EFFECT OF ALKALI ON THE SIZE DISPERSITY OF MAMMALIAN DNA MEASURED BY FILTER ELUTION

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ABSTRACT DNA from unirradiated and irradiated cultured 9L rat brain tumor cells was held for varying times in low ionic strength solutions at pH 11.0, 12.3, or 12.9. The effect of this exposure to alkali on the DNA size distribution was determined by comparing the DNA filter elution profiles obtained experimentally with those theoretically predicted for monodispersed and random distributions. At pH 12.3 or 12.9, DNA from cells irradiated with 300 rad eluted with first-order kinetics corresponding to a random DNA size distribution. The median size of the distribution decreased if the irradiated DNA was exposed to pH 12.3 for 24 h. At pH 12.3 or 12.9, DNA from unirradiated cells eluted initially with complex kinetics that later became linear (18–21 h for pH 12.3 or 13–15 h for pH 12.9), characteristic of a monodispersed DNA size distribution. Holding either unirradiated or irradiated DNA at pH 11.0, below the critical unwinding pH, produced no effect on the elution profiles. Analysis of these filter elution data indicated that after sufficient exposure to pH 12.3 or 12.9, undamaged DNA molecules from mammalian cells elute as a single-stranded monodispersed size distribution of $\sim 1 \times 10^{10}$ daltons. While the possibility cannot be completely eliminated that this monodispersed size represents an upper limit determined by physical forces, these results, in conjunction with those obtained using other techniques, lend credence to the existence of a nonrandom higher-order structure in mammalian chromosomal DNA.

INTRODUCTION

While mammalian chromatin structure at the level of the nucleosome is rapidly becoming defined and information is accumulating on how nucleosomes are organized into 100-Å filaments, the higher-order structure of mammalian chromosomal DNA still remains a matter of conjecture (1–3). The average mammalian chromosome contains $\sim 1 \times 10^{11}$ daltons of DNA. It has been proposed that this DNA may exist as an array of high molecular weight subunits joined through either DNA or non-DNA linkages (4–22). The largest of these subunits have been estimated to have single-stranded molecular weights of 1×10^8–1×10^{10} daltons (5, 12–16, 18–20). The primary limitation to the complete acceptance of this proposal has been the lack of techniques capable of (*a*) quantitatively measuring the size distribution of high molecular weight DNA that may be as large as 1×10^8 to 5×10^{10} daltons (23, 24) and (*b*) making measurements that are not ultimately determined by essentially the same hydrodynamic properties of the DNA.

The principal methods previously used to provide size measurements of mammalian DNA have been alkaline sucrose gradients (10, 25, 26), neutral sucrose gradients (17–19), or viscoelastometry (20, 24). Alkaline or neutral sucrose gradient analysis is subject to numerous physicochemical complications such as anomalous sedimentation due to turnover effects, droplet sedimentation, gel formation, or speed dependence that often become a problem with DNA molecules that are larger than 1×10^8 daltons (14, 23, 27–29). This makes it difficult to correctly quantitate their strand length or molecular weight. Although independently sedimenting nonlinear chain polymers are extremely useful for quantitating damage induced by low doses of drugs or radiation (30–34), there is no known mathematical relationship between their sedimentation coefficients and their strand length or molecular weight (34–36). The viscoelastic retardation time (τ) is determined predominantly by the largest molecules in the solution so viscoelastometry does not readily measure alterations in a continuous size distribution of DNA molecules. In addition, gel formation and nonlinear chain polymers can subtly influence interpretation of viscoelastometry data.

Size measurements of high molecular weight DNA (1×10^8–4×10^{10} dalton) obtained by alkaline sucrose gradients, neutral sucrose gradients, and viscoelastometry are also based on essentially the same hydrodynamic properties of the DNA, so these estimates may be subject to errors of similar type and magnitude. For example, due to

the negative charge of the DNA, changes in the ionic strength of the solvent affect the conformation of the DNA molecule and therefore both its sedimentation coefficient (s) and its intrinsic viscosity (η). As s and η vary with ionic strength they remain related over a wide range of ionic strengths as described by the Mandelkern and Flory equation for both native and single-stranded T7 DNA in alkaline or neutral solutions (37). Therefore, the size determinations of mammalian DNA by alkaline sucrose gradients (10, 25, 26), neutral sucrose gradients (17–19), and viscoelastometry (20, 24) are based on related parameters that can be similarly affected by changes in the hydrodynamic properties of the DNA molecule.

The alkaline filter elution technique is theoretically capable of measuring DNA strand length distributions with maximal sensitivity for DNA molecules of $>3–4 \times 10^8$ dalton (38–40). Although subject to its own set of potential artifacts, most of these involve properties that are different from those predominantly affecting the previously mentioned techniques. In general, large double-stranded DNA molecules to not elute from filters, whereas single-stranded molecules elute at a rate inversely proportional to their strand length (40). The DNA elution kinetics for a given DNA size distribution can be defined mathematically (K. Kohn, personal communication; Materials and Methods section). When the percentage of DNA retained is plotted vs. elution time, first-order kinetics should result for random DNA size distributions. Monodispersed distributions should result in linear kinetics; a constant percentage of the initial DNA on the filter elutes with time. From the linear elution rate an estimation of the size of the monodispersed DNA molecules can be obtained.

In the studies reported here, DNA from unirradiated and irradiated 9L rat brain tumor cells was exposed to low ionic strength solutions of pH 11.0, 12.3, or 12.9 for varying lengths of time, and the effect of this exposure to alkali on the DNA size distribution was determined using the alkaline elution technique in order to provide information on the higher-order structure of mammalian chromosomal DNA.

MATERIALS AND METHODS

Cell Maintenance, Labeling, and Irradiation

9L/Ro cells from an *N*-methylnitrosourea-induced rat brain tumor (41) were grown in monolayer cultures using Eagle's basal medium supplemented with 10% newborn calf serum as previously described (42, 43). Asynchronous exponentially growing cultures with a doubling time of 18–20 hr were labeled with 0.05 μCi/ml [2-^{14}C]-thymidine for 24–30 h. The cells were then trypsinized and resuspended in phosphate-buffered saline (PBS) (131 mM NaCl, 9.0 mM Na_2HPO_4, 0.75 mM NaH_2PO_4, 1.50 mM KH_2PO_4, pH 7.4). Single cell suspensions were irradiated with 0, 300, or 600 rad of ^{137}Cs γ-rays (588 rad/min) at 4°C and stored on ice until placed on the filters.

Alkaline Filter Elution

Alkaline filter elution was performed using a slight modification of Kohn's Procedure B (44). ~8×10^5 9L cells suspended in 20 ml ice-cold PBS were loaded onto 2-μm porosity, polycarbonate filters (Nucleopore Corp., Pleasanton, CA) supported by Swinnex filter holders (Millipore/Continental Water Systems, Bedford, MA). For some experiments, 8×10^5 unirradiated cells plus 4×10^5 cells irradiated with 600 rad were loaded onto each filter. The cells were rinsed with 10 ml PBS, lysed with 5 ml of a solution of 2% wt/vol sodium dodecyl sulphate (SDS, specially pure, BDH Chemicals Ltd., Poole, England), and 0.025 M ethylenediaminetetraacetic acid (EDTA, acid form), pH 9.7 and then washed with 5 ml of 0.025 M EDTA pH 10.0, leaving the DNA and <2% of the cellular protein on the filters. The flow for these solutions was by gravity and the meniscus was kept above the surface of the filters to avoid shearing the DNA. The DNA was eluted through the filters at a constant flow rate of ~2 ml/h with a solution of 0.025 M EDTA, 0.1% wt/vol SDS and sufficient tetra-*n*-propyl ammonium hydroxide (Pr_4NOH) for a final pH of 11.0, 12.3, or 12.9. The filter units were shielded from light because DNA in alkali often seems to be degraded by visible light (45). The use of an eight channel peristaltic pump and the introduction of stopcocks between the pump and filter holders allowed the DNA on four filters to be eluted while the DNA on the other four filters was held in the presence of eluting solution for up to 48 h before resuming elution.

Data Analysis

90-min fractions were collected and weighed. Aliquots of 1 ml from each fraction were neutralized with 1 ml of 0.4 M HCl and counted with 2 ml of scintillation fluid (PCS, Amersham Corp., Arlington Heights, IL). The average weight for the fractions eluted from each filter was determined and multiplied by the radioactivity per aliquot to calculate the total DNA in each fraction (f). This corrected for small variations in flow rate between filter units. The total DNA per filter before elution (T) was determined as the sum of the radioactivity: (a) in all of the fractions (Σf), (b) on the filter, and (c) in the post-elution NaOH (0.4 M) rinses of the elution apparatus. The percentage of the DNA retained on the filter (R) as a function of time (t) was calculated as

$$R(t = x) = 100\left[1 - \left(\sum_{t=0}^{x} f/T\right)\right].$$

All data points are the average of four filters per experiment for two or more experiments.

Elution profiles of DNA from mixed cell populations (0 and 600 rad) were separated into their constitutent profiles of unirradiated and irradiated DNA. Since DNA from cells irradiated with 600 rad completely elutes in 15 h, fractions 11–28 (elution times of 16.5–42.0 h) of the mixed profile were assumed to contain DNA from only unirradiated cells. Fractions 1–10 (0–15 h) of the mixed profile contained DNA from both irradiated and unirradiated cells. The contribution of the latter to the radioactive counts in each fraction was determined from the control profile (fractions 1–10) of DNA from an equal number of unirradiated cells (8×10^5) eluted alone. The calculated contribution of the counts due to unirradiated DNA in fractions 1–10 was then subtracted from the total counts in each fraction to obtain the number of counts for the irradiated DNA in these fractions. The total unirradiated or irradiated DNA was calculated by summing the relative contributions of each in the fractions corresponding to their respective elution profiles. The remainder of the calculations were as previously described.

DNA Size Determination

The linear rate of elution for a monodispersed DNA distribution can be used to estimate the size of its molecules (K. Kohn, personal communication). Radiation randomly breaks DNA molecules (36, 46–49). If the initial distribution of DNA lengths is monodispersed, ~5 breaks per

molecule are required to change the initial monodispersed distribution into a random distribution (36, 46). A dose of 300 rads produces ~10 breaks per 10^{10} daltons of DNA in 9L cells, so for doses of 300 rads or more a random distribution of DNA molecules exists. The rate of elution of such a random distribution of DNA molecules is described by

$$-\mathrm{d}R(t)/\mathrm{d}t = APe^{-APt}, \quad (1)$$

where R is the fraction of DNA retained on the filter at time t, P is the probability of a break at any given nucleotide, and A is a constant equal to the slope of the elution rate plotted against radiation dose. The fraction of DNA molecules (F) of chain length L and range ΔL can be approximated by

$$F = P^2L_0e^{-PL}\Delta L, \quad (2)$$

where L_0 is the initial chain length (48, 49). Since the rate of elution for linear chain DNA molecules is proportional to their strand length

$$-\mathrm{d}R(t)/\mathrm{d}t = \int_0^\infty P^2L_0e^{-PL}\,E(L, t)\,\mathrm{d}L, \quad (3)$$

where $E(L, t)$ describes the probability of eluting a molecule of length L in time t. Eqs. 1 and 3 can be combined and rearranged to yield

$$e^{-KP}/P = \int_0^\infty G(L)e^{-PL}\,\mathrm{d}L, \quad (4)$$

where $K = At$ and $G(L) = (L_0/A)\,E(L, t)$ for simplification. $G(L)$ can be solved by use of the appropriate LaPlace Transform to show that

$$G(L) = 0 \quad \text{for} \quad 0 < L < K \quad (5)$$

$$= 1 \quad \text{for} \quad L > K \quad (6)$$

For a monodispersed DNA size distribution of constant length L, $f = -\mathrm{d}R(t)/\mathrm{d}t$, where f is the fraction of the initial DNA on the filter eluting per unit time. Combining the solution for $E(L, t)$ from Eq. 6 with Eq. 3 for a monodispersed DNA size distribution yields

$$f = A/L. \quad (7)$$

Therefore, since A can be experimentally determined, the value of L can be calculated for an observed constant f.

RESULTS

The elution at pH 12.3 of DNA from cells irradiated with 300 rad followed first-order kinetics characteristic of a random DNA size distribution (Fig. 1 *A*). When the DNA from irradiated cells was held at pH 12.3 for 24 h prior to elution, the rate of elution increased but still remained first order. The elution of DNA from unirradiated cells that was not held resulted in complex kinetics, neither first order (Fig. 1 *A*) nor linear (Fig. 1 *B*). However, when the DNA from unirradiated cells was held at pH 12.3 for 24 h prior to elution, the elution kinetics were linear, indicative of a monodispersed DNA size distribution (Fig. 1 *B*).

To determine when the monodispersed DNA was first released during elution at pH 12.3, DNA from unirradiated cells was eluted at pH 12.3 for extended periods. The elution kinetics became linear after 18–21 h (Fig. 2 *A*). The slope (-2.77 ± 0.04) of the linear portion of the elution profile for DNA eluted at pH 12.3 without prior exposure to pH 12.3 was not statistically different

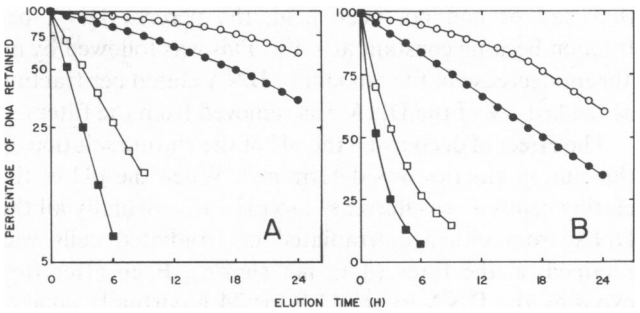

FIGURE 1 Effect on the elution kinetics of holding DNA at pH 12.3 for 24 h prior to elution at pH 12.3. Data plotted on semilogarithmic axis (*A*) or on linear axis (*B*). ○,●, DNA from unirradiated cells. □, ■, DNA from cells irradiated with 300 rad at 4°C. Open symbols (○, □), unheld DNA, eluted at pH 12.3. Closed symbols (●, ■), DNA held 24 h at pH 12.3 before elution at pH 12.3. The points are the means of measurements from 16 filters in four independent experiments. Error bars (±SEM) are within the points.

($p > 0.5$) from the slope (-2.78 ± 0.06) of the profile produced by DNA held at pH 12.3 for 24 h prior to elution. The percent DNA eluted per 90 min fraction for DNA that was not held varied during the time in which the initial complex elution kinetics occurred (Fig. 2 *B*). During the time corresponding to the linear elution kinetics for DNA

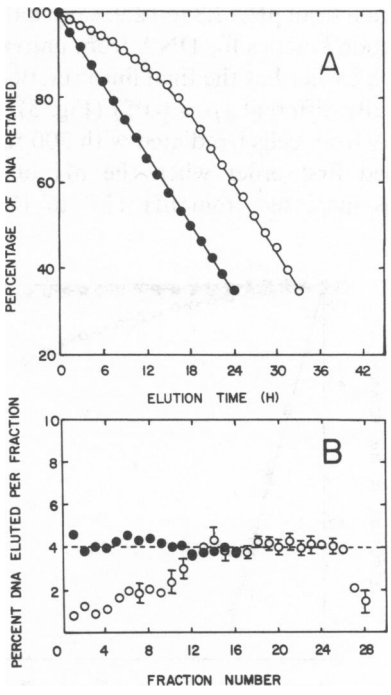

FIGURE 2 Comparison of the elution kinetics of DNA from unirradiated cells eluted at pH 12.3 (○) or held 24 h at pH 12.3 prior to being eluted at pH 12.3 (●). Elution kinetics plotted as (*A*) percentage of the DNA retained vs. elution time and (*B*) percent DNA eluted per fraction vs. fraction number. The straight portion of each curve was fit by a least-squares linear regression analysis of the appropriate data. All points represent the mean ± SEM of measurements from 16 filters in four independent experiments.

that had or had not been held, the percent DNA per fraction became constant at ~4%. This was followed by an abrupt decrease in the amount of DNA eluted per fraction as the last 5% of the DNA was removed from the filters.

The effect of decreasing the pH of the eluting solution on the elution kinetics was determined. When the pH of the eluting solution was decreased to pH 11.0, virtually all the DNA from either unirradiated or irradiated cells was retained on the filter (data not shown). Even after first exposing the DNA to pH 11.0 for 24 h, virtually none of the unirradiated or irradiated DNA eluted from the filter (Fig. 3). Holding the DNA from unirradiated or irradiated cells for 24 h at pH 11.0 before elution at pH 12.3 produced no statistically significant ($p > 0.05$) change in the elution rate compared with DNA immediately eluted at pH 12.3 (Fig. 3) and did not alter the slope of the final linear elution kinetics (Fig. 4). The 1.5-h delay in the onset of linear elution (Fig. 4) was due to the time necessary to equilibrate the eluting solution from pH 11.0 to 12.3 since the final 2–3 ml of pH 11.0 solution could not be removed before adding the pH 12.3 solution without shearing the DNA. Thus, a long exposure to pH 11.0 did not affect the final linear elution rate at pH 12.3.

The effect of increasing either the pH of the eluting solution or the length of exposure to alkali on the elution kinetics was also determined. Increasing the pH of the eluting solution from pH 12.3 to 12.9, caused the transition to linear elution kinetics for DNA from unirradiated cells to occur ~6 h earlier but the final linear elution rates were not statistically different ($p > 0.05$) (Fig. 5). The elution rate of DNA from cells irradiated with 300 rad increased but remained first order when the pH of the eluting solution was increased from pH 12.3 to 12.9 (Fig. 5).

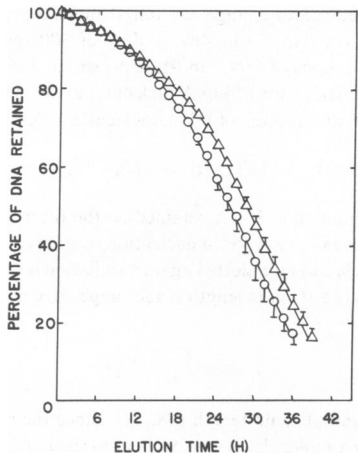

FIGURE 4 Effect on the DNA elution kinetics of exposing the DNA to pH 11.0 for 24 h prior to elution. DNA from unirradiated cells was eluted at pH 12.3 (O) or held for 24 h at pH 11.0 prior to elution (△). The linear portion of the curves was fit by a least-squares linear regression analysis. All points represent the mean ± SEM of measurements from eight filters in two independent experiments.

Exposure of DNA from unirradiated cells to pH 12.3 for 48 h before elution at pH 12.3 resulted in the reappearance of first-order kinetics typical of a random DNA size distribution (Fig. 6). Therefore, exposure to the higher pH (12.9) did not change the final linear elution rate although it did affect the time of its appearance. However, prolonged exposure to pH 12.3 prior to elution at pH 12.3 caused the loss of linear elution kinetics (Fig. 6).

DNA from irradiated and unirradiated cells was eluted simultaneously to determine whether damaged and undamaged DNA molecules elute independently. The elution profile of DNA from a mixed cell population (8×10^5

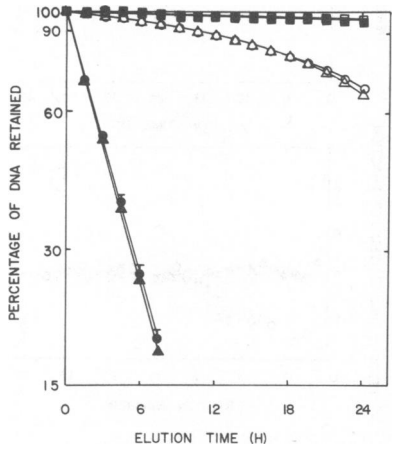

FIGURE 3 Comparison of elution kinetics for DNA. O, ●, DNA eluted at pH 12.3; △, ▲ DNA held at pH 11.0 for 24 h prior to elution at pH 12.3, or □, ■, DNA held at pH 11.0 for 24 h prior to elution at pH 11.0. Open symbols (O, △, □), DNA from unirradiated cells. Closed symbols (●, ▲, ■), DNA from cells irradiated with 300 rad at 4°C. The lines for ●, ▲ were fit by a least-squares linear regression analysis using the natural log transform of the data. All points represent the mean ± SEM of measurements from 12 filters in three independent experiments.

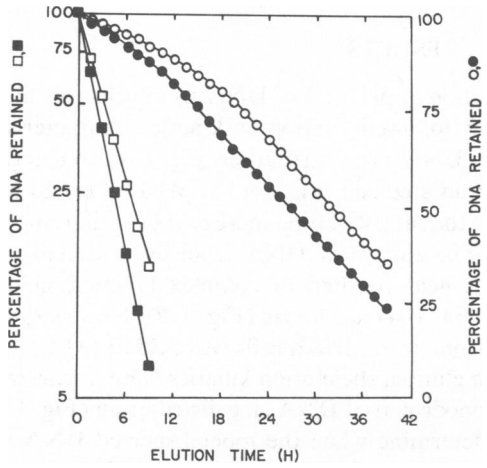

FIGURE 5 DNA elution kinetics as a function of eluting solution pH. O, ●, elution of DNA from unirradiated cells plotted on linear axis; □, ■, elution of DNA from cells irradiated with 300 rad plotted on semilogarithmic axis. Open symbols (O, □): DNA eluted at pH 12.3. Closed symbols (●, ■): DNA eluted at pH 12.9. The straight portion of the curves was fit by a least-squares linear regression analysis. All points represent the mean ± SEM of measurements from at least four filters.

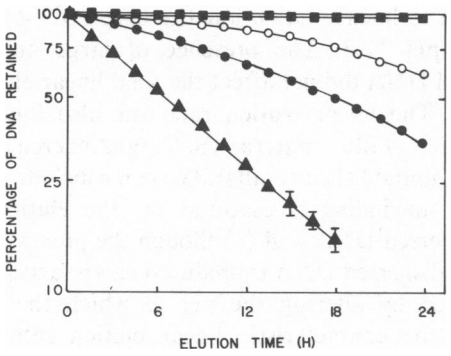

FIGURE 6 Elution kinetics for DNA from unirradiated cells exposed for varying times to pH 11.0 or 12.3. O, DNA eluted at pH 12.3; ●, DNA held 24 h at pH 12.3 before elution at pH 12.3; ▲, DNA held 48 h at pH 12.3 before elution at pH 12.3; ■, DNA held 48 h at pH 11.0 before elution at pH 11.0. All points represent the mean ± SEM of measurements from 12 filters in three independent experiments.

unirradiated cells plus 4×10^5 cells irradiated with 600 rad per filter) was separated into its constituent profiles of unirradiated and irradiated DNA as previously described (Materials and Methods, Data Analysis). The shorter irradiated DNA molecules eluting first from the mixed population accounted for 33% of the total DNA retained, while the longer unirradiated molecules eluting later accounted for 66% of the total DNA retained, as expected for the 2:1 mixture described (Fig. 7). The elution rate

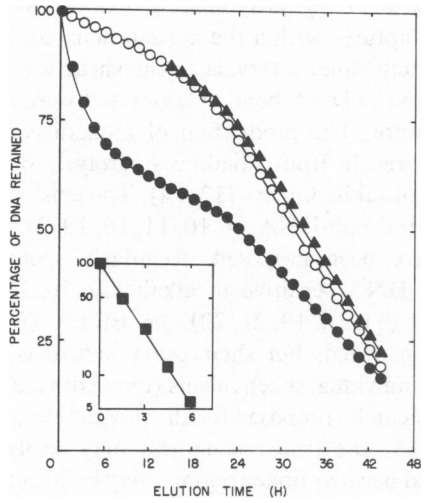

FIGURE 7 Elution kinetics of DNA from unirradiated and irradiated cells eluted simultaneously. Data plotted on linear axis with insert on semilogarithmic axis. O, control DNA from unirradiated cells eluted alone. ●, DNA from a mixed population of unirradiated cells and cells irradiated with 600 rad, eluted simultaneously. ▲, DNA from unirradiated cells eluted as part of the mixed population but analyzed as a separate component. Elution rates for linear portions: O(-2.6 ± 0.1), ●(-1.8 ± 0.1), ▲(-2.7 ± 0.1). ■, DNA from cells irradiated with 600 rad, eluted as part of the mixed population but analyzed as a separate component. Elution rate (■) -0.47 ± 0.10. The straight portion of each curve was fit by a least-squares linear regression analysis of the appropriate data. All points represent the mean ± SEM of measurements from 12 filters in three independent experiments.

from the constituent profile of irradiated DNA ($m = -0.472$, Fig. 7, insert) was not statistically different ($p > 0.05$) from that of DNA from cells irradiated with 600 rad, which was eluted alone. The constituent profile of unirradiated DNA showed a linear elution rate ($m = -2.69$) by 21 h, which was not statistically different ($p > 0.1$) from that of unirradiated DNA eluted alone ($m = -2.64$). Therefore, damaged and undamaged DNA molecules eluted simultaneously but independently.

DISCUSSION

Measurement of Alterations in DNA Size Distributions by Alkaline Elution

The alkaline filter elution technique can provide a means of studying the higher-order structure of mammalian DNA. Although its measurements are affected by some of the same properties of the DNA as the other techniques, the magnitude of these effects should differ. The alkaline elution technique functions by discriminating single-stranded sizes of mammalian DNA through the utilization of filters that act to mechanically impede the passage of long DNA strands (44). There are two basic premises governing the removal of DNA from filters during alkaline elution. First, large double-stranded DNA molecules do not elute from filters, whereas large single-stranded molecules do (40). This is confirmed by the data in Figs. 3 and 6 since virtually all the DNA is retained on the filter even after total exposures of up to 72 h to pH 11.0; a pH that is alkaline but below the critical unwinding pH (35). Second, it has been demonstrated that the rate of DNA elution is proportional to the radiation dose and therefore should be inversely proportional to strand length (40). As a result of the theory governing alkaline elution, a random DNA size distribution should elute with first-order kinetics. For a monodispersed DNA size distribution, linear elution kinetics should result when the percentage of DNA retained vs. time is plotted. This corresponds to a step function when plotted as the percent DNA eluted per fraction (see Materials and Methods, DNA Size Determination).

Evidence for the Linear Elution of Monodispersed DNA

These theoretical predictions are supported by comparison with the experimental data. The random nature of radiation-induced DNA strand breaks (36, 46–49) should yield a random size distribution for the DNA from irradiated cells. The irradiated DNA in Fig. 1 did elute with first-order kinetics. First-order kinetics should also result from the random damage of DNA by alkaline hydrolysis during prolonged exposure to alkali as is seen with long lytic storage times on alkaline sucrose gradients (12). This is demonstrated in Fig. 1 for irradiated DNA where after exposure to pH 12.3 for 24 h before elution, the elution rate increased but remained first order and in Fig. 6 where the

elution rate of unirradiated DNA changed from linear to first order when the DNA exposure to pH 12.3 before elution was increased to 48 h. Therefore, regardless of how random DNA damage is produced, first-order elution kinetics result, supporting the theoretical model for a random DNA size distribution. While it can be shown mathematically that monodispersed DNA should elute with linear kinetics, note that the concave down elution profile for undamaged DNA will probably pass through a somewhat linear intermediate as it converts to the concave up profile for damaged DNA (Fig. 1 *B*). Although a primary monodispersed DNA standard is not available in the size range required to prove that the linear elution kinetics observed in these experiments represents a monodispersed distribution of DNA molecules, this interpretation is probably correct since both the basic premises and the predictions for the elution of random DNA size distributions by the model have been confirmed. Also, not only the linear elution rate but the theoretically predicted abrupt cessation of elution could be experimentally demonstrated for this DNA distribution (Fig. 2).

A third type of elution kinetics also results for unirradiated DNA. We have designated these kinetics as complex since the kinetics are neither linear nor first order over the entire elution time (Figs. 1 and 2). The initial portion of the elution profile for DNA from unirradiated cells that was not held is probably composed of damaged molecules and short DNA strands from replication forks that would elute first (40), while the longer undamaged molecules eluted later. Fig. 7 demonstrates that the damaged and undamaged DNA, from a mixed population of irradiated and unirradiated cells, elute simultaneously but independently. Therefore, it is most likely that the undamaged nonreplicating DNA molecules, which might be as large as 1×10^{11} daltons, undergo the alkali-induced transition to a monodispersed DNA size distribution.

Characteristics of the Production of Monodispersed DNA During Alkaline Elution

The production of the monodispersed DNA size distribution when unirradiated DNA was exposed to alkali was examined under a variety of conditions to try to determine its mechanism. When the DNA was exposed for 24 h to pH 12.3 before elution, the DNA elution profile changed from complex to linear indicative of the production of monodispersed DNA (Fig. 1). Eluting the DNA at pH 12.3 without previous holding showed that the transition from complex to linear elution kinetics occurred after 18–21 h of exposure to pH 12.3, and the final linear elution rate was not significantly different ($p > 0.05$) from that of the held DNA (Fig. 2). Neither exposure for 24 h to pH 11.0 before elution at pH 12.3 (Fig. 4) nor elution at pH 12.9 (Fig. 5) altered the final linear elution rate although its onset was affected. When the DNA was eluted at pH 12.9, the transition to linear elution kinetics occurred ~6 h earlier than at pH 12.3. The presence of large amounts of irradiated DNA did not affect the final linear elution rate (Fig. 7). The linear elution rate was also found to be independent of filter material (S. C. vanAnkeren and K. T. Wheeler, unpublished results). We can conclude from this that (*a*) unwinding is essential for the elution of the monodispersed DNA and (*b*) though the process whereby the monodispersed DNA is produced can be accelerated or decelerated by altering the pH to which the DNA is exposed, the characteristic linear elution rate remains unchanged. The size of the monodispersed DNA distribution can be determined from the percentage of the DNA eluted per fraction and corresponds to a single-stranded size of 9.5×10^9 (SEM = 0.8×10^9) daltons (see Materials and Methods).

Mechanisms for the Production of Monodispersed DNA

There are at least two mechanisms by which monodispersed DNA could be produced upon exposure to alkali: (*a*) denaturation and strand separation in alkali may produce breaks from shear forces, setting an upper limit to the size of single-stranded DNA obtainable at high pH (50) and (*b*) alkaline hydrolysis of regularly spaced alkali-labile linkers (12–14). The shear forces created by the viscous drag opposing strand rotation during DNA unwinding could cause strand rupture within the normal DNA helix or at regularly spaced sites of less structural stability. Ruptures within the normal helix could occur at approximately equal intervals as the shear forces became greater than the DNA bond energies and were relieved by strand rupture. The production of monodispersed DNA could also result from alkaline hydrolysis of regularly spaced alkali-labile linkers (12–14). The existence of both DNA (9) and non-DNA (4, 10, 11, 16, 19, 21, 22) linker regions have been proposed. Regularly spaced regions within the DNA sensitive to alkali (12, 16, 21), various proteinases (11, 16, 19, 21, 22), or EDTA (9, 10) have been demonstrated, but their exact nature is unknown. While two individual mechanisms (unwinding and alkaline hydrolysis) can be proposed for the production of monodispersed DNA, the true mechanism may easily be more complicated because linker regions may be structurally less stable during unwinding as well as being alkali-labile. Of the possible mechanisms for producing monodispersed DNA, all but that of shear forces overcoming the normal bond energies of the DNA at repeated intervals would be indicative of a naturally-occurring subunit structure in the DNA.

The experimentally determined characteristics of the process by which the monodispersed DNA is produced can be compared with these theoretical mechanisms to determine which are plausible. The pH dependence of the onset of linear elution could result from an increased rate of

either DNA unwinding or alkaline hydrolysis of regularly spaced alkali-labile linkers since both processes are pH dependent (24, 35, 51–53). An exposure to alkali of 18–21 h at pH 12.3 or 13–15 h at pH 12.9 is necessary to produce the elution of the monodispersed DNA. Could this correlate with either the time necessary to unwind a double-stranded DNA molecule of 2×10^{10} daltons or to hydrolyze a linker per 1×10^{10} daltons of DNA?

Support for the Role of DNA Strand Separation in Alkali

A number of theoretical models with some experimental corroboration are available for the rate of DNA strand separation (51, 54–59). The model of Freese and Freese (51) for DNA unwinding from one end, with the other end free to rotate, correlates well with the rate of inactivation of transforming DNA. This model predicts an unwinding time of ~6 h for a double-stranded molecule of 2×10^{10} daltons. This is a minimum value since the model calculates a mean unwinding time rather than the time necessary to unwind all DNA molecules, and the estimate was for conditions of 2 M NaCl at pH 13. Under the conditions of alkaline elution (lower pH and ionic strength), the unwinding time could be substantially increased (35, 57).

The DNA unwinding conditions of Davison (58) more closely resemble the conditions DNA would be exposed to during alkaline elution in terms of pH, ionic strength, and temperature but the model is calibrated only in the range of $2–20 \times 10^7$ daltons making extrapolation to 2×10^{10} daltons (a predicted unwinding time of 8 d) questionable. Extrapolation of the DNA size derived by Ahnstrom and Erixon for the complete denaturation of mammalian DNA during a 20-min exposure to pH 13 (59) predicts, assuming unwinding is proportional to the square of the molecular weight (56), at least 21 h for the unwinding of a DNA molecule of 2×10^{10} daltons. The results of Linn and Wheeler (60) indicate that 4–6 h were necessary for complete strand separation for untreated DNA from 9L cells as measured by S1 nuclease sensitivity under conditions of high ionic strength. Although the dependence of the rate of strand separation on pH, temperature, and ionic strength make it impossible to predict the actual time necessary for the unwinding of a double-stranded DNA molecule of 2×10^{10} daltons under the conditions of alkaline elution, a value of hours is reasonable. Our values of 18–21 h at pH 12.3 and 13–15 h at pH 12.9 for the production of monodispersed DNA are, therefore, consistent with unwinding as a possible mechanism of production.

Support for the Role of Alkaline Hydrolysis

There is less information available for actual rates of alkaline hydrolysis for untreated DNA. Slow but constant rates of alkaline hydrolysis of untreated DNA were measured for up to 80 h at 37°C at a variety of pH values (53). In 0.1 M NaOH (approximate pH of 12.5) a rate equivalent to 112 strand breaks per hour per 1×10^{10} daltons was measured. In a similar study the same authors found that the rate of alkaline hydrolysis at 20°C was 1/20 the rate at 37°C yielding ~11 strand breaks per hour per 1×10^{10} daltons at 20°C (61). There is difficulty in extrapolating these results to alkaline elution since: (*a*) the chemical reactions involved in the alkaline hydrolysis of DNA in tetra-*n*-propyl ammonium hydroxide may not be the same as those in NaOH and (*b*) these rates are for random alkaline hydrolysis. Alkaline hydrolysis at alkali-labile sites need not have the same kinetics. A half-time of hours for alkaline hydrolysis during exposure to eluting solution is therefore also consistent with the experimental data as a possible mechanism for production of monodispersed DNA.

Comparison with DNA Subunits Identified by Other Techniques

While both unwinding and alkaline hydrolysis remain possible mechanisms for the production of monodispersed DNA in alkali, comparison of the size of the monodispersed DNA derived by alkaline elution with the size of the DNA subunits determined by other techniques decreases the probability that shear forces overcoming the normal bond energies of the DNA is the mechanism by which unwinding may produce monodispersed DNA. Monodispersed DNA has been reported with a double-stranded molecular weight of $\sim 2 \times 10^{10}$ daltons obtained by neutral sucrose gradients (20) and confirmed by viscoelastometry (19). This corresponds well to our double-stranded molecular weight of 1.9×10^{10} daltons. Since the double-stranded molecules were isolated under neutral conditions, denaturation forces probably did not play a role in their production supporting the possibility that the monodispersed DNA observed by alkaline elution is a structural subunit of mammalian chromosomal DNA rather than a product of shear generated during unwinding.

It has been reported that the 2×10^{10} daltons monodispersed DNA obtained by neutral sucrose gradients may be comprised of ~21 double-stranded subunits of 8×10^8 daltons each, connected by protein linkers (19). These 8×10^8 double-stranded subunits may correspond to the 5×10^8 daltons single-stranded subunits identified by alkaline sucrose gradients (12, 13). It has been suggested that the mammalian chromosome is constructed of eight duplex circular DNA molecules arranged in a side-by-side array (62–64) and that the monodispersed size of $\sim 2 \times 10^{10}$ daltons may correspond to these eighth-of-a-chromatid pieces (19). The exact relationship between the 5×10^8 daltons subunit of alkaline sucrose gradients and the 1×10^{10} daltons monodispersed DNA demonstrated by alkaline elution is still unclear since both sizes are isolated in alkali. However, it is clear that if 1×10^{10} daltons single-stranded DNA can be obtained in alkali as we have

shown here, then it is highly unlikely that the 5×10^8 daltons single-stranded DNA molecules observed in alkaline sucrose gradients (12, 13) arose because of shear forces generated by the unwinding process (50). The possibility remains that the combination of 1 M NaCl with alkali used in the lytic zone of alkaline sucrose gradients causes cleavage at protein linkers proposed for connecting the neutral sucrose gradient subunits. This is supported by the observation that the rate of alkaline hydrolysis in 0.3 M NaOH, 0.7 M NaCl at 37°C of alkylated DNA was greater than the rate measured for 0.4 N NaOH without NaCl (53, 61). However, it is not yet definitively known how these DNA subunits identified by different techniques relate to each other and ultimately to the structure of mammalian chromosomal DNA.

CONCLUSION

The previously reported large mammalian DNA subunits were measured with techniques that were difficult to extrapolate to DNA sizes in the range of 1×10^8–5×10^{10} daltons (5, 12–16, 18–20). The data presented here used an independent technique, alkaline filter elution, to demonstrate the possible existence of a single-stranded DNA subunit of 9.5×10^9 daltons. While the possibility cannot be eliminated at present that this size represents an upper limit determined by physical forces governing DNA unwinding, these results in conjunction with those obtained by other techniques help support the concept of a nonrandom higher-order structure for mammalian chromosomal DNA. Further work is in progress in an attempt to determine the relative contributions of DNA unwinding and alkaline hydrolysis in the production of this monodispersed DNA.

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