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Partial Denaturation of Mouse DNA in Preparative CsCl Density Gradients at Alkaline pH†

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ABSTRACT: A new technique—partial denaturation of DNA in equilibrium CsCl density gradients at pH 11.4—is used to determine the distribution of intermediate states in the melting of mouse DNA. When the technique is applied in the preparative ultracentrifuge, the DNA is fractionated according to stability. Neutralization of the partially denatured fractions results in the recovery of most of the DNA in its native form. The individual fractions are more homogeneous than the total DNA: they have decreased density heterogeneity (smaller band widths), neutral CsCl buoyant densities that differ from the average, and more homogeneous melting profiles with melting temperatures that differ from the average.

We have studied intermediate states in the helix-coil transition or "melting" of DNA by partial denaturation in analytical CsCl density gradients at alkaline pH (Wiesehahn et al., 1976). This method gives high resolution because the alkaline titration of DNA is accompanied by a large increase (58 mg/cm^3) in the DNA buoyant density (Baldwin and Shooter, 1963; Vinograd et al., 1963). Of the DNAs we studied, mouse DNA gave a particularly broad distribution of intermediate states during its denaturation. Here we use a preparative version of the same technique to fractionate the mouse genome according to stability. The same partially denatured DNA distributions seen in analytical CsCl gradients are reproduced in the preparative gradients.

After fractionation, the alkaline DNA solutions are neutralized. Any DNA molecules which have not been completely denatured (i.e., strand-separated) will still have their complementary strands in register. These molecules are expected to undergo rapid and complete "type I reversibility" (Geiduschek, 1962) or "rezippering" when the denaturing conditions are removed. To test this expectation, the reversibility of the partial denaturation is investigated by S_1-nuclease resistance, electron microscopy, and denaturation experiments performed on the isolated mouse DNA fractions.

The components of mouse DNA that are isolated by the partial denaturation technique are more homogeneous than the total genome. This increased homogeneity is evident both from neutral CsCl density gradient profiles and from melting profiles of the DNA fractions.

These experiments allow some conclusions to be drawn about the range of molecular weight at which base-compositional heterogeneity occurs in the mouse genome. In addition, the partial denaturation technique is a useful method for fractionating the heterogeneous mouse genome. It gives better resolution of density components than either neutral CsCl or Ag^+-Cs_2SO_4 density gradient centrifugation.

Materials and Methods

DNA Preparation. DNA was extracted from [^{3}H]thymidine-labeled SVT2 mouse tissue culture cells, obtained from Theodore Gurney, Jr. (University of Utah). Purification steps included chloroform-isoamyl alcohol extractions, ethanol precipitations, and Pronase and RNase treatments (Cech et al., 1973). The specific activity of the DNA preparation used in most of the experiments described here was 4.0×10^4 cpm/μg. It contained 10.2% satellite DNA, as determined by analytical CsCl density gradient centrifugation. The DNA had a native molecular weight of 6.2×10^7 and a single-stranded molecular weight of 1.5×10^7. A few experiments were done with Balb/c mouse DNA, isolated according to the procedure of Flamm et al. (1966a). Molecular weights were determined

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by neutral or alkaline sedimentation in the analytical ultracentrifuge as described in Wiesehahn et al. (1976).

Neutral CsCl Density Gradient Centrifugation. Centrifugation was performed at 40 000 rpm at 25 °C in the Spinco Model E. Solutions contained Harshaw optical-grade CsCl and 0.01 M Tris buffer–0.001 M EDTA , pH 7.5. Buoyant densities were measured relative to *Micrococcus lysodeikticus* marker DNA, which had a measured density of 1.733 g/cm^3 relative to that of *Escherichia coli* DNA, ρ_0 = 1.710 g/cm^3. Density differences between bands were calculated from the density gradient $(d\rho/dr)_{buoyancy} = 9.35 \times 10^{-10}\omega^2\bar{r}$ according to Schmid and Hearst (1971).

DNA Denaturation Curves in Analytical CsCl Density Gradients. DNA samples were dialyzed against 0.015 M NaCl–0.0015 M sodium citrate–0.001 M EDTA, pH 7.6. Solutions for centrifugation were prepared by mixing 0.460 ml of saturated CsCl, 0.050 ml of 0.5 M phosphate buffer (Na_2HPO_4–Na_3PO_4), 0.034 ml of H_2O, 0.011 ml of 0.05 M EDTA, and 0.050 ml of DNA. The DNA solution was added last, and the entire procedure was carried out in an ice bucket to avoid denaturation. The stated buffer ratios (acid/salt = Na_2HPO_4/Na_3PO_4) were used to give reproducible pHs. Measured pHs (Radiometer pH meter, Gk2321C electrode) are also stated. The precision of the readings was ±0.01 pH unit in a single experiment, with a reproducibility of ±0.05 pH unit over a period of many months.

Details of our methods for the determination of a DNA melting curve in an alkaline CsCl density gradient are given in Wiesehahn et al. (1976).

Partial Denaturation of DNA in Preparative CsCl Density Gradients. Solutions for preparative centrifugation were prepared exactly as described above for analytical samples, except that each volume was nine times larger. Each sample contained 1.2 $OD_{260nm}{}^{1cm}$ units of DNA, which was mixed with the alkaline CsCl solution at ice-bath temperature. Each sample (5.35 ml final volume) contained 0.001 M EDTA and 0.04 M phosphate buffer and had a refractive index of 1.4012 at 25 °C. This corresponds to a density of 1.720 g/cm^3, assuming that the buffer makes an insignificant contribution to the refractive index and density. Samples were mixed in polyallomer centrifuge tubes, which were then filled with Dow Corning 200 silicon oil. Centrifugation in the Spinco fixed-angle 65 rotor was at 40 000 rpm, 26.5 °C, for about 40 h. Accurate temperature control was provided by the Spinco L2-65B ultracentrifuge. A calibration experiment showed that the temperature indicated on the centrifuge was accurate to within ±0.2 °C. At the end of the centrifugation, before the rotor was allowed to coast to a stop, the temperature setting was reduced by 1 °C to protect against small temperature fluctuations that might occur during deceleration. Each tube was punctured with a sawed-off 22-gauge needle. The gradient was dripped through the needle, resulting in constant drop sizes and therefore constant fraction sizes. The fractions were allowed to stand for several hours at room temperature, which was 5 °C below the centrifugation temperature, and then neutralized with NaH_2PO_4. The purpose of the room temperature incubation was to allow partially denatured molecules to rezipper into their native conformation. This incubation treatment either worked perfectly or was unnecessary, because we never observed molecules containing partially denatured regions in neutralized samples. We avoided refrigeration of the fractions before neutralization, because refrigeration resulted in bimolecular renaturation of totally denatured strands. Such products would be mistaken for native DNA in an S_1-nuclease assay. For a DNA fraction that is half melted at 26.5 °C at high pH, refrigerator temperature corresponds to about T_m[1] −25 °C, and is therefore optimal for bimolecular renaturation (Marmur and Doty, 1961).

Nuclease S_1 Treatment. Our method for isolating and assaying the single-strand specific nuclease S_1 is described by Cech et al. (1973). In the present study, assay samples (0.20 ml) contained 0.30 M NaCl, 0.025 M potassium acetate buffer, pH 4.5, 0.0015 M $ZnSO_4$, 10 μg/ml heat-denatured calf thymus DNA, and 0.2–1.2 μg/ml of the ^{3}H-labeled mouse DNA to be assayed. Incubation was at 37 °C. In the experiments presented here, each assay sample contained 12 units (1 μl) of S_1 enzyme in 50% glycerol. When 6 units were used, the reaction were slower but reached approximately the same final value. With 36 units of enzyme, the digestion was at first rapid, followed by a slow linear decrease in Cl_3CCOOH precipitability with time. Extrapolation of this second phase of the reaction to time zero gave a level of S_1 resistance equal to the plateau level of the assays done with 12 units of S_1. The slow secondary reaction seen at high enzyme concentrations may be due to a contaminating nuclease activity.

Electron Microscopy. DNA was spread in 50% formamide according to the isodenaturing technique of Davis et al. (1971). Grids were stained with uranyl acetate and shadowed with 80% Pt–20% Pd. A Phillips 201 electron microscope was used.

Results and Discussion

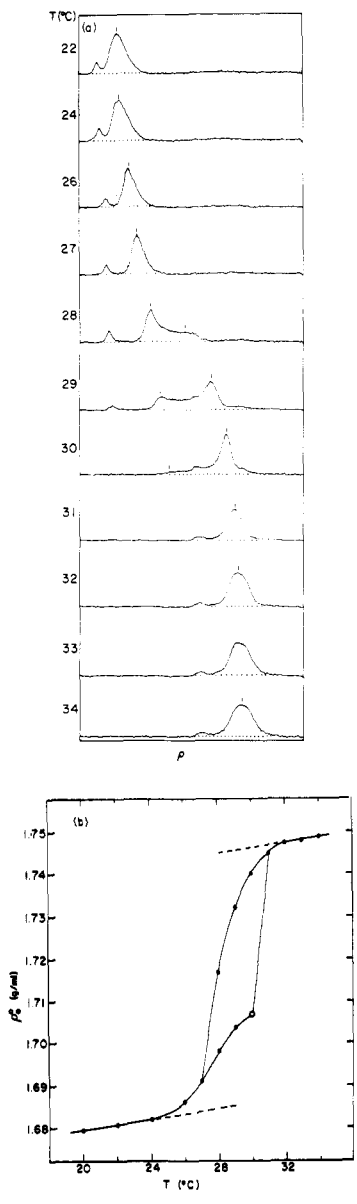
Thermal Denaturation of Mouse DNA in an Analytical CsCl Density Gradient at High pH. Figure 1a shows a series of equilibrium CsCl density gradients of total mouse DNA at high pH. The temperature range of 24–32 °C spans the helix–coil transitions for all components of the DNA. Even when the temperature is raised by fairly large increments, several partially denatured equilibrium distributions of the main-band DNA are seen. The satellite DNA melts in a much sharper transition than the main-band, but with about the same T_m. This is expected from the spectrophotometric melting curves of these DNAs (Flamm et al., 1966b; Corneo et al., 1968). There is evidence of some heterogeneity in the thermal stability of the satellite: at 29 °C, part of the satellite is fully denatured and strand separated while part is at its native density.

The peak maxima from Figure 1a are plotted in the form of a melting curve in Figure 1b. The density of a partially denatured DNA band, relative to the extrapolated densities of fully native and fully titrated DNA at that temperature, is almost directly proportional to the fraction of base pairs that have been titrated (Wang, 1974; Vinograd et al., 1968).

The standard type of DNA melting curve, with denaturation monitored by optical hyperchromicity, is shown in Figure 1c. The denaturation curve at pH 11.4 is similar to that at pH 6.8, except that in the alkaline solution the denaturation occurs at a temperature that is approximately 70 °C lower. The 10–90% transition breadths of the neutral and alkaline denaturation curves are similar: 4.5 ± 0.2 °C. The high ionic strength of the solutions results in these relatively sharp transitions (Doty et al., 1959).

Comparison of the density gradient melting curve (Figure 1b) with the optical melting curve (Figure 1c), done under the same conditions of ionic strength and buffer, reveals two major differences. (1) The 10–90% transition breadth for the denaturation of mouse DNA in an alkaline CsCl density gradient

[1] Abbreviations used: T_m, temperature corresponding to the midpoint of the helix–coil transition; A, adenine; T, thymine; G, guanine; C, cytosine; AT-rich and GC-rich, relative abundance of the indicated base pairs.



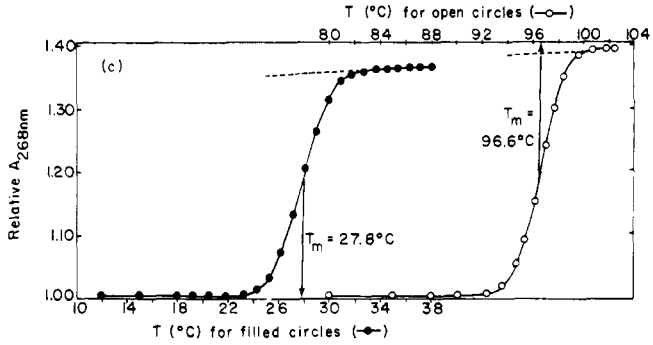


FIGURE 1: Thermal denaturation of mouse DNA in an analytical CsCl density gradient at pH 11.42 (acid/salt buffer ratio = 10/4.8). After each temperature change, about 16 h was required for the DNA to again reach equilibrium. The whole curve required 9 days of centrifugation. (a) Buoyant density profiles. Vertical lines designate peak positions, the densities of which are plotted in b. The light peak at 22–29 °C is untitrated satellite DNA. The fully titrated light strand of satellite can be seen in the 30–34 ° scans. The heavy strand is hidden under the main band, except in the 30 °C scan. The optical density (y axis) scale was adjusted to keep the peaks on scale; the total area of the scans increased during the melt due to hyperchromism. (b) Thermal denaturation graph. Filled circles designate the densities of the peak maxima from a. The open circle at 30 °C gives the density of the extreme edge of that distribution; little of the DNA is at this density. No marker DNA was used, the densities being calculated from initial solution densities as described in Wiesehahn et al. (1976). Because of the Na^+ in the buffer, these densities are 10 mg/cm^3 lighter than the neutral $\rho_0°$ values, which are determined relative to a marker DNA. Relative buoyant density shifts are more accurate than the absolute density scale because of the uncertainty in the initial solution density. The linear increase in the apparent buoyant density with temperature before and after the titration is due to expansion of the solution and dehydration of the DNA (Vinograd et al., 1965), for which no corrections have been made. (c) Optical melting curves of unfractionated, high molecular weight Balb/c mouse DNA. Absorbance at 268 nm and temperature were monitored by a Beckman DU spectrophotometer equipped with Gilford accessories. Both DNA samples contained 0.04 M phosphate buffer, 0.001 M EDTA, and CsCl to give a density of 1.72 g/cm^3. The data were normalized to the absorbance at low temperature and were not corrected for thermal expansion. (○) Phosphate buffered at pH 6.8; two samples gave almost identical melting curves so the average of the two is plotted here; 10–90% breadth = 4.3 °C. (●) Phosphate buffered at pH 11.4 (acid/salt buffer ratio = 10/5.4); a single determination is plotted; 10–90% breadth = 4.6 °C. The dashed lines show the extrapolation of the high temperature absorbance points; these lines were used in the calculation of T_m and 10–90% breadth.

is 3.3 ± 0.3 °C (average of five determinations ± maximum difference). This transition breadth is 1.2 °C sharper than that seen without the density gradient present. There is an instability gradient, probably a pH gradient, associated with the alkaline CsCl density gradient (Wiesehahn et al., 1976; Wang, 1974). Partial denaturation of the DNA results in its movement to a higher density, where it is less stable and therefore denatures further. The melting transition is thereby sharpened. (2) The optical melting curves give only the fraction of the base pairs in the whole sample that are denatured. At T_m, one-half of the base pairs are denatured, but the data give no information about the distribution of these denatured bases in the DNA. The density gradient melting curve, on the other hand, gives the distribution of partially denatured states present in the sample at each point in the transition. Near T_m (29 °C in Figure 1a,b), about one-half of the main-band DNA is 80% partially denatured, and a smaller fraction is about 30% denatured. About one-half of the satellite DNA is still native, while the other half is obscured by the main-band DNA and is therefore presumed to be largely or totally denatured.

Preparative Fractionation of Mouse DNA according to Thermal Stability. Figure 2 shows two preparative alkaline CsCl density gradient profiles of partially denatured mouse DNA. The profile shown in Figure 2a corresponds approximately to the 29 °C scan in Figure 1a, the profile in Figure 2b to the 30 °C scan. Six other preparative profiles (not shown) spanned the whole transition, from slightly to fully titrated.

The mouse DNA preparation included a broad range of fragment sizes, so it seemed possible that the distribution of partially denatured states might represent different molecular weight classes. To test this possibility, single-strand molecular weights were determined for the DNA in regions 2, 4, and 7 of Figure 2a, which had been 79, 55, and 24% denatured, respectively (Table I). These three samples were indistinguishable in molecular weight from the unfractionated DNA, which had never been exposed to alkali. Therefore, the melting profile is not significantly affected by fractionation according to molecular weight, and the DNA is not detectably degraded by the fractionation.[2]

[2] Degradation was observed in an earlier experiment done without EDTA in the solutions. In that experiment, three regions of a partially denatured distribution also had equal molecular weights, three times smaller than the initial single-strand molecular weight. The importance of EDTA was confirmed in analytical alkaline CsCl studies, where degradation was monitored by DNA band width.

Table I: Properties of Mouse DNA after Alkaline CsCl Fractionation.

Fraction[a]	% of DNA[b]	% ds in Gradient[c]	After Neutralization: Av $\rho_0°$ (CsCl)[d]	After Neutralization: $M_3^{app} \times 10^{-6}$ [e]	After Neutralization: % ds (S_1)[f]
Native, unfrac Gradient a			mb 1.698_7 (sat. 1.686_8)	2.1 (33.0)	100
1	9.2	0	1.720		5
2	15.6	21	1.698_3 (1.714_5)		59
3	19.4	33	1.698_5	4.9	79
4	13.9	45	1.699_5	10.3	[77][g]
5	13.1	55	1.701_6	6.5	95
6	14.6	64	1.703_6	3.9	98
7	11.1	76	1.703_9		96
	96.9				Wt av: 76 → 85[h]
Gradient b					
1	21.4	10	1.716_6 (1.699) (1.707)		29
2	35.5	24	1.702_2 (1.715)		56
3	17.6	41	1.702_4 (1.697)	5.6	[79][g]
4	10.1	55	1.704_4	7.6	97
5	9.1	69	1.707_0	4.5	97
	93.7				Wt av: 59 → 66[h]

[a] Gradients a and b are those shown in Figure 2. [b] Because the regions of each gradient that were analyzed did not include all fractions, the numbers add to less than 100%. [c] The percent double-strandedness of the DNA in the gradient was calculated as $[(\rho_D - \rho)/(\rho_D - \rho_N)] \times 100$, where ρ_D is the buoyant density of denatured, fully titrated mouse main-band DNA (1.750); ρ_N is the buoyant density of the native DNA (1.692). Each value has an expected maximum uncertainty of ±3 due to uncertainty in refractive index measurement. [d] Neutral buoyant density measured at band center, which equals the density of the peak maximum if the peak is symmetric. Reproducibility between two determinations was ±0.0002 for the satellite (sat.) density. Main band (mb) densities were identical. Similar uncertainties are expected for the high pH gradient fractions. Numbers in parentheses are for minor peaks. [e] Apparent molecular weight of the Na-DNA, calculated from band width according to Hearst and Schmid (1973). The values were not extrapolated to zero concentration, but were determined at similar concentration and are therefore useful relative values. Reproducibility for two determinations was ±0.1 for mb, ±2 for sat. Values are not given for distributions that included components with widely different densities. [f] Percent double-strandedness is calculated from the plateau level of S_1-nuclease resistance (Figure 4), with small corrections for the 2% S_1 susceptibility of native DNA and the 4% S_1 resistance of denatured DNA. Native DNA, 98% S_1 resistant, is defined as 100% ds. Precision of these measurements is ±2%. [g] These numbers are lowered because of the single-stranded satellite DNA (light strand) which bands in these gradient regions. If the denatured satellite is mostly degraded by the S_1 nuclease, the main band DNA in these fractions must be about 100% ds to give the observed values. [h] The weight average values of percent ds for each gradient are calculated from the data in columns 2 and 6. The arrows represent a correction for single-stranded satellite DNA.

It seemed most probable that the distributions of partially denatured states seen in the preparative gradients reflected heterogeneity in the average GC content of the DNA, in fragments with an average molecular weight of 6.2×10^7. The least denatured fractions should then have the highest GC contents and, if they rezippered upon neutralization, the highest neutral buoyant densities (Schildkraut et al., 1962; Flamm et al., 1969; Schildkraut and Maio, 1969).

Figure 3 shows neutral CsCl density gradients of the fractions from the two preparative gradients. The heaviest density fractions from the preparative gradients contain largely denatured DNA, $\rho_0° = 1.717$ for DNA of the average GC content. Fractions from less denatured regions of the gradients have largely native densities. The average buoyant densities of the fractions in neutral CsCl are an increasing function of the percent double-strandedness in the alkaline preparative gradients, as summarized in Table I. This is consistent with fractionation on the basis of GC content.

The neutral profiles of Figure 3a (1–7) were added, each weighted according to its percentage of the total DNA. The resulting distribution is given in Figure 3c. It fits the unfractionated DNA distribution ("N" in Figure 3) quite well. There are several differences: the satellite peak is missing since it has strand-separated and therefore cannot rezipper; the whole distribution is shifted to heavier densities by 2 mg/cm^3, reflecting complete melting of some AT-rich fragments; and the DNA that has been melted past the point of reversibility forms a peak at heavy density. The density distribution resulting from addition of the peaks in Figure 3b would be shifted even more from the native DNA distribution.

The Extent to Which the Partially Denatured DNA Reforms a Native Structure after Neutralization. Although many of the neutral CsCl profiles in Figure 3 have native-like densities, these may be shifted to higher densities if the DNA contains molecules with single-stranded gaps or tails. Such single-stranded regions could be produced by denaturing all of the DNA between two single-strand breaks, or between a break and the end of a molecule. (There was an average of two single-stranded breaks per molecule in the native DNA.) The double-strandedness of the neutralized fractions was investigated by treatment with the single-strand specific nuclease S_1, and by electron microscopy under conditions in which both single-stranded and double-stranded DNA can be visualized.

A sample of the S_1 nuclease data is given in Figure 4, and the results are summarized in Table I. Denatured DNA became almost totally acid soluble after 15 min of enzyme treatment, while the effect on native DNA was barely detectable after 30 min. DNA from each region of the preparative alkaline CsCl gradients showed a resistance to the enzyme that correlated with the amount of native DNA seen in the

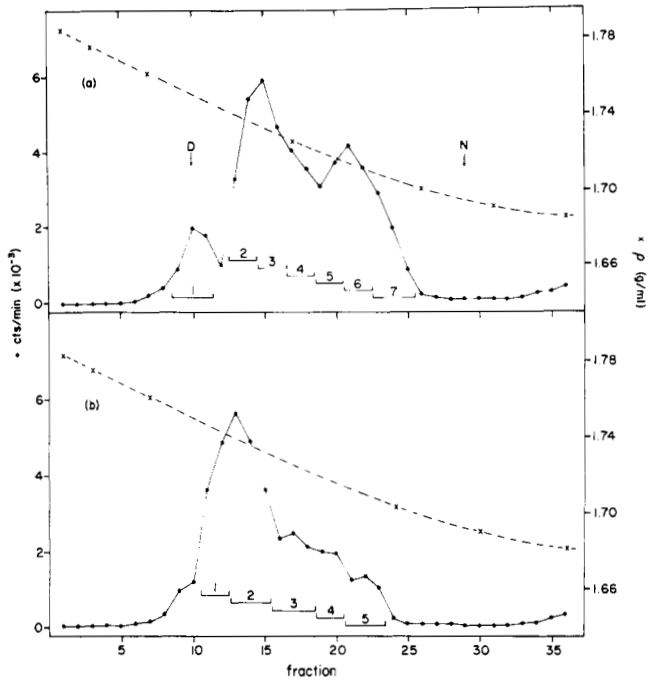


FIGURE 2: Isolation of partially denatured mouse DNA in preparative CsCl density gradients at high pH. (a) pH 11.40 (acid/salt buffer ratio = 10/4.8); (b) pH 11.42 (ratio = 10/5.1). (●) Mouse DNA, net ^3H cpm in a small aliquot of each fraction; (X) solution density at 25 °C, determined from refractive index; *D*, ρ = 1.750, density of fully titrated main-band DNA determined by a similar preparative gradient at pH 11.47; *N*, ρ = 1.692, calculated density of native main-band DNA. Fractions in various regions of each gradient were pooled for further analysis as indicated by the numbered brackets. Both gradients contain 10% strand-separated satellite DNA; the two strands are expected to have peak centers in fractions 9 and 17 of both gradients.

corresponding neutral CsCl density gradient. Some of the fractions contained separated single strands of satellite DNA, and a correction for this contribution was made. Fractions that were 60% or less denatured in the preparative gradients were 97 ± 2% resistant to S_1, and therefore must have rezippered very efficiently upon neutralization. Fractions that were more than 60% denatured showed increasing susceptibility to S_1. The results are graphed in the form of a reversibility plot (Geiduschek, 1962) in Figure 5.

Regions 5, 6, and 7 of Figure 2a and regions 4 and 5 of Figure 2b contained no significant amount of denatured main-band DNA when banded in neutral CsCl, yet were 2–5% single stranded as judged by S_1 nuclease treatment. Electron microscopy suggested an explanation for this low level of single-strandedness. There was an increased frequency of molecules with single-stranded tails in the DNA that had been fractionated at high pH. (See Figure 6 and Table II.) Tails were also seen in the unfractionated DNA, but were less frequent and considerably shorter than those seen in the DNA that had been partially denatured. Internal single-stranded gaps were seen in all of the DNA samples, but their frequency never exceeded 14% of the "ds with ss tails" category.

The electron microscopy showed no evidence of partially zippered strands, e.g., double-stranded regions that forked into two single strands, each stabilized by intrastrand base pairing. Thus, type I reversibility of DNA denaturation is an all-or-nothing process: molecules that are still in register recover all of their double-strandedness (in the absence of single-strand breaks) when melting conditions are reversed. These observations are consistent with the conclusions of Geiduschek (1962).

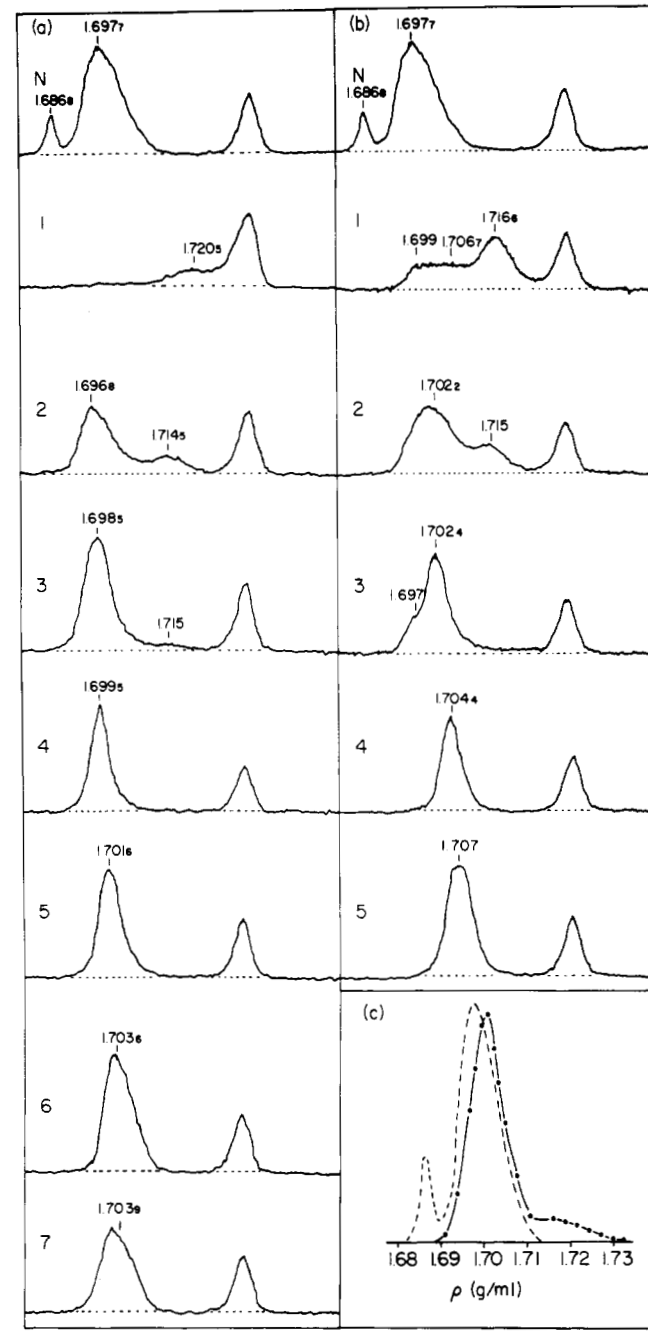


FIGURE 3: Neutral CsCl density gradient profiles of mouse DNA fractionated by partial denaturation. (a) Regions of Figure 2a. (b) Regions of Figure 2b. N, the native, unfractionated total mouse DNA, included for comparison. The heavy density peak in each scan is marker DNA, ρ_0° = 1.733. (c) The sum of the seven fractionated DNA profiles in a, each weighted according to its contribution to the total as given in Table I. (●) The fractionated DNA, including the points used in the calculation; (- - -) the unfractionated DNA profile, as in N above.

The effect of S_1 nuclease on one DNA fraction, region 3 of Figure 2b, was examined in more detail. After S_1 treatment, electron microscopy showed no evidence of single-stranded DNA—tails, gaps, and denatured single strands had been totally degraded. The molecular weight of the remaining double-stranded regions was not noticeably smaller than that of the untreated DNA. The same results were found when the DNA was treated with a threefold higher concentration of S_1 enzyme. Thus, partially denaturing and then neutralizing the DNA did not produce large numbers of S_1-sensitive regions

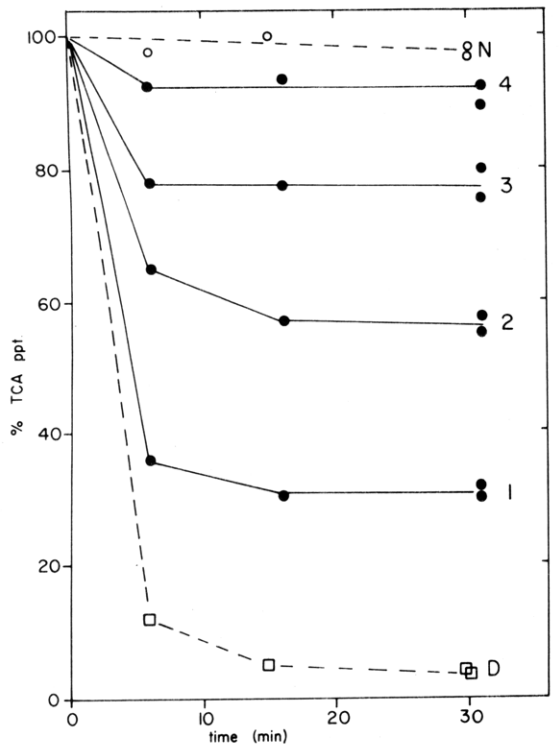


FIGURE 4: S_1 nuclease assay of the double-strandedness of mouse DNA fractionated by partial denaturation. Resistance to the nuclease is taken as the percent of the initial Cl_3CCOOH (TCA) precipitable cpm that remained Cl_3CCOOH precipitable after S_1 treatment. Zero time values were determined on duplicate samples, either incubated at 37 °C for 30 min or not, but with no S_1 added. These duplicates differed by an average of 4%, so each point has an uncertainty of about ±2%. (- - O - -) Native, unfractionated mouse DNA; (- - □ - -) same DNA denatured at low ionic strength by heating in a boiling water bath for 10 min, followed by quenching in ice. The 4% S_1 resistant DNA in this sample is expected from intramolecular renaturation of foldback DNA (Cech et al., 1973; Wilson and Thomas, 1974). (— ● —) DNA from regions of the high pH preparative gradient in Figure 2b. The fractions were neutralized and dialyzed before the assay.

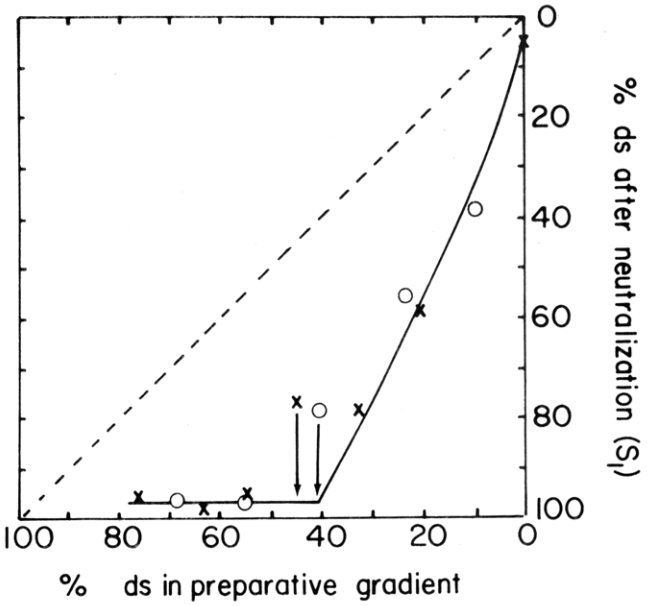


FIGURE 5: Reversibility of the partially denatured mouse DNA fractions. The line (- - -) represents the curve expected for no reversibility—i.e., if DNA that was 50% denatured in the preparative high pH gradient remained 50% double stranded after neutralization. (X) Regions of the gradient in Figure 2a; (O) in Figure 2b. Arrows correct for the presence of single-stranded mouse satellite DNA in two of the fractions.

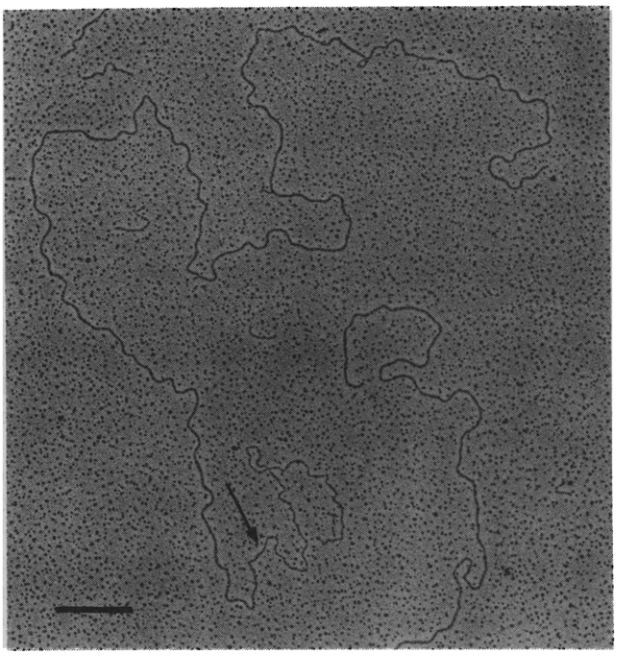


FIGURE 6: Formamide electron microscopy of partially denatured DNA after neutralization. DNA is from region 2 of Figure 2a. The arrow indicates the approximate beginning of a single-stranded tail on a predominantly double-stranded molecule. The bar has a length corresponding to a DNA molecular weight of 1.0×10^6 double stranded, 0.6×10^6 single stranded, determined by measurement of circular phage DNAs on separate grids.

in addition to the single-stranded regions identified by electron microscopy.

The neutral CsCl density gradient profiles of this DNA fraction before and after S_1 treatment are shown in Figure 7. The light shoulder, thought to be the light strand of the satellite DNA, disappeared after S_1 treatment. The buoyant density of the major peak decreased by 0.6 mg/cm^3, which corresponds to 4% of the density difference between native and denatured DNA. Therefore, the partially denatured DNA reformed an average of 96% base pairs upon neutralization. This conclusion is supported by the small number of single-stranded gaps and tails seen in this DNA prior to S_1 treatment (data not shown).

The conclusions of this section can be summarized as follows: (i) the neutral CsCl buoyant densities of the rezippered DNA are essentially native buoyant densities, although they may be slightly increased due to single-stranded tails and gaps; (ii) DNA obtained from a region of the alkaline gradient that corresponds to ≤60% denaturation is most similar to native DNA since the denaturation is about 97% reversible up to that point.

Melting Curves of DNA Fractionated by Partial Denaturation. The DNA from two regions of Figure 2a, representing early-denaturing (region 3) and late-denaturing (region 6) sequences, was melted in analytical alkaline CsCl gradients to determine how much homogeneity had been gained by the fractionation. The alkaline CsCl gradient method was preferred over spectrophotometric melting, because it gives the distribution of partially denatured states as well as the T_m. The results are plotted in Figure 8. During the melt, both fractions showed a smaller range of partially denatured states than the unfractionated DNA. The late-melting DNA (region 6) remained very homogeneous during the early titration, and later formed a broad peak at the same density as the late-melting

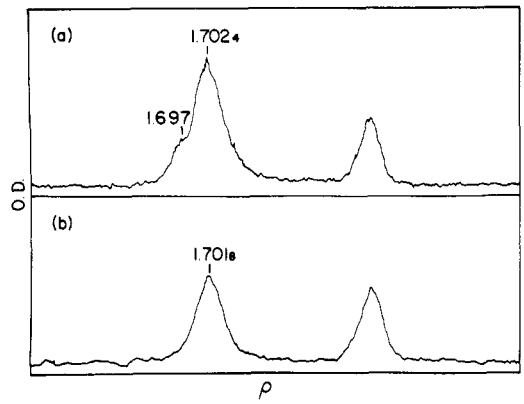


FIGURE 7: Neutral CsCl density gradient profiles before and after S_1 nuclease treatment. DNA is from region 3 of Figure 2b. (a) Untreated; (b) treated with S_1 for 30 min (76 ± 1% resistant, duplicate samples), then extracted with chloroform-isoamyl alcohol and dialyzed before centrifugation.

Table II: Electron Microscopy of Partially Denatured DNA Fractions after Neutralization.[a]

Fraction	n^b	% of Molecules by Wt[c] ds	ds with ss Tails or Gaps	ss	% ds (em)[d]	% ds (S_1)[e]
Native, unfrac	117	85	14	1	99	100
Gradient a, region 2	291	29	44	27	64	59
a, region 5	132	66	33	1	96	95
b, region 4	119	73	23	4	94	97

[a] Abbreviations: ds, double stranded; ss, single stranded; em, electron microscopy. [b] Number of random molecules scored. [c] Calculated from number-percent data by assuming that ss molecules contained ¼ the DNA of molecules in the other two categories, because the ss molecular weight of this DNA preparation was ¼ of the ds molecular weight. [d] Calculated from "% of molecules by weight" by assuming that ds molecules were 100% ds, that ss molecules were 0% ds, and that ds molecules with ss tails or gaps were 80–100% ds, depending upon the extent of partial denaturation of the fraction. [e] From Table I, included for comparison.

shoulder seen in the melt of the total DNA (Figure 1, 29–30 °C). The region 6 sample had a T_m that was higher by 0.6 °C than the average for the unfractionated DNA. This corresponds exactly to the late part of the unfractionated DNA melt. The region 3 DNA had a T_m that was 2.4 °C lower than the average. This is about 0.8 °C lower than expected for the early part of the unfractionated DNA melt. Partial single-strandedness of this sample (which had been 67% denatured in the preparative gradient) could be the cause of its slightly premature melt.

Distribution of Partially Denatured States as a Function of Molecular Weight. The preparative alkaline gradient experiments were performed at a high molecular weight, and distinctive partially denatured distributions were seen. Shearing the DNA could conceivably separate AT-rich regions from GC-rich regions, allowing them to melt independently. This might give noticeably altered partially denatured profiles in an alkaline CsCl melting experiment. Spectrophotometric melting curves would of course be insensitive to these changes in seuqence linkage.

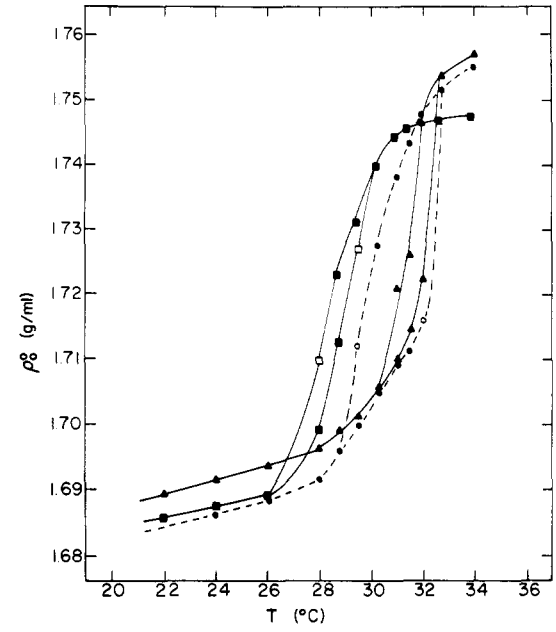


FIGURE 8: Thermal denaturation curves of mouse DNA fractionated by partial denaturation. Thermal denaturation was done in alkaline CsCl density gradients as in Figure 1. The three samples were dialyzed against the same buffer and diluted into the same CsCl-phosphate buffer stock solution (pH 11.35, acid/salt buffer ratio = 10/4.8) before centrifugation. As explained in Figure 1b, the densities plotted here are expected to be 10 mg/cm³ lighter than the neutral $\rho_0°$ values. (- - ● - -) Unfractionated total mouse DNA, neutral $\rho_0° = 1.697_7$ g/cm³. (— ■ —) DNA from region 3 of Figure 2a, neutral $\rho_0° = 1.698_5$ g/cm³. (— ▲ —) DNA from region 6 of the same gradient, neutral $\rho_0° = 1.703_6$ g/cm³. Only main-band DNA peak positions are plotted. Open symbols refer to minor components, as in Figure 1b.

Table III: The Thermal Denaturation of Mouse DNA in Alkaline CsCl Gradients as a Function of Molecular Weight.

Source of mouse DNA	ds Mol Wt. (×10⁻⁶)	ss Mol Wt (×10⁻⁶)	T_m (°C)	10–90% Breadth (°C)
SVT2[a]	62	15	28.6	3.3 ± 0.3
Balb/c[a]	nd[d]	5.2	28.7	3.2 ± 0.3
Balb/c[b]	nd	4.9	28.8	3.0 ± 0.3
Balb/c[c]	nd	1.9	28.6	3.5 ± 0.3

[a] DNA not deliberately sheared. [b] DNA sheared by forcing the solution through a 22-gauge needle at maximum thumb pressure, using a 1-ml plastic tuberculin syringe. [c] Sheared with a 27-gauge needle. [d] nd, not determined.

Four DNA samples were prepared, with single-strand molecular weights ranging from 1.5×10^7 to 1.9×10^6. The results of the melting experiment on these samples are summarized in Table III. The T_m's of the samples were equal within experimental error. The distributions of partially denatured DNA were virtually identical for the four samples, and looked the same as those seen in the many previous experiments with mouse DNA. Therefore, reducing the DNA molecular weight from 6.2×10^7 to 4×10^6 (double-strand) does not result in further scission of early-melting from late-melting sequences.

Further Discussion

Heterogeneity of Mouse DNA with Respect to Thermal Stability. The helix-coil transition of both procaryotic and eucaryotic DNAs occurs over a wide temperature range

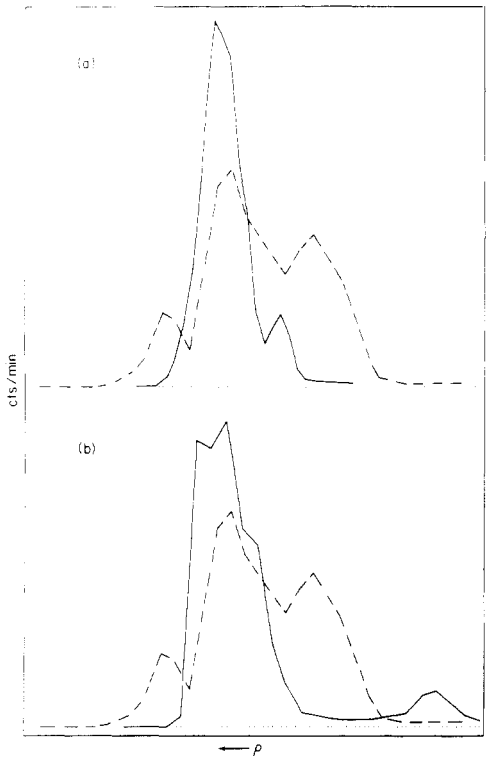


FIGURE 9: The resolution of total mouse DNA into density components in three types of preparative density gradients. (a) DNA banded in neutral CsCl gradient (—); alkaline CsCl gradient from Figure 2a (- - -). (b) DNA banded in Ag^+-Cs_2SO_4 gradient according to Corneo et al. (1968) (—); alkaline CsCl gradient from Figure 2a (- - -). Baseline (. . .) indicates zero cpm above background. All centrifugation was in the fixed angle 65 rotor, at 40 000 rpm for the alkaline CsCl gradient and at 35 000 rpm for the neutral CsCl and Ag^+-Cs_2SO_4 gradients. The three distributions are normalized to the same number of fractions per gradient and to approximately the same total area.

(Marmur and Doty, 1959). This could be due to base-compositional heterogeneity at any of several levels. For instance, regions containing hundreds of base pairs could have base compositions significantly different from the average, as in bacteriophage λ DNA (Inman, 1966; Miyazawa and Thomas, 1965). The large amount of density heterogeneity seen when high molecular weight eucaryotic DNA is banded in CsCl density gradients (Meselson et al., 1957; Sueoka et al., 1959) suggests that there are much larger regions of differing base composition.

Unbroken phage DNA melts homogeneously in alkaline CsCl gradients: all molecules go through the same partially denatured states, although the melt is broadened depending upon the amount of intramolecular base compositional heterogeneity (Wiesehahn et al., 1976). Mouse DNA, on the other hand, contains classes which exhibit a wide range of melting transitions. This heterogeneity with respect to thermal stability is observed even at the high DNA molecular weight of 6.2×10^7. Therefore, fragments of DNA long enough to contain about 100 average genes (assuming 1000 base pairs per gene) have thermal stabilities quite different from the average for total mouse DNA. When these fragments are sheared about 15-fold, the distribution of partially melted states shows little change. This suggests that sequences of a given base composition are clustered (tandemly arranged) in the genome, forming much longer blocks of the same average base composition. Such a view is supported by the work of Thiery, Macaya, and Bernardi (Thiery, 1974). They observed that the density components which make up many mammalian main-band DNAs do not vary over a molecular weight range of $(1\text{–}65) \times 10^6$.

The large regions of similar base composition, which have a more homogeneous distribution of partially denatured states than total mouse DNA, must still contain a shorter range segregation of bases into GC-rich and AT-rich regions. This can be seen from the alkaline CsCl melt of total mouse DNA (Figure 1a,b)—except for the satellite, the density components of the total DNA melt over a broad temperature range. When isolated density components are remelted in alkaline CsCl (Figure 8), the transitions become more homogeneous but only slightly sharper (i.e., smaller in 10–90% breadth). Each density component melts like unbroken phage DNA containing intramolecular base-compositional heterogeneity. From the work presented here, the upper limit for this shorter range segregation is $\sim 4 \times 10^6$ molecular weight. The work of Cohen and Crothers (1970) gives a smaller upper limit for the case of calf DNA. They find that fragments with a molecular weight of 1×10^5, fractionated according to GC content, still have broad melts.

Fractionation of Mouse DNA in Preparative CsCl Density Gradients at High pH. The base-compositional heterogeneity seen here by the partial denaturation of mouse DNA in alkaline CsCl gradients is expected from the broad band formed by the DNA in neutral CsCl. Previous workers have used neutral CsCl density gradients to fractionate the bulk DNA from higher organisms into buoyant density classes (Flamm et al., 1969; McConaughy and McCarthy, 1970). Mouse DNA and many other eucaryotic DNAs have a neutral CsCl gradient profile that extends over a 15–20 mg/cm^3 density range, excluding satellite components. Partial denaturation in alkaline CsCl gradients spreads the same DNA over a 58 mg/cm^3 range, giving much better resolution of components. The two types of gradients are compared in Figure 9a. The partially denatured profile used in this comparison is the one obtained near the T_m of mouse DNA; other partially denatured profiles would allow better resolution of very early-melting and very late-melting mouse DNA components.

Centrifugation of DNA in Ag^+- and Hg^{2+}-Cs_2SO_4 gradients (Nandi et al., 1965; Corneo et al., 1968; Filipski et al., 1973) and antibiotic CsCl gradients (Kersten et al., 1966; Brown et al., 1971; Peacock et al., 1974; Hearst et al., 1974) has been widely used to separate different DNA components. They give improved resolution over that obtained in neutral CsCl gradients. As shown in Figure 9b, partial denaturation in an alkaline CsCl gradient has much higher resolution for mouse main-band DNA than Ag^+-Cs_2SO_4, although the latter gives better resolution between the satellite and main-band DNAs.

Although partial denaturation in alkaline CsCl gives better resolution than the other techniques discussed above, it does have two major disadvantages. The first is the sensitivity of the extent of denaturation to the pH. A pH change of 0.1 corresponds to a 6.5 °C shift in T_m, which is greater than the 10–90% breadth of the mouse DNA melt in alkaline CsCl gradients. Therefore, accurate reproducibility of pH is required. In our experience, volumetric mixing of 0.5 M Na_2HPO_4 and Na_3PO_4 stock solutions gives more reliable pHs than adjustments made using a pH meter. In preparative isolations, a useful initial experiment is the centrifugation of a series of samples in the same rotor, each differing by about 0.02 in pH. The desired partially denatured profile can then be reproduced in subsequent experiments by using the same buffer ratio.

The alkaline CsCl technique is also at a disadvantage for applications in which strictly native DNA is required. The

more denatured regions of the gradient may contain DNA that has strand-separated and, therefore, cannot rezipper upon neutralization. If the DNA initially contains single-strand breaks, fractions from less denatured regions of the gradient may contain single-stranded tails and gaps after neutralization. In the experiments described here, only 2–5% of such DNA was single stranded by the S_1-nuclease criterion. Therefore, this fractionation technique is most useful for isolating GC-rich, late-melting sequences in native form.

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