dent of the fragment size thus making the reaction rate proportional only to (mol wt)$^{-1/2}$ (Hutton and Wetmur, 1973a; Chilton, 1973). Therefore, the results obtained by Sutton should not be generalized to apply to all redundant DNA renaturation kinetics. Nevertheless, in some interspecies DNA renaturation experiments where very large degrees of mismatching occur, it is necessary to consider a sizable decrease in rate of heterologous reaction over the homologous before attempting to quantitate the absolute degree of homology.

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Role of Base Composition in the Electrophoresis of Heat-Treated Deoxyribonucleic Acid from HeLa and Mouse Cells in Composite Polyacrylamide Gels†

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ABSTRACT: Heat-treated DNA from HeLa and mouse cells could be electrophoretically resolved into two components representing the native and denatured fractions. In 1.7% polyacrylamide–0.5% agarose gels heat-denatured DNA migrated in a discrete band of significantly slower mobility than did native DNA. Spectrophotometric gel scanning allowed a quantitation of the per cent denaturation at various temperatures and thus permitted calculation of a T_m, which agreed well with that determined by the optically measured thermal transition profile method of Marmur and Doty (1962). In addition, fully heat-denatured mouse satellite DNA electrophoresed as two discrete bands, presumably as a result of compositional differences between the complementary strands. The role of base composition in the electrophoretic behavior of DNA was studied by labeling the cytosine or guanine bases of HeLa DNA with tritiated nucleoside precursors while simultaneously labeling the thymine bases with thymidine-^{14}C. The ratio of $^{3}H/^{14}C$ incorporation within the native HeLa DNA band indicated a linear gradient in GC content. These studies further demonstrated the ability of polyacrylamide gel electrophoresis to fractionate DNA molecules on the basis of their base composition.

In a recent series of experiments we have examined some of the electrophoretic characteristics of DNA in polyacrylamide gels (Zeiger *et al.*, 1971, 1972a; Dingman *et al.*, 1972). It was found that high molecular weight DNA from the mouse and crab could be separated by polyacrylamide gel electrophoresis into satellite and main band DNA species. The separation was possible because the dA-dT rich crab and mouse satellite DNAs had lower electrophoretic mobilities than their corresponding main band DNAs. Subsequent studies using microbial DNAs of differing GC content showed that DNAs with progressively greater GC content had correspondingly greater electrophoretic mobilities in polyacrylamide gels. Within the range of base compositions studied (25–67% mole per cent G + C) the relationship between base composition and electrophoretic mobility was linear. In the present study we examined the electrophoretic behavior of DNA, denatured to a varying extent, and found that the native and denatured fraction could be easily resolved. We again found that the electrophoretic mobility of high molecular weight DNA is dependent on base composition. We also observed that polyacrylamide gel electropho-

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resis can separate the light (L) and heavy (H) strands of mouse satellite DNA.

Materials and Methods

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. Cesium chloride, optical grade, and adenosine-*8*-^{14}C (47 Ci/mol) were obtained from Schwarz/Mann. NCS, Spectrofluor, thymidine-*2*-^{14}C (57 Ci/mol), and cytidine-^{3}H (G) (2.5 Ci/mmol) were purchased from Amersham/Searle. Deoxycytidine-^{3}H (G) (5.49 Ci/mmol) and guanosine-^{3}H (G) (1.8 Ci/mmol) were obtained from New England Nuclear Corp. Unlabeled adenosine, guanosine, deoxyadenosine, and deoxyguanosine were all A grade and purchased from Calbiochem. Electrophoretic cells, the design of Raymond (1962), were the products of the E-C Apparatus Corp.

Double Labeling of the GC and AT Base Pairs in HeLa Cell DNA. To HeLa cells, Type S3-1 (0.7–2 × 10^6 cells/ml), grown in spinner flasks in 100 ml of Eagle's Minimal Essential Medium supplemented with 5% horse serum, and 100 units/ml each of penicillin and streptomycin, were added cytidine-^{3}H and thymidine-*2*-^{14}C to levels of 10 and 0.2 μCi/ml, respectively, and the unlabeled nucleosides adenosine, guanosine, deoxyadenosine, and deoxyguanosine each in a concentration of 25 μg/ml; 24 hr later the cells were harvested and DNA was extracted.

Isolation and Characterization of DNA. Unfractionated DNA from HeLa cells or 36-hr old whole C57Bl mice was extracted by a modified sodium dodecyl sulfate–chloroform–isoamyl alcohol technique as previously described (Marmur, 1961; Zeiger *et al.*, 1971). DNA was sheared to *ca.* 7–8 × 10^6 daltons (determined by zone sedimentation in linear sucrose gradients (Dingman, 1972)) by passing the DNA solution maintained at 4° ten times through a 27 gauge, 1 in. long needle (Zeiger *et al.*, 1972a). Mouse DNA (100 μg) was further fractionated by CsCl ultracentrifugation into satellite and main band fractions as described earlier (Brunk and Leick, 1969; Zeiger *et al.*, 1971). The separated satellite and main band DNA fractions were heat treated and analyzed by electrophoresis as described below.

The specific activities of HeLa DNA labeled with the ^{3}H and ^{14}C precursors were approximately 27,500 dpm of ^{3}H/μg of DNA and 4800 dpm of ^{14}C/μg of DNA; the ratio of $^{3}H/^{14}C$ for this double-labeled DNA was 5.7. This ratio ($^{3}H/^{14}C$) in HeLa DNA was taken to represent the relative proportion of label in the GC (labeled with cytosine-^{3}H) and the AT (labeled with thymidine-*2*-^{14}C) moieties. Fractions of HeLa DNA with a $^{3}H/^{14}C$ ratio greater than 5.7 indicates a relatively greater content of GC base pairs than in unfractionated HeLa DNA, while a $^{3}H/^{14}C$ ratio less than 5.7 indicates a relatively greater content of AT base pairs. The ratio $^{3}H/^{14}C$ was determined for both the entire DNA and for the various electrophoretic fractions. The "total DNA-weighted average" $^{3}H/^{14}C$ ratio was calculated from the sums of the total ^{3}H and ^{14}C counts in both the denatured and native DNA electrophoretic fractions. This value should be equal to the $^{3}H/^{14}C$ ratio of 5.7 in unfractionated DNA if there were no preferential losses.

^{14}C-labeled mouse DNA was the kind gift of Dr. M. Ishizawa.

Irreversible Thermal Denaturation of DNA. A DNA preparation (mouse or HeLa cell DNA) containing 100 μg of DNA/ml in 0.1 × SSC (SSC = 0.15 M sodium chloride–0.015 M sodium citrate (pH 7.0)) was heated in a thermostatically controlled water bath and after a 5-min equilibration at a given temperature, aliquots (300 μl) were removed and immediately quenched by placing in test tubes immersed in ice. To determine thermal denaturation profiles, 200-μl samples (20 μg) from the above quenched aliquots were diluted with 800 μl of 0.1 × SSC and the absorbance (*A*) of this solution at 260 nm at 25° was measured in a Beckman Model DU spectrophotometer (Geiduschek, 1962). For the electrophoretic fractionation the remaining 100-μl aliquots were diluted with 25 μl of a bromophenol–sucrose solution (Peacock and Dingman, 1968) and from 40 to 60 μl (*ca.* 3–5 μg of DNA) of this solution was applied to the composite gels.

Electrophoresis of DNA. Preparation of gels and electrophoretic techniques have been described elsewhere (Peacock and Dingman, 1968; Dahlberg *et al.*, 1969; Zeiger *et al.*, 1971). Specific electrophoretic conditions are described in the legends to the figures. Gels were stained with "Stains-all," destained in running tap water, and photographed immediately. Stained gel strips were scanned at 585 nm (the wavelength which was 96% of the maxima for both native and denatured DNA) in a Gilford 240 recording spectrophotometer with a Gel Scan attachment. Double-stranded polynucleotides, whether DNA or RNA, stain bluish, while single-stranded polynucleotides, whether DNA or RNA (and including denatured DNA), stain purplish to pinkish blue (Dahlberg *et al.*, 1969; Dingman *et al.*, 1972). The percentage of DNA in the denatured band (or native band) after treatment at different temperatures was assumed to be equal to the percentage of bound stain found associated with that band relative to the total stain associated with both the DNA bands (Zeiger *et al.*, 1971, 1972b). For radioactivity determinations, gel strips were cut into 1-mm slices, placed in counting vials containing 1.0 ml of NCS and 0.2 ml of 0.5 N NaOH, and incubated overnight. Toluene–Spectrofluor solution (10 ml) was then added (Zeiger *et al.*, 1971) and 18 hr later the vials were counted in a Beckman LS-250 liquid scintillation counter.

Chromatography of Bases in HeLa Cell DNA. ^{3}H- and ^{14}C-labeled HeLa cell DNA were hydrolyzed for 60 min in 70% perchloric acid at 100°. The bases were separated (using standards as markers) by descending paper chromatography on Whatman No. 1 paper for 24 hr using 2-propanol–concentrated HCl–water (170:41:39 by volume) as solvent (Kaye *et al.*, 1967). The radioactivity present in the bases was determined by placing *ca.* 35 1 × 4 cm paper strips in counting vials and incubating them with 0.5 ml of water for 4 hr and then overnight with agitation following the addition of 2.0 ml of NCS; 10 ml of a toluene–Spectrofluor solution was then added and the radioactivity counted in a Beckman LS-250 liquid scintillation counter. The distribution of ^{3}H and ^{14}C among the bases of HeLa DNA is shown in Table I. Almost 100% of the ^{14}C from thymidine-*2*-^{14}C was incorporated into the thymine moiety. Thus almost all ^{14}C counts in DNA represent only AT pairs. The incorporation of ^{3}H into GC pairs was slightly less specific. Approximately 90% of the ^{3}H from cytidine-^{3}H was incorporated into either cytosine (68%) or methylcytosine (22%). Swindlehurst *et al.* (1971) made similar observations. Although the 10% conversion of cytidine into thymine affects the absolute quantitation of ^{3}H in GC base pairs, it is small enough not to affect the qualitative changes in base composition that will be demonstrated in the experiments to follow. In these experiments the tritium label will be considered therefore to represent GC bases. Attempts to use other precursors of GC bases were unsuccessful.

TABLE I: Percentage Distribution of Label from Nucleoside Precursors into the Bases of HeLa Cell DNA.

Precursor	Guanine	Adenine	Cytosine	Methylcytosine	Thymine
Cytidine-^3H			68	22	10
Deoxycytidine-^3H[a]		1	11	<0.5	87.5
Deoxyguanosine-^3H[b]	97	3			
Thymidine-2-^{14}C					>99
Adenosine-8-^{14}C	40	60			

[a] Similar results were found by Firshein *et al.* (1967). [b] Depurination during heat treatment of the DNA caused loss of label.

Results

Electrophoretic Properties of Heat-Treated HeLa Cell DNA. Representative scans of electrophoretic patterns of heat-treated HeLa cell DNA are shown in Figure 1. On this 1.7% acrylamide–0.5% agarose gel there were two major DNA bands: a blue staining band at *ca.* 50 mm and a purplish band at *ca.* 23 mm. The DNA band at 50 mm is native undenatured

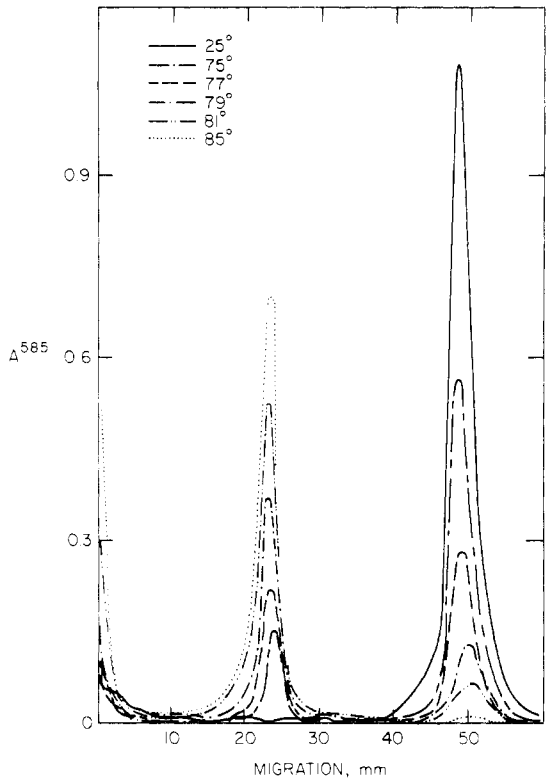

FIGURE 1: Electrophoregrams of heat-treated HeLa DNA on composite gels. HeLa DNA previously heated to various temperatures and then quenched was applied to the slots of a 3-mm slab gel in a large Raymond Model EC 490 vertical electrophoretic cell. The slab was composed of 1.7% polyacrylamide–0.5% agarose, catalyzed with *N,N,N',N'*-tetramethylethylenediamine (0.031% final concentration), and gelled overnight at 20°. A prerun at 200 V was performed while the temperature of the gel was lowered to 10° and the current stabilized. A Tris-EDTA–borate buffer (pH 8.3) was used both during the prerun and the electrophoresis but was not recirculated (Peacock and Dingman 1967). HeLa DNA was electrophoresed at 300 V and 85 mA (average) for 120 min with the circulating coolant at 0°. Gels were stained overnight with "Stains-all," placed in tap water to remove excessive stain, and then scanned at 585 nm. The temperature to which each DNA sample had been heated is noted on the figure. Native DNA stained blue and appears at *ca.* 50 mm; denatured DNA stained purplish and appears at *ca.* 23 mm.

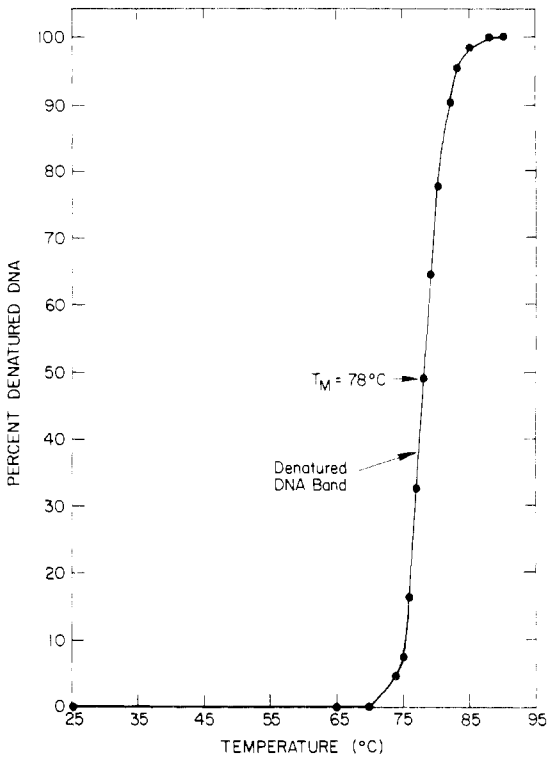

FIGURE 2: Thermal denaturation as measured by the percentage of denatured HeLa DNA found on electrophoresis of heat-treated HeLa DNA in 1.7% polyacrylamide–0.5% agarose gel at 200 V and 44 mA (average) for 3 hr (see Figure 1 for details). Stained gel strips were scanned at 585 nm in a Gilford recording spectrophotometer. The relative percentage of DNA within the denatured band was determined by quantitating the areas under the peaks of the tracings of the stained gel strips as described in the Methods.

double-stranded DNA as determined by its bluish coloration (see Methods) in "Stains-all" and by its presence as the only band when unheated HeLa cell DNA is electrophoresed (Figure 1, 25°). The band at *ca.* 23 mm represents heat-denatured DNA. It stained a characteristic purplish color and was found only after heating the DNA at temperatures greater than 71°. The electrophoresis of heated HeLa DNA showed that, as the temperature of the heat treatment was increased, there was a progressive increase in the amount of the denatured DNA and a concomitant decrease in the native DNA (Figure 1). Quantification of these and other gels showed that the percentage of DNA within the denatured band increased as a function of temperature (Figure 2). The temperature at which 50% of the DNA was denatured (T_m) was approximately 78° for this heLa DNA preparation in 0.1 × SSC. The "electrophoretic" T_m and the T_m which we found by the conventional spectrophotometric irreversible thermal denaturation method (Geiduschek, 1962) were identical.

Effect of Temperature on the Base Composition of Native Fractions of HeLa DNA. We have previously shown, using microbial DNAs of different base composition but similar molecular weight, that the electrophoretic mobility of DNA is linearly related to its GC content. Moreover the stability of the double helix has been correlated with the GC content of the DNA as measured by melting temperatures of native DNAs (Marmur and Doty, 1962). The following experiments examine the relationship between these two properties of DNA employing gel electrophoresis of heat-denatured HeLa DNA labeled in the GC moiety with tritium (employing cytidine-^3H as a precursor) and in the AT moiety with ^{14}C (employing thymidine-2-^{14}C as a precursor) (see Methods). The ratio of $^3H/^{14}C$ in

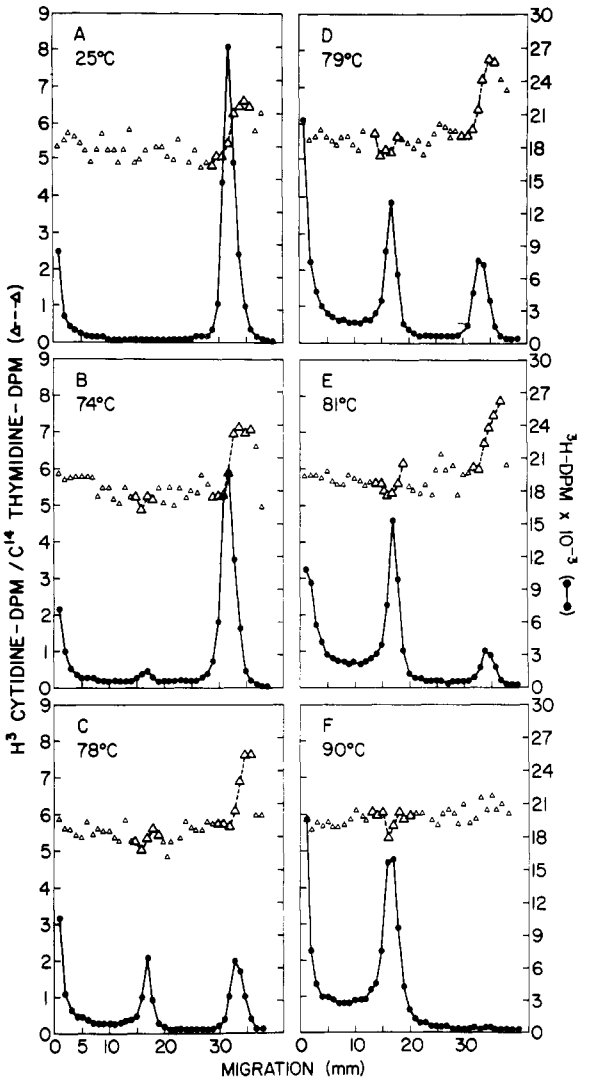

FIGURE 3: Ratio of incorporated cytidine-^3H to thymidine-2-^{14}C ($^3H/^{14}C$) within the electrophoretic bands of heat-treated HeLa DNA. Radioactivity was determined on representative samples of electrophoresed DNA (250 V, 140 min) by counting 1-mm cut gel slices by liquid scintillation. The $^3H/^{14}C$ ratio was measured along an entire gel strip. The large triangles represent significant ratios calculated from significant counts within each DNA band, while the smaller triangles are ratios computed from extremely low counts retained within the substance of the gel but not associated with any stainable DNA band. The distribution of incorporation of cytidine-^3H (●——●) is included to highlight areas of significant radioactivity.

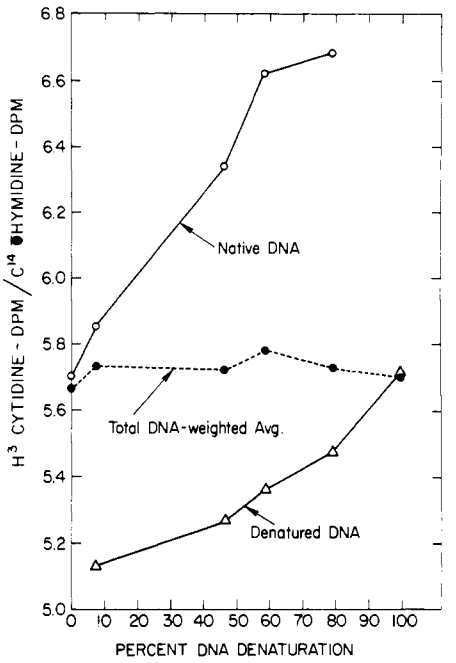

FIGURE 4: The relationship between the $^3H/^{14}C$ ratio and DNA denaturation. The average (mean) $^3H/^{14}C$ ratio for each electrophoretic band in Figure 3 was calculated and plotted against the per cent of denatured DNA.

the extracted, unfractionated HeLa cell DNA was 5.7. This double-labeled HeLa DNA was heated to various temperatures and analyzed electrophoretically, as described above. The gels were sliced into 1-mm sections, the ^3H and ^{14}C radioactivities in each section were counted, and the $^3H/^{14}C$ ratio wihin each section was calculated. The $^3H/^{14}C$ ratio and total ^3H radioactivity in the various electrophoretic bands was compared (Figure 3). The larger triangles represent ratios determined from significant ^3H counts; the smaller triangles represent ratios calculated from rather insignificant counts not associated with either stained DNA region. Accordingly, only the ratios depicted by the larger triangles are important here. The $^3H/^{14}C$ ratio increases within the native DNA band (at 30–35 mm) from about 5 in the trailing portion (30 mm) to about 7 in the leading edge (35 mm) of the DNA band (Figure 3A). This increase in the $^3H/^{14}C$ ratio was found along the native DNA band region in each of the heated samples (Figure 3). The average $^3H/^{14}C$ ratio within a DNA band was calculated by summing all the pertinent ^3H and ^{14}C counts, and forming the ratio. For native DNA this average ratio increased with increasing temperature of heat treatment from a low of 5.7 (original DNA ratio) to a maximum of 6.8 (Figure 4). Within each denatured DNA band the average $^3H/^{14}C$ ratio increased with increasing temperature of heat treatment from a low of 5.1 at 74° to a high of 5.7 at 90° (Figure 4). The internal consistency of these experiments is shown by the fact that the $^3H/^{14}C$ ratio for total DNA (determined by combining the ^3H and ^{14}C counts within both native and denatured DNA regions) for the various heat-treated DNA preparations was 5.7, equal to that found for both completely native and totally denatured DNA (Figure 4).

These data indicate that for high molecular weight HeLa DNA (1) denatured DNA has a slower electrophoretic mobility than native DNA; (2) within the band of native DNA electrophoretic mobility in composite gels is a function of GC content; (3) portions of the HeLa genome which melt (denature) at lower temperatures, as determined by this electrophoretic technique, are AT rich, in agreement with previous thermal denaturation studies (Marmur and Doty, 1962); and (4) portions of the genome rich in AT can be separated from the remainder by the electrophoretic fractionation of heat-treated DNA samples in composite gels.

Further Electrophoretic Fractionation of Mouse Satellite DNA. The species of HeLa DNA denaturing at 74°, present in very small amount, consistently contained a faint duplex band on composite polyacrylamide gel electrophoresis. We thought it possible that this denaturation duplex represented two complementary strands of an AT-rich satellite DNA, which might denature at a lower temperature than other, more GC-rich, fractions. This possibility was examined using mouse DNA which contains an AT-rich satellite DNA comprising 10% of the total DNA (Kit, 1961). We demonstrated previously that mouse DNA can be fractionated by polyacrylamide gel electrophoresis into a slower migrating satellite DNA band and a faster moving main band DNA.

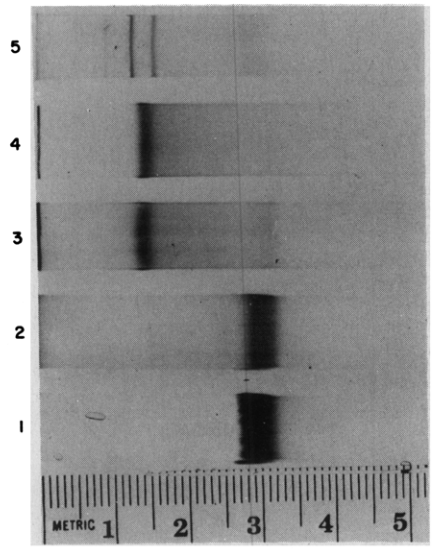

FIGURE 5: Electrophoregram of heat-treated mouse satellite and main band DNA in 1.7% polyacrylamide–0.5% agarose gels. The purified mouse fractions (obtained by isopycnic centrifugation in CsCl) were electrophoresed at 300 V and 35 mA (average) for 80 min in a Raymond Model EC 470 vertical electrophoretic cell. Samples: slots 1–4 are mouse main band DNA heated to 25, 73, 80, and 90°, respectively; slot 5, mouse satellite DNA heated to 73°. (A trace of mucopolysaccharide appears at 10 mm in slot 5.)

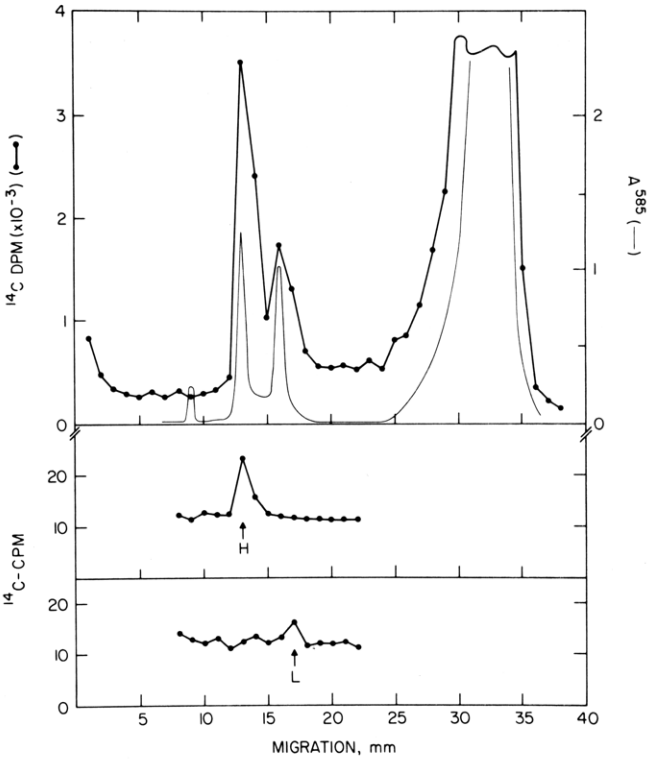

FIGURE 6: (Top) Mouse DNA (labeled with thymidine-2-^{14}C) was heated to 73° in 0.1 × SSC, denaturing the satellite DNA, but not the main band DNA, as shown in Figure 5 and analyzed by electrophoresis, as in Figure 2. The gel scanned at 585 nm (——) using a Disc integrator attachment to compute the areas under the three DNA peaks and then sliced for estimation of radioactivity (O——O). The two peaks at 13 and 16 mm are the denatured satellite DNA. (The small peak at 9 mm is a mucopolysaccharide; main band DNA is at 30–35 mm.) (Middle and bottom) Labeled mouse satellite DNA, isolated by isopycnic centrifugation, was separated into heavy (H) and light (L) strands by centrifugation on an alkaline CsCl gradient and, after dialysis, run in parallel with the gel shown in the top panel.

Purified mouse satellite and main band DNAs were isolated by CsCl fractionation as previously described (Zeiger *et al.*, 1971). These purified mouse DNA fractions were heated at various temperatures and electrophoresed on composite polyacrylamide gels (Figure 5). Heat-treated purified satellite DNA heated to 73° was converted to DNA which migrated in two discrete bands at 13 and 16 mm (Figure 5, slot 5), while at the same temperature, 73°, the purified main band was not altered (Figure 5, slot 2). At 80 and 90° purified mouse main band DNA electrophoresed in a band at *ca.* 14 mm (Figure 5, slots 3 and 4), intermediate in mobility to the two satellite DNA denaturation bands. To investigate the possibility that the separation of the denatured satellite bands might be an artefact resulting from the use of borate-containing buffer, these experiments were repeated using a different buffer (Tris-HCl 0.005 M, EDTA 0.0025 M, pH 7.4), with identical results.

These experiments demonstrate that in mouse the duplex denaturation bands are derived from the heat denaturation of satellite DNA. It seemed likely that the denatured satellite duplex represents the light and heavy complementary strands of which mouse satellite DNA is composed (Flamm *et al.*, 1969). Supportive of this suggestion is the finding that denatured main band DNA (which has a density intermediate between the L and H satellite strands (Flamm *et al.*, 1969) migrates electrophoretically between the two bands derived from denatured satellite DNA.

Two experiments were performed to find out which of these two electrophoretically separated bands corresponded to the more dense of the satellite strands. Balb/C3T3 cells were labeled by growing the cells for 2.3 days (approximately 2.3 generations) in medium containing thymidine-2-^{14}C and satellite DNA isolated by isopycnic centrifugation (Zeiger *et al.*, 1971). The heavy and light strands were separated by centrifuging the satellite DNA in an alkaline CsCl gradient (Hanawalt and Cooper, 1971). Fractions containing heavy or light strands were pooled, dialyzed, and subjected to electrophoresis, using conditions of Figure 5. Because the radioactivity was low, the counting was prolonged for 50 min, thereby achieving ±10% accuracy. No background correction was made. Clear evidence for a peak for the heavy fraction at the position of the slow migrating band is shown in Figure 6, bottom panels. The second experiment depends on the fact that the percentage of thymidine is 2.04 times greater in the heavy strand than in the light strand (Flamm *et al.*, 1967). The same thymidine-2-^{14}C labeled DNA was heated to 73°, at which temperature, see Figure 5, the satellite is essentially denatured completely, and the main band is essentially native. After electrophoresis the gel was scanned at 585 nm to estimate the amount of DNA in each of the two bands of satellite DNA and the main band DNA, and then sliced for radioactivity measurements (Figure 6, top panel). Both the radioactivity and absorbance in the composite satellite region (sum of both satellite DNA bands) were 10% of the total radioactivity and absorbance, in agreement with previous results. Considering only the region of the gel containing the satellite DNA, 70% of the radioactivity, but only 54% of the area, were associated with the slower band. The relative specific activity (% DPM/% DNA) of the slower and faster migrating bands were 1.3 and 0.65, respectively. Thus the specific activity of thymidine in the slower band is 2.0 times that of the faster band. The results of both experiments indicated that the more slowly migrating band of denatured satellite DNA is the strand having the higher buoyant density in CsCl, while the faster migrating band is the strand having the lower buoyant density.

Discussion

These experiments confirm and extend our previous findings

that the electrophoretic mobility of high molecular weight native DNA in composite gels is dependent in part on its base composition. Specifically, we have shown by labeling the cytosine or guanine bases of HeLa cell DNA with ^{3}H (employing cytidine-^{3}H or deoxyguanosine-^{3}H as precursors) while simultaneously labeling the thymine bases with ^{14}C (using thymidine-2-^{14}C as precursor) that there was an increasing gradient in GC content within the native undenatured DNA electrophoretic band, and to a lesser extent within the heat-denatured DNA band (Figures 1, 3, and 4). Moreover, native and heat-denatured DNA were sufficiently separated by electrophoresis, Figures 1 and 3, to permit calculation of a T_m for a particular species of DNA (Figure 2). Consistent with the findings of Flamm *et al.* (1969) that mouse satellite DNA fractionated in alkaline CsCl into two major peaks, our experiments demonstrate that heat-denatured mouse satellite DNA migrates electrophoretically as two discrete denaturation bands, representing the L and H complementary satellite strands (Figure 6). Similar results have been obtained with denatured phage DNAs using agarose gels by Hayward (1972). Unlike native DNAs which we have studied, the denatured H strand (*i.e.*, the denser fraction) migrated slower than the denatured L strand, indicating the importance of variables other than density in determining electrophoretic migration of denatured DNA. These experiments point out the potential of polyacrylamide gel electrophoresis for conveniently and rapidly separating and studying regions of the genome differing in GC content. It may ultimately permit dissection of an individual genome to the extent of uncovering those regions most susceptible to carcinogenic attack, hormone action, antigen binding, and perhaps even repressor control.

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