Isolation of Mouse Satellite Deoxyribonucleic Acid by Composite Polyacrylamide Gel Electrophoresis*

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ABSTRACT: Mouse DNA was separated into a major fast-moving and minor slow-moving band by electrophoresis on a composite 2.5% polyacrylamide–0.5% agarose slab gel. The slow-moving band migrated 0.5 mm/hr slower than the fast-moving band at 200 V and 0° and comprised 9% of the total cellular DNA. Mouse satellite and main-band DNA separated by isopycnic cesium chloride (CsCl) centrifugation were shown to be identical on electrophoresis with the slow-moving and fast-moving bands, respectively. The buoyant density of DNA isolated from the slow-moving band was found to be 1.693 g/cm^3; DNA from the fast-moving band had a buoyant density equal to 1.701 g/cm^3. These values agree with the densities reported for satellite and main-band DNA, respectively. The versatility of composite polyacrylamide gel electrophoresis to study functional and metabolic characteristics of satellite DNA was examined. The methylation of satellite DNA as studied by gel electrophoresis was found to be about three times greater than that of main-band DNA. The electrophoresis of mouse DNA on composite polyacrylamide gels therefore provides a rapid, convenient, and inexpensive alternative to isopycnic CsCl centrifugation for analytical studies of satellite DNA.

Polyacrylamide gel electrophoresis has become a powerful and widely used tool for fractionation of different species of RNA (Loening, 1967; Peacock and Dingman, 1967; Richards and Gratzer, 1964; McPhie *et al.*, 1966; Dahlberg and Peacock, 1971) but has been infrequently employed for DNA. On the other hand, isopycnic CsCl centrifugation (Kit, 1961; Flamm *et al.*, 1966; Brunk and Lieck, 1969) and hydroxylapatite column chromatography (Flamm *et al.*, 1967; Corneo *et al.*, 1970; Graham, 1970) have been traditionally the procedures used for DNA fractionation. Mouse DNA, which is composed of two species of nuclear DNA (satellite DNA comprising 10% and main band DNA comprising 90% of the genome) (Kit, 1961), seemed a convenient source of DNA to fractionate by polyacrylamide gel electrophoresis. Physically, satellite DNA of mouse is characterized by a lower density (in CsCl) than the main band DNA and by its ability to reassociate rapidly after denaturation. The latter property suggests that mouse satellite DNA is composed of repeated copies (10^6) of a rather short nucleotide sequence (300–600 base pairs) (Waring and Britten, 1966). Moreover, base compositional differences exist between satellite and main-band DNA (Corneo *et al.*, 1968). Such differences in physical characteristics led us to evaluate the possibility of fractionating DNA by gel electrophoresis The present study demonstrates the ability of composite polyacrylamide gels to fractionate mouse DNA electrophoretically both into satellite and main-band DNA.

Mouse satellite DNA is more than two times richer than main-band DNA in molar concentration of 5-methylcytosine (Salomon *et al.*, 1969). The study of methylation of mouse satellite DNA by gel electrophoresis, therefore, provides a convenient and important test of the versatility of the technique to delineate metabolic and functional differences among species of DNA.

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Experimental Section

Materials. Pancreatic ribonuclease A (salt-free, lyophilized) and pancreatic deoxyribonuclease I (electrophoretically purified, RNase-free) were the products of Worthington Biochemical Corp. Acrylamide, *N,N'*-methylenebisacrylamide, *N,N,N',N'*-tetramethylethylenediamine, and the stain, 1-ethyl-2-(3-(1-ethylnaphtho[1,2*d*]thiazolin-2-ylidene)-2-methylpropenyl)naphtho[1,2*d*]thiazolium bromide ("Stains-all") were obtained from Eastman Kodak, Distillation Products Industries, Rochester, N. Y. SeaKem agarose was purchased from Marine Colloids, Inc. Reagent grade crystalline phenol was obtained from Analar. Sodium dodecyl sulfate was purchased from K&K Laboratories, Inc., Kankakee, Ill. Cesium chloride, optical grade, tris(hydroxymethyl)aminomethane, and [*methyl*-^{3}H]thymidine (16.4 Ci/mmole) were obtained from Schwarz Mann. NCS, Spectrofluor, and [*methyl*-^{3}H]methionine (5.2 Ci/mmole) were purchased from Amersham/Searle. Eagle's minimal essential medium, buffered saline solution, and Liebovitz medium (without methionine) were obtained from Grand Island Biological Co. Pronase was obtained from Calbiochem, and *Micrococcus lysodeikticus* from Sigma Chemical Co. The electrophoretic cell and elution apparatus of Raymond were the products of the E-C Apparatus Corp. The microfuge (Spinco) was purchased from Beckman Instruments, Inc.

Methods. Preparation of Mice for [methyl-^{3}H]Thymidine Incorporation. Mouse DNA was labeled with thymidine by injecting subcutaneously 0.1 ml of a solution containing 50 μCi of [*methyl*-^{3}H]thymidine into C57/Bl newborn mice less than 16 hr old (Salomon *et al.*, 1969). Twenty-four hours later DNA from the whole body or liver was extracted by the rapid phenol or Marmur methods. Specific activities of DNA from whole body and from liver were 2900 dpm/μg of DNA and 11,300 dpm/μg of DNA, respectively.

Preparation of Fibroblasts for in Vitro DNA Methylation. Primary cultures were derived from 16-day embryos from three C57/Bl pregnant mice. Secondary cultures were made after adequate growth had occurred. Twenty-four hours later, medium deficient in methionine (Liebovitz), but containing serum, antibiotics, 2 mg/ml of sodium formate

(Salomon *et al.*, 1969), and 200 μCi of [*methyl*-^{3}H]methionine, was added to each plate. Forty hours later the cells were harvested and extracted by the rapid phenol procedure.

Isolation of DNA. (1) RAPID PHENOL METHOD BASED ON GREEN *et al.* (1971). Tissue (0.1 volume) in 1 volume of 0.5% sodium dodecyl sulfate in a Tris–EDTA–borate buffer (pH 8.3) (TEB)[1] (Peacock and Dinghan, 1967) was homogenized manually with a glass homogenizer or a Waring Blendor for 0.5 min. After the addition of 1 volume of water-saturated phenol, the mixture was shaken thoroughly on a Vortex shaker and then centrifuged in a microfuge (Spinco) for 2 min. From 10 to 60 μl of the aqueous phase was applied to the gel. The exact amount of solution applied depended on the tissue extracted, and therefore, for any experiment it was best to apply graded volumes to obtain optimal resolution. Usually from 1 to 10 μg of DNA was applied to each of eight slots in a 3-mm gel; the lower quantities gave higher resolution. Extraction procedures performed at room temperature usually degraded high molecular weight RNA, with DNA remaining intact. To remove contaminating RNA, the aqueous phase was incubated at 37° for 1 hr with 0.1 volume of RNase (6 mg/ml). DNase digestions were performed as described earlier (Peacock and Dingman, 1967).

(2) MARMUR METHOD. DNA extraction was essentially identical with that described by Marmur (1961), except Pronase (2 mg/ml) was added after the sodium dodecyl sulfate, incubation with RNase was prolonged to 1 hr, and the precipitation step with isopropyl alcohol was omitted.

Fractionation of DNA. DNA (50–150 μg) obtained by the Marmur method was centrifuged to equilibrium in CsCl. Step gradients were prepared after the method of Brunk and Leick (1969). Polyallomer tubes were coated with bovine serum albumin to eliminate DNA absorption to the tubes (Billen and Hewitt, 1966). The gradients were centrifuged in the type 40 fixed-angle (26°) rotor in a Beckman Model L or L2-65B ultracentrifuge at 25° for 60–66 hr. After puncturing the bottom of the tubes with a 23-gauge needle, 5-drop fractions were collected and diluted with 0.4 ml of water. The absorbance of these fractions was measured at 260 nm in 1-cm path-length cells in a Beckman DU spectrophotometer. One absorbance unit was assumed equal to a concentration of 50 μg of DNA/ml. Buoyant density determinations were based on an internal standard of ρ = 1.731 g/cm^3 for *M. lysodeikticus* DNA (Schildkraut *et al.*, 1962) and ρ = 1.701 g/cm^3 for mouse main-band DNA (Kit, 1961). Radioactivity was determined after diluting the samples with 2 ml of water, mixing with 4 ml of NCS, and finally adding 10 ml of toluene–Spectrofluor solution (3 l. of toluene plus 126 ml of Spectrofluor). The efficiency for counting tritium was 38%. For gel electrophoresis of CsCl fractions, DNA fractions (satellite and main bands) were combined separately and dialyzed against water overnight. After lyophilization the satellite and an aliquot of main DNA were dissolved in 50 μl of TEB buffer. The samples were applied to the composite gels and electrophoresed as described below.

Electrophoresis of DNA. The preparation of the gels and the electrophoretic techniques have been described previously (Raymond, 1962; Peacock and Dingman, 1968; Dahlberg *et al.*, 1969). Composite 3-mm slab gels containing 2.5% acrylamide and 0.5% agarose were used. The TEB buffer was used for electrophoresis. All gels were prerun 1 hr at 200 V, and the temperature of the circulating coolant was lowered to 0° before sample application. The same buffer was employed for the prerun and electrophoresis without recirculation. DNA (1–10 μg) was applied to the slots and electrophoresed at 0° and 200 V for 6–7 hr. Gels were stained overnight with "Stains-all" (Dahlberg *et al.*, 1969). After destaining in running tap water (with normal laboratory illumination), the gels were photographed, and the strips were scanned in the Gilford 240 recording spectrophotometer to determine relative DNA concentrations. The strips were frozen on Dry Ice for 2–3 min and then cut into 1-mm slices into counting vials containing 1 ml of NCS. After adding 10 ml of toluene–Spectrofluor solution, the vials were counted in a Beckman LS-100 liquid scintillation counter. The efficiency of counting tritium under these conditions was 40–42%.

Electrophoretic Elution of DNA from Composite Preparative Gels. Aqueous phase (100 μl) obtained by rapid phenol extraction of whole body mouse DNA labeled with [*methyl*-^{3}H]thymidine was diluted with 200 μl of TEB buffer (containing 18 mg of sucrose) and applied to each of four slots of a 6-mm 2.5% acrylamide–0.5% purified agarose gel (by ethanol precipitation from formamide) (A. C. Peacock and S. L. Bunting, in preparation). Electrophoretic conditions were as described above except that the time of electrophoresis was increased to 11 hr. DNA bands were identified using uv light and separated from one another by slicing the gel with a sharp knife. After soaking in TEB buffer for 1 hr, the separated bands from four gels were placed in the Raymond elution apparatus (Raymond and Jordan, 1966) and electrophoresed at 20 V and 20° for 6 hr. The TEB buffer was recirculated during electrophoresis. The DNA migrated from the gel into the collecting tubules beneath. The fluid from the tubules was collected, pooled, dialyzed extensively against 0.01 SSC (SSC = 0.15 M sodium chloride–0.015 M sodium citrate), and lyophilized. The powder was dissolved in 200 μl of water and centrifuged in a microfuge for 5 min to remove some particulate impurities. The supernatant from both DNA bands had about 16,000 dpm (about 5 μg of DNA), while the precipitate had essentially none. The eluted labeled DNA was added to 100 μg of unlabeled mouse DNA (extracted by Marmur method) and centrifuged to equilibrium in CsCl as described above. DNA (28 μg) from *M. lysodeikticus* was added as a marker for density determinations.

Results

Electrophoretic Analysis of Unfractionated DNA. An electrophoretic analysis of DNA derived from the aqueous phase of a phenol–sodium dodecyl sulfate extract of mouse skin is shown in Figure 1 (slot 1). On this 2.5% acrylamide–0.5% agarose gel there was a minor slow-moving band at 38 mm and major fast-moving DNA band at 41–48 mm. These bands were resolved on composite gels with an acrylamide concentration ranging from 2.0 to 3.0%, but 2.5% acrylamide gel gave optimal resolution and separation. The slow-moving band migrated about 0.5 mm/hr slower than the fast-moving band at 200 V and 0° and comprised about 9% (range 7–11%) of the total cellular DNA as measured by quantitating the areas under the peaks of tracings of stained gels scanned by recording spectrophotometry. Although these two bands were easily discernible in all mouse tissues studied (newborn and adult mouse DNA and mouse tissue DNA *in vitro*), no slow-moving band was seen from guinea pig, rat, and human DNA. The resolution or separation of the bands was not altered by mild shearing or sonication of the DNA. However, after treatment of DNA with 200-atm shearing force (ob-

[1] Abbreviation used is: TEB, Tris–EDTA–borate buffer.

TABLE I: Comparison of DNA Concentration and Isotope Incorporation into Mouse Slow-Moving Band.[a]

	DNA (%)[b]		Dpm (%)[c]		Rel sp act.[d]	
^{3}H isotope (see text)	Mean	Range	Mean	Range	Mean	Range
[*methyl*]Thymidine	9.0	7–11	9.2	7–11	1.0	0.9–1.2
[*methyl*]Methionine	9.7	8–11	30.9	26–36	3.2	3.0–3.3

[a] The slow-moving band was isolated by composite gel electrophoresis from unfractionated DNA extracted by phenol or Marmur methods. [b] Percentage of the total cellular DNA contained by slow-moving band. [c] Percentage of the total radioactivity in the slow-moving band. [d] Relative specific activity defined as % dpm/% DNA in the slow-moving band.

tained in a French press), most of the electrophoresed DNA migrated in a multidisperse zone faster than the fast-moving band. The DNA in the slow-moving band incorporated [*methyl*-^{3}H]thymidine proportionally to its concentration (between 7 and 11%) as shown in Table I. Both the slow-moving and fast-moving bands were degraded by DNase but not by RNase or Pronase. Heat-denatured DNA was totally excluded from the gels. This was true even with heat-denatured and partially renatured mouse L cell satellite DNA (obtained about 85% pure after one hydroxylapatite fractionation). Gel electrophoresis, thus, may prove to be a valuable tool to evaluate qualitatively the extent of mismatching and incompleted reassociation of hybridized nucleic acid preparations.

Characterization and Identification of the Slow-Moving Band with Satellite DNA. (a) ELECTROPHORETIC ANALYSIS OF SATELLITE AND MAIN-BAND DNA. Whole body mouse DNA, extracted by the Marmur method, was centrifuged once to equilibrium in CsCl. The satellite and main-band DNA fractions were combined separately and electrophoresed on composite gels. Figure 1 shows the electrophoretic patterns of DNA obtained from the low-density (satellite) fractions (slots 2 and 3), DNA obtained from main-band fractions (slot 5), and DNA obtained from a region of intermediate density, slightly heavier than satellite DNA (slot 4) after CsCl centrifugation. The slow-moving fraction contained 82% (slot 2) and 84% (slot 3) of the total DNA derived from the satellite DNA fraction of two separate gradients (containing 54 and 81 μg of DNA, respectively), while no slow-moving band was resolved from main-band DNA (slot 5). One centrifugation in CsCl using less than 45 μg of DNA was reported by Flamm *et al.* (1966) to provide a low-density fraction containing about 90% satellite DNA. Thus, the finding that the satellite DNA fraction contained about 85% slow-moving band strongly supports the identity of satellite DNA and the slow-moving band. The absence of any slow-moving band in the main DNA fraction further strengthens this identity. Moreover, the intermediate region of the gradient contained about equal proportions of slow-moving band and fast-moving band, indicating that the diffusion of DNA at equilibrium was probably responsible in part for contaminating the satellite fraction with main-band DNA.

(b) BUOYANT DENSITY ESTIMATION OF DNA FROM THE SLOW-MOVING BAND AND FAST-MOVING BAND. Newborn mice were treated with [*methyl*-^{3}H]thymidine to label DNA which was then isolated by the phenol method. After electrophoretic separation of the labeled DNA, sections of the gel containing only the slow-moving band or fast-moving band were eluted electrophoretically from preparative polyacrylamide gels, as described in the Methods section. About 5 μg of labeled DNA (having approximately 16,000 dpm) from each of these bands was added separately to solutions containing 100 μg of unlabeled mouse DNA (extracted by the Marmur method) and 28 μg of *M. lysodeikticus* DNA, the latter serving as a density marker. The mixtures were then centrifuged to equilibrium in isopycnic CsCl gradients. The labeled DNA from the slow-moving fraction banded primarily at $\rho = 1.693$ (Figure 2), while the labeled DNA from the fast-moving fraction banded at $\rho = 1.701$ (Figure 3) (the buoyant densities of mouse satellite and main DNA, respectively). The presence in the slow-moving band of some DNA of density 1.701 g/cm^3 (Figure 2) was attributable to a large extent to the imprecision of slicing unstained gels under uv light and in part to trailing of the fast-moving band. The observations that almost all of the labeled DNA in the fast-moving band had a density equivalent to main-band DNA, while the DNA from the slow-moving band had a density equivalent to satellite DNA, confirmed the identification of the slow-moving band with satellite DNA and the identification of the fast-moving band with main-band DNA.

Metabolic Studies. Methylation of DNA. The high resolution obtained by composite polyacrylamide gel electrophoresis of DNA raised the possibility of examining some functional and metabolic properties of the two species of mouse DNA.

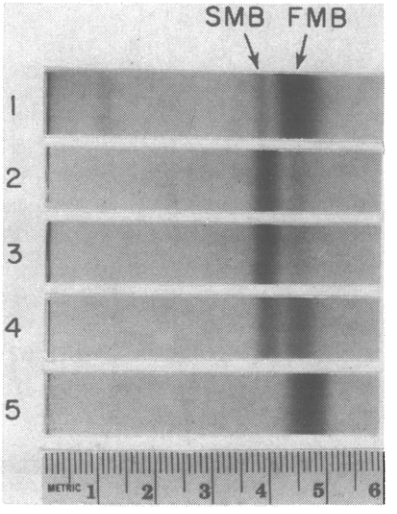

FIGURE 1: Electrophoregrams in 2.5% polyacrylamide–0.5% agarose gels of mouse DNA. The samples applied were satellite (slots 2 and 3), intermediate (slot 4), and main-band (slot 5) DNA fractions obtained from CsCl centrifugation gradients of whole body DNA, and unfractionated skin DNA (slot 1). (SMB and FMB are abbreviations for slow-moving band and fast-moving band.) (A trace of mucopolysaccharide appears at 11 mm in slot 1.)

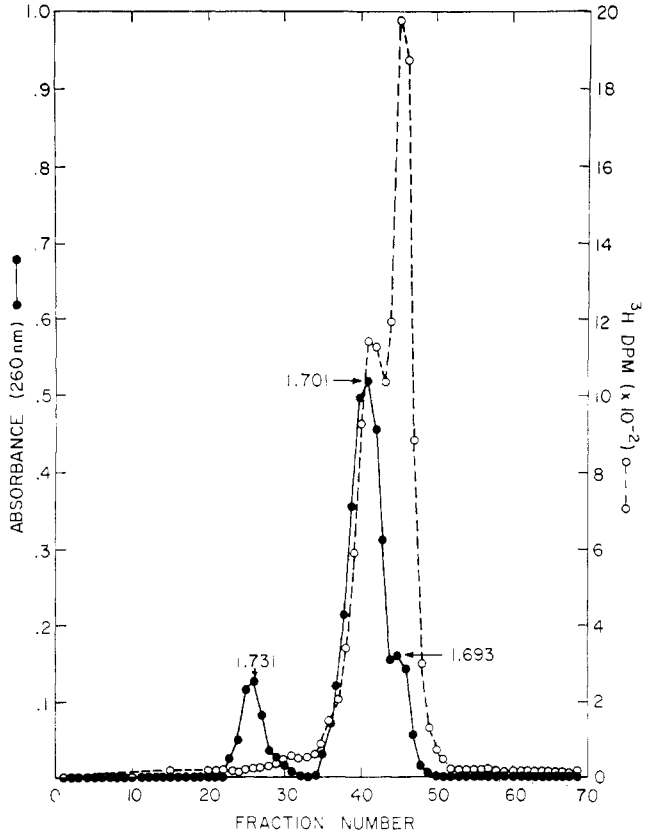

FIGURE 2: Fractionation of DNA from mouse slow-moving band in a preparative CsCl density gradient. Approximately 16,000 dpm (5 μg) of [*methyl*-^{3}H]thymidine-labeled DNA from slow-moving band plus 100 μg of unlabeled mouse DNA were centrifuged to equilibrium in CsCl. The peak on the left corresponds to DNA from the density marker, *M. lysodeikticus* (ρ = 1.731 g/cm^3).

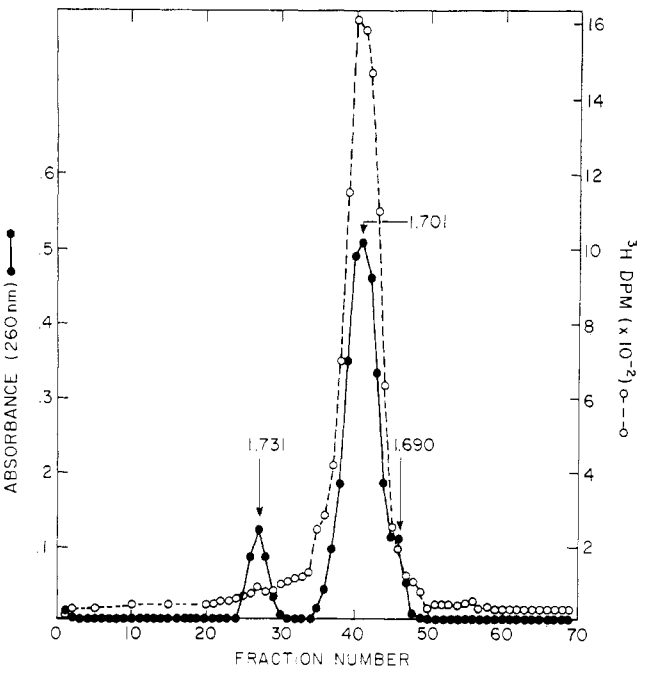

FIGURE 3: Fractionation of DNA from mouse fast-moving band in a preparative CsCl density gradient. Approximately 16,000 dpm (5 μg) of [*methyl*-^{3}H]thymidine-labeled DNA from fast-moving band plus 100 μg of unlabeled mouse DNA were centrifuged to equilibrium in CsCl. The peak on the left corresponds to DNA from the density marker, *M. lysodeikticus* (ρ = 1.731 g/cm^3).

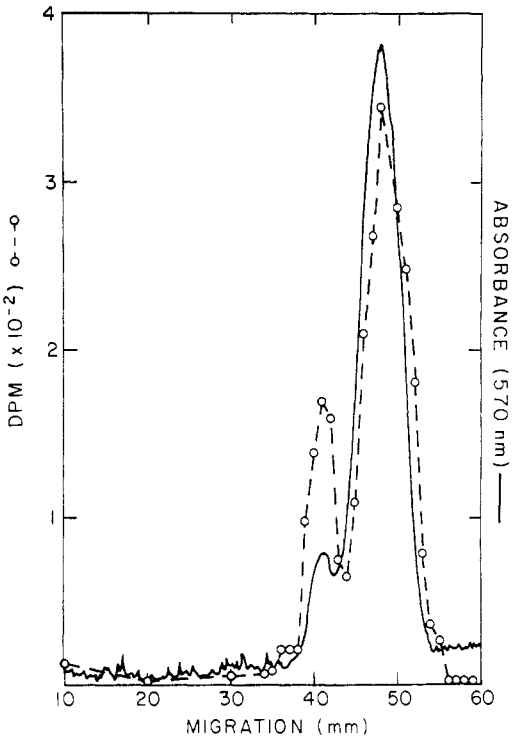

FIGURE 4: Methylation of mouse DNA. DNA was extracted by the phenol method from secondary embryo cultures 40 hr after incubation with 200 μCi of [*methyl*-^{3}H]methionine in medium deficient in methionine with added sodium formate (2 mg/ml) and electrophoresed on composite gels. Absorbance was determined by scanning stained gels by recording spectrophotometry at 570 nm; radioactivity was determined by counting 1-mm cut gel slices by liquid scintillation.

The methylation of DNA was chosen since previous studies showed that satellite DNA had a molar concentration of 5-methylcytosine more than twice as high as did main-band DNA (Salomon *et al.*, 1969). As described in the Experimental Section, secondary mouse embryo cultures were grown in methionine-deficient medium with added sodium formate. By diluting the one-carbon pool, sodium formate blocked incorporation of the one-carbon fragment of the methyl group of [*methyl*-^{3}H]methionine into DNA and thus permitted almost exclusive uptake of the label by transmethylation (Salomon *et al.*, 1969). Forty hours after [*methyl*-^{3}H]methionine administration, DNA was extracted from the cultured fibroblasts by the rapid phenol method. The satellite band (slow-moving band) averaged 9.7% of the total DNA, as determined by spectrophotometric scanning of gels. However, as seen in Table I and Figure 4, the satellite fraction incorporated between 26 and 36% of the total DNA label. The relative specific activity of [*methyl*-^{3}H]methionine incorporation (% dpm/% DNA) into satellite DNA was in all cases about three times higher than that of main-band DNA.

Discussion

This report demonstrates the fractionation of mouse DNA by electrophoresis on polyacrylamide gels into a minor slow-moving band and major fast-moving band. These two bands have been shown to be equivalent to the satellite and main-band DNA, respectively, as these are defined by isopycnic CsCl centrifugation. This conclusion is based on the demon-

stration that (a) satellite DNA isolated from the low-density region of the CsCl gradient migrated electrophoretically with the slow-moving band, while DNA from the main band migrated electrophoretically with the fast-moving band, and (b) the density of DNA isolated from the slow-moving band was the same as the density of satellite DNA, while the density of DNA from the fast-moving band was the same as main-band DNA. The effects of molecular weight, configuration, and base composition on the electrophoretic mobility of DNA in gels are now under investigation in an attempt to understand more fully the properties of DNA which permit its fractionation in gels.

The experiment describing methylation of DNA was selected to demonstrate the real potentialities of the fractionation of mouse DNA by polyacrylamide gel electrophoresis. In the first case, the preferential methylation of satellite DNA (Table I, Figure 4) was shown, confirming earlier studies (Salomon *et al.*, 1969). Additionally this demonstrated that the method was sensitive enough to discern metabolic differences between species of DNA using only microgram amounts of DNA.

These experiments show that composite polyacrylamide gel electrophoresis of mouse DNA provides a method with exceptional resolution to study microgram quantities of satellite and main-band DNA. In addition, multiple samples can be evaluated simultaneously with little additional effort. It may therefore provide a convenient, rapid, and inexpensive supplement to techniques currently available for the study of satellite DNAs.

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