fraction and its specific activity recovered in three typical experiments. For comparison, the specific activity of DNA from the NP40 pellet from each experiment is listed. Using a conversion factor of 50 μg of DNA per OD_{260} and assuming 5.7 pg of DNA/animal cell, we found that the amount of DNA which was recovered from NP40 supernates was 1–3% of the total initial cell DNA. Comparison of the low percentage of total cell DNA in the fraction with the high percentage of incorporated [^{3}H]TdR as well as the specific activities of DNA from NP40 supernates and pellets indicated that the former was metabolically active as defined by Pelc (Stroun et al., 1967; Pelc, 1968). We shall refer to the DNA isolated from the NP40 supernatant fraction as metabolically active detergent-soluble (MADS) DNA.

Physical properties of chromosomal and MADS DNA were compared. After cesium chloride gradient centrifugation, the density pattern of MADS DNA was broad, centering around ρ = 1.69 g/mL (Figure 2). This pattern contrasted with that of chromosomal DNA, which banded with a sharp peak at ρ = 1.69 g/mL (Figure 2), and with λ DNA with a sharp symmetric peak at ρ = 1.70 g/mL (data not shown).

After denaturation and digestion of chromosomal and MADS DNA with venom phosphodiesterase, base ratios were analyzed directly by means of HPLC. Earlier attempts to

Table I: Recovery and Specific Activity of DNA from Detergent Fractions[a]

expt	total[b] (dpm × 10^{-6})	radiolabel[c] (dpm × 10^{-6})	DNA[d] (μg)	sp act.[e] (dpm × 10^{-5}/μg)	sp act.[f] (dpm × 10^{-5}/μg)
I	21.5	5.1 (23.7)	50.4	1.01	0.53
II	25.9	7.3 (28.1)	50.1	1.45	0.62
III	33.0	7.2 (21.8)	33.0	2.18	0.57

[a] In each experiment, splenocytes [5 × 10^6/mL, (3.1–4.5) × 10^8 total cells] were incubated for 2 h at 37 °C in [^{3}H]TdR, washed twice at 4 °C, resuspended in half the original volume, and lysed by the addition of NP40 to a final concentration of 0.5%. Pellets (chromosomal DNA) were separated from supernates containing detergent-soluble DNA (MADS DNA) by rapid centrifugation at 13 000 rpm for 2 min. [b] Total dpm in the NP40 supernate. The radioactive material in the NP40 supernate is found in the form of [^{3}H]TMP, and MADS DNA. [c] Radiolabel recovered in MADS DNA after purification as described under Materials and Methods. The numbers in parentheses refer to the percentage of radiolabel in the NP40 supernate recovered as purified DNA. [d] Amount of DNA recovered as MADS DNA. [e] Specific activity (dpm × 10^{-5}/μg of DNA). [f] Specific activity of nuclear DNA (dpm × 10^{-5}/μg of DNA) obtained from NP40 pellets in each preparation.

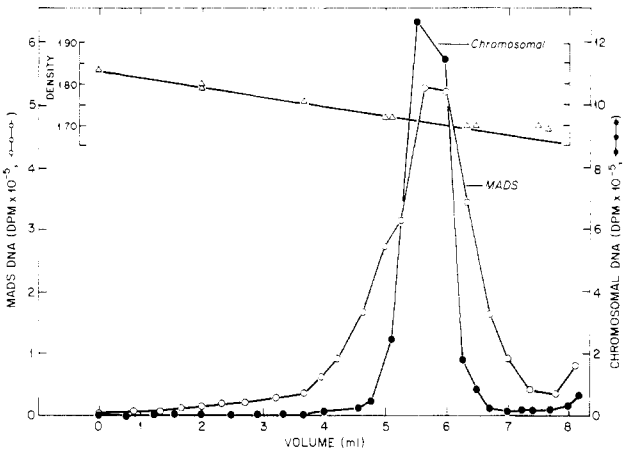

FIGURE 2: Equilibrium cesium chloride centrifugation of chromosomal and MADS DNA. DNA from NP40 supernates (MADS DNA) and NP40 pellets (chromosomal DNA) was isolated as described under Materials and Methods. DNA from each source was analyzed by means of equilibrium gradient centrifugation in the presence of cesium chloride (66 h, 15 °C, 36 000 rpm). Fractions were collected by dripping from the bottom of the tube and analyzed for the presence of radiolabeled material and for density. MADS DNA dpm per fraction (○); chromosomal DNA dpm per fraction (●); density (△).

examine base ratios with cells labeled with [^{33}P]phosphate had proved unsuccessful. While the cells took up [^{33}P]phosphate into high and low molecular weight materials during the 2-h incubation period with [^{3}H]TdR, the ratio of incorporated ^{33}P to ^{3}H was not constant for all size classes. Therefore, direct analysis was necessary. The G + C content of nuclear DNA obtained from seven independent experiments was 39.9 ± 0.7% (SE). The G + C content of MADS DNA isolated during 10 different experiments was 43.4 ± 1.3% (SE). Recovery of the tritium label as [^{3}H]TMP generally was 95% with the remainder eluting with the solvent front. In addition, samples analyzed for tritium distribution by means of paper and thin-layer chromatography prior to HPLC showed that all the radioactivity had the mobility of [^{3}H]TMP. Therefore, MADS DNA contained slightly more deoxyguanosine and deoxycytidine than nuclear DNA. Note, however, that this was an average base composition for material with a broad distribution on cesium chloride (see above) gradients and heterogeneous sizes (see below).

The size distributions of MADS and chromosomal DNA were compared by means of agarose gels under native (1.2%) and denaturing (30 mM NaOH–1% agarose) conditions (Figure 3). MADS DNA was heterogeneous, ranging in size from 4.2 to 0.16 kilobase(s) as determined by gel electrophoresis under native conditions and assuming MADS DNA to be completely double stranded in comparison with *Hae*III-digestion fragments of φX174RF. Most of the chromosomal DNA failed to enter the gel. When the size distribution of MADS DNA was examined by means of alkali

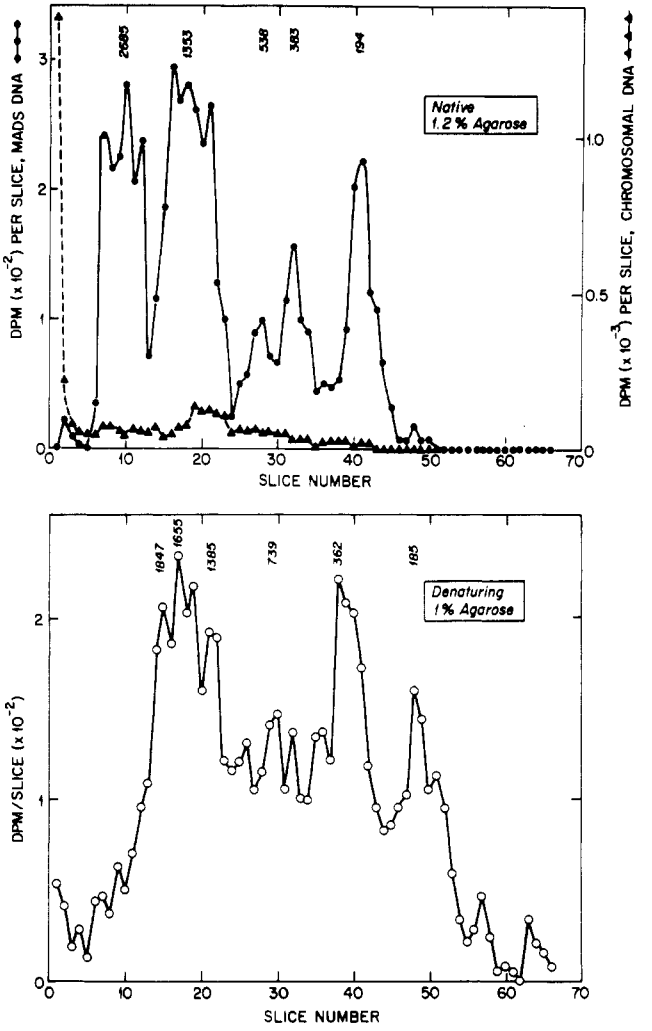

FIGURE 3: Size distribution of MADS and chromosomal DNA. MADS and chromosomal DNAs were analyzed by gel electrophoresis under native conditions (1.2% agarose). MADS DNA was also analyzed under denaturing conditions (0.1 N NaOH, 1% agarose). The positions of the products were determined by slicing the gel and counting the dissolved slices by means of scintillation spectrometry. The positions of standards (φX174RF/*Hae*III and λ/*Hin*dIII) were determined by means of ethidium bromide staining. (Top panel) Native gel: chromosomal DNA (▲); MADS DNA (●). (Bottom panel) Denaturing gel: MADS DNA (○).

gels, the size range observed was 1.8 to 0.14 kb (Figure 3). Therefore, MADS DNA is not completely double stranded.

In order to determine the distribution of ^{3}H as a function of DNA species, sufficient material was loaded onto the gel as to visualize the bands by means of ethidium bromide staining (Figure 4). Perhaps the most striking feature was the periodicity of the size classes, the smallest of which was about 200 base pairs with a repeat of about 160–220 base

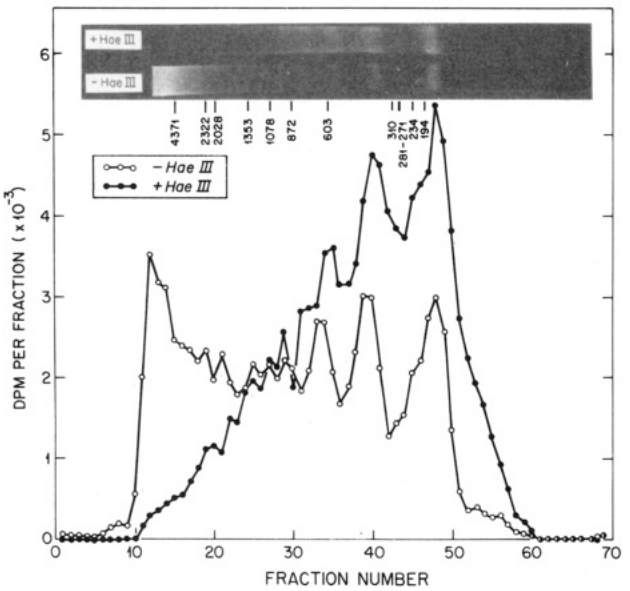

FIGURE 4: Correlation of ethidium bromide visualized bands with distribution of [^{3}H]thymidine. MADS DNA (1.6 μg) was treated with *Hae*III (8 units) for 2 h at 37 °C and analyzed on a 1.4% agarose gel (standards run on the same gel were φX174/*Hae*III and λ/*Hin*dIII). The top of the figure shows the ethidium bromide staining pattern. The lower part of the figure shows the distribution of radiolabeled material in each slice. MADS DNA treated with buffer alone (○); MADS DNA treated with *Hae*III (●).

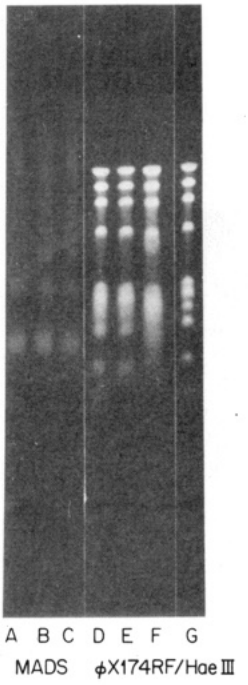

FIGURE 5: Effect of nuclease S-1 on MADS DNA. MADS DNA (1.2 μg, lanes A–C) or φ174RF/*Hae*III DNA (1.0 μg, lanes D–F) was treated with nuclease S-1 at 37 °C for 1 h. Products were analyzed by electrophoresis under denaturing conditions (1% agarose in 30 mM NaOH). Lanes A and D, 0 units of S-1; lanes B and E, 10 units of S-1; lanes C and F, 50 units of S-1; lane G, φ174RF/*Hae*III untreated.

pairs. Distribution of the fragments of MADS DNA corresponded to the distribution of [^{3}H]TdR (Figure 4). When the lower molecular weight bands were analyzed on 6% polyacrylamide gels, each band contained a spread of at least 40 base pairs. On the basis of the intensity of the bands visualized by ethidium bromide, one would estimate that roughly similar amounts of DNA are found in the smaller classes and that a large amount of larger DNA remains unresolved toward the top of the gel under native conditions. If [^{3}H]TdR were uniformly incorporated into the different size classes, more radiolabeled tritium per slice should be visualized closer to the origin. As this is decidedly not the case, we conclude that [^{3}H]TdR is not uniformly incorporated into MADS DNA.

Enzyme Treatments. To determine the presence of single-stranded regions in MADS DNA, native and heat-denatured samples were exposed to nuclease S-1 and resolved by means by alkaline agarose electrophoresis (Figure 5). φX174RF/*Hae*III DNA was treated in a similar fashion to serve as the control. Heat-denatured DNA from both sources was hydrolyzed completely under the conditions employed (not shown). Neither MADS DNA nor φX174RF/*Hae*III DNA was affected by nuclease S-1. Thus, MADS DNA is not a simple collection of single-stranded fragments.

In order to determine whether MADS DNA was representative of a particular class of repetitive DNA, MADS DNA was treated at 37 °C for 2 h with *Hae*III (4 and 20 units) and analyzed on a 1.2% agarose gel (Figure 4). *Hae*III treatment resulted in digestion of species larger than 1500 base pairs to shorter fragments of variable sizes. However, the shorter polynucleotides were still evident. Since similar results were obtained with 20 units of *Hae*III for 24 h, it can be emphasized that the *Hae*III reaction was driven to completion. Also, *Hae*III treatment of MADS DNA in the presence of φX174RF resulted in the expected φX bands superimposed over the MADS DNA pattern (data not shown).

Inhibition Experiments. Attempts were made to determine whether ongoing DNA synthesis was required for the labeling of MADS DNA. In five separate experiments, we treated splenocytes with 2 mM HU or 5 μg/mL APHI, which inhibits template-directed nuclear DNA synthesis by different mechanisms (Pedrali-Noy et al., 1980; Pedrali-Noy & Spadari, 1980; Reichard, 1972), or with 5 μg/mL EB, which inhibits mitochondrial DNA synthesis (Slonimski et al., 1968). Under these conditions, the viabilities of treated and control cells were not significantly different. After pretreatment of cells for 30 min with the designated agent, [^{3}H]TdR was added, and incubation was continued for an additional 2 h. Cells were then washed in the usual fashion and lysed with NP40. While EB was without effect on incorporation into both MADS and chromosomal DNA, both HU and APHI inhibited the incorporation of [^{3}H]TdR into MADS DNA, HU by 83% and APHI by 96%.

Sequence Homology to Mitochondrial DNA. To further exclude the possibility that MADS DNA was related to mitochondrial DNA, mouse mitochondrial DNA cloned in pBR322 was labeled by nick translation and hybridized to a blot prepared from MADS DNA resolved by means of 1.2% agarose gel electrophoresis (Figure 6). Homology to mitochondrial DNA was detected in two minor bands from the detergent-soluble fraction. The molecular weights of these DNA species were greater than those of MADS DNA visualized by ethidium bromide (Figure 6).

Sequence Homology to the Smallest Size Class. Because of the striking periodicity in MADS DNA, we chose to determine whether common sequences were present. Therefore, sequence homology of the larger fragments of MADS DNA to the nick-translated smallest 200 base pair fragment of MADS DNA isolated from 3.5% polyacrylamide gels was examined (Figure 7). Hybridization to most of the larger fragments was observed. Poor hybridization to the smallest species was probably due to loss during the blotting procedure.

Discussion

In this paper, we report the initial characterization of metabolically active detergent-soluble (MADS) DNA in

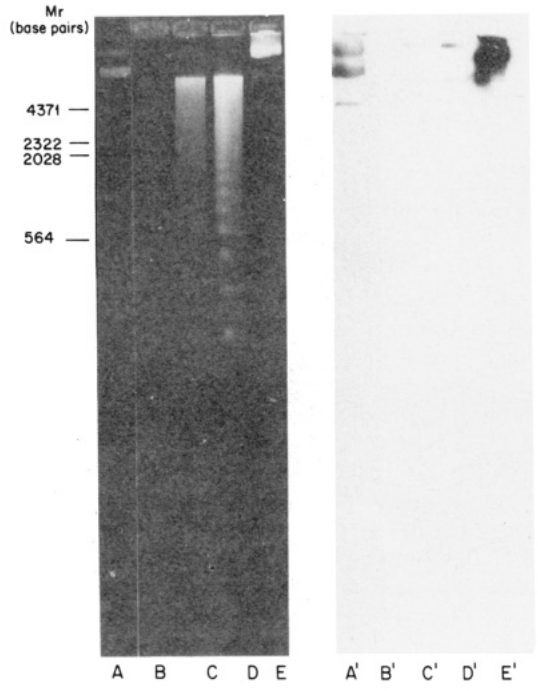

FIGURE 6: Detection of mitochondrial DNA in MADS DNA. MADS DNA, pBR322, and mitochondrial DNA cloned in pBR322 were resolved by means of agarose gel electrophoresis (1.2% gel) and stained with ethidium bromide to locate the individual components. λ/*Hin*dIII and φX174/*Hae*III were run as standards. The DNA was then transferred to nitrocellulose for Southern blot analysis using the nick-translated mitochondrial genome in pBR322 as the probe. The ethidium bromide staining pattern is shown on the left; the corresponding autoradiogram is shown on the right. Lanes A and A′, 0.6 μg of pBR322; lanes B and B′, 1.4 μg of MADS DNA; lanes C and C′, 14 μg of MADS DNA; lanes D and D′, 34 μg of MADS DNA; lanes E and E′, 0.62 μg of mitochondrial DNA in pBR322. Chromosomal DNA included on the same blot contained no detectable sequences homologous to mitochondrial DNA.

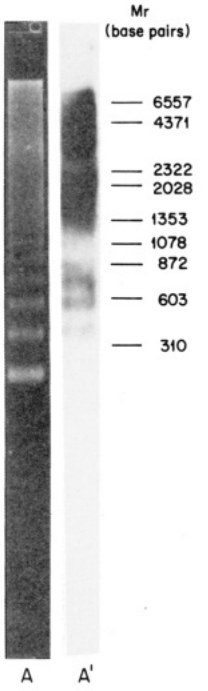

FIGURE 7: Sequence homology of the smallest fragment of MADS DNA with larger species. The smallest MADS fragment was isolated after 3.5% polyacrylamide gel electrophoresis of MADS DNA, nick translated, and used to probe MADS DNA (3.25 μg) resolved by means of agarose gel electrophoresis. See the legend to Figure 6 for experimental details.

splenocytes from Con A stimulated mice. The four features which stand out are the rapid nonuniform labeling with [^{3}H]TdR, the periodocity of the bands, the requirement for ongoing chromosomal DNA synthesis, and the detergent solubility. Detergents are used in the preparation of nuclei from cell types as diverse as chicken erythroblasts (Hewish, 1977) and human lymphocytes (Lerner et al., 1971) to *Tetrahymena thermophila* (Gottschling et al., 1983). Nevertheless, little is known about the basis for this method of fractionation.

We consider several explanations for the presence of MADS DNA. Lymphocyte death induced by glucocorticoids (Wyllie & Morris, 1982; Vedekis & Bradshaw, 1983) or γ irradiation (Umansky et al., 1981) has been associated with apparent fragmentation of DNA. We exclude cell death because under the experimental protocol employed here the cells are obtained from animals exposed to an immunological stimulant 24–48 h prior to sacrifice. Since stimulation is not performed in culture, the cells are more than 90% viable. In addition, several unstimulated cell lines including S49 murine thymoma cells, P3X63-Ag8.6.5.3 murine B lymphoma cells, and Jurkat human lymphoma cells contain MADS DNA (unpublished results). On the other hand, mouse stomach cells obtained by the method of Romrell et al. (1975) lack MADS DNA.

While cell death is an unlikely cause for the production of MADS DNA, nuclease activation during preparation or incubation of cells is possible. We exclude this source, because unincubated cells and cells from unfractionated spleens of Con A stimulated mice also contain detergent-soluble DNA with a similar size distribution. We have also excluded the possibility that the size heterogeneity arises at or after cell lysis, since lysis has been performed at 4 °C in the presence or absence of 25 mM EDTA without change in the results.

Even though nuclease activation has not occurred during cell preparation or lysis, endogenous nucleases may have been activated during the process of stimulation in the animal with Con A, especially since splenocytes from unstimulated mice contain 15–20% the amount of MADS DNA on a per cell basis (unpublished results). Indeed, Slor et al. (1976) reported the presence of a unique DNase associated with rat lymphocytes activated with Con A in vitro. However, MADS DNA cannot be merely a degradation product due to an endogenous nuclease for two reasons. First, the specific activity of MADS DNA exceeds that of bulk chromosomal DNA. Second, the labeling pattern visualized after 30-min exposure to [^{3}H]TdR is maintained for at least 3 h in the absence of exogenous radiolabeled pyrimidine (unpublished results). The source of the size distribution and the extent of informational heterogeneity remain to be determined.

Since ongoing DNA synthesis is required, MADS DNA either is a direct product of polymerase α or requires such a product. Even so, it is unlikely that MADS DNA is newly synthesized single-stranded Okazaki fragments (Okazaki et al., 1968) because native MADS DNA is resistant to nuclease S-1. We note also that aphidicolin has been reported to inhibit repair of DNA in UV-irradiated human fibroblasts (Snyder & Regan, 1981) so that a role for MADS DNA in repair synthesis is possible. If MADS DNA is a replication intermediate, the size of the radiolabeled fragments should increase with time. This possibility remains to be explored.

The size distribution of MADS DNA is reminiscent of DNA obtained from isolated lymphocyte nuclei incubated with divalent cations [reviewed by Burgoyne & Hewish (1978)] now

commonly referred to as nucleosomal DNA. In that case, the nonrandom digestion pattern is ladderlike with the smallest fragment being 185–200 base pairs. It is not seen until nuclei are exposed to divalent cations so that certain partly characterized nucleases are activated (Ishida et al., 1974) or unless chromatin is treated with micrococcal nuclease or DNase I. The involvement of metabolic activity as seen in the case of MADS DNA is usually not considered or, when recognized, not considered to be significant. Despite similarities in size distribution between MADS and nucleosomal DNAs, it is difficult to visualize how only a small percentage of nucleosomal fragments would be solubilized by a neutral detergent. This class of nucleosomes would have to be special, containing already cleaved DNA so that it is separable from chromatin by detergents. Transcriptionally active chromatin is also susceptible to endogenous nucleases which leave a DNA size distribution similar to the nucleosome ladder (Igo-Kemenes et al., 1982). How one would account for rapid [^{3}H]TdR incorporation and aphidicolin sensitivity is unclear. Finally, MADS DNA might represent a special, rapidly turning over segment much like that described by Rogers & Rucinsky (1982). Whatever the source, the segments are not anchored in chromosomal DNA since they are detergent soluble. The possibility of a nonrandom process of physiological significance can be entertained.

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