MOLECULAR WEIGHT ANALYSIS OF MAMMALIAN DNA≠

M.W. McBurney and G.F. Whitmore

Department of Medical Biophysics, University of Toronto, and The Ontario Cancer Institute, 500 Sherbourne Street, Toronto, Ontario.

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SUMMARY. It has been suggested that DNA in mammalian nuclei may exist as discrete molecular subunits joined by non-nucleotide linkers. In the present experiments single strands of mammalian DNA of greater than 2 x 10^9 daltons have been isolated and analysed by velocity sedimentation in alkaline sucrose gradients. Thus, the DNA units in mammalian cells appear to have molecular weights in excess of 4 x 10^9. This result increases the probability that the entire DNA complement of a chromosome may exist as a single molecular entity.

A detailed description of the molecular form of DNA in mammalian nuclei is a prerequisite for the understanding of chromosome structure, genetic linkage, and the control of transcription. The data obtained from autoradiographs of labelled DNA from human lymphocytes suggests that strands may be as long as 2.2 cm (1); however, techniques for sedimentation of mammalian DNA in alkaline sucrose gradients have yielded single stranded molecules of much smaller dimensions (about 2 to 5 x 10^8 daltons (2-6)). We have recently attempted to refine the existing technique for the isolation and sedimentation of mammalian DNA in alkaline sucrose gradients and succeeded in obtaining DNA with sedimentation coefficients of about 500 S (7). The sedimentation patterns observed indicated that the 500 S DNA was not single stranded. However, the data obtained could be explained if it was assumed that the DNA in the 500 S peak was so long, perhaps of the order of 10^{10} daltons, that complete unwinding and separation of sister strands did not occur during the 16 hours of exposure to alkali prior to centrifugation. This explanation suggests that mammalian chromosomes contain DNA molecules

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much larger than has been postulated from data obtained using conventional techniques (2,6).

In this communication we describe experiments designed to obtain the DNA from the 500 S material in single stranded form. These experiments are based on the assumption that single strand breaks present in only one strand of a large DNA molecule should, on exposure to alkali, facilitate unwinding and separation of this strand from the unbroken strand which may then be analysed with respect to sedimentation coefficient and molecular weight in alkaline sucrose gradients. The experimental procedure involved L-cells cultured for several generations in the presence of ^{14}C-TdR to uniformly label the DNA. These cells were then grown for one generation in the presence of BUdR to label one strand of each DNA molecule. The cells were then exposed to fluorescent light to induce single strand breaks in the BU-substituted strand (8). In alkali, the BU-strand separated from the ^{14}C-labelled DNA molecules which now appeared to have single stranded molecular weights in excess of 2×10^9.

MATERIALS AND METHODS

Mouse L-cells were maintained in exponential growth at 37°C in medium CMRL 1066 (9) minus nucleosides supplemented with 10% fetal bovine serum. Cellular DNA was labelled by growing cells in the presence of ^{14}C-TdR (0.05 μCi/ml, 60 mCi/mmole) for 48 hours. Cells were then centrifuged and resuspended in medium containing BUdR (2 μgm/ml) and ^{3}H-BUdR (0.5 μCi/ml) and maintained for approximately one generation time (18 hours) before being washed and resuspended at about 4×10^5 cells/ml in cold PBS (10) for x-irradiation. Neither ^{14}C-TdR labelling nor BUdR labelling affected the generation time of these cultures. Following x-irradiation at 4°C, one ml of the cell suspension was placed in a 35 mm Petri dish situated less than 3 cm beneath two 18 inch fluorescent bulbs (15 watt) in a lamp in a cold room (4°C). Finally about 10^5 cells in 0.25 ml were loaded at 4°C on top of a 2 ml layer of 2% sucrose which had been placed over 36 ml of a 10 to 30% linear sucrose gradient made up in 0.3 N NaOH, 0.5 M NaCl, and

0.01 M EDTA. Following a 16 to 18 hour interval at 4°C, the tubes were centrifuged at 8,000 rpm in the SW27 rotor for 22 hours at 4°C. Centrifugation was carried out at low speed in an attempt to reduce anomalous sedimentation effects observed earlier for large single stranded DNA molecules centrifuged under high speed (11). Following centrifugation, fractions were collected from the top and acid precipitable material collected on glass fiber filters for scintillation counting.

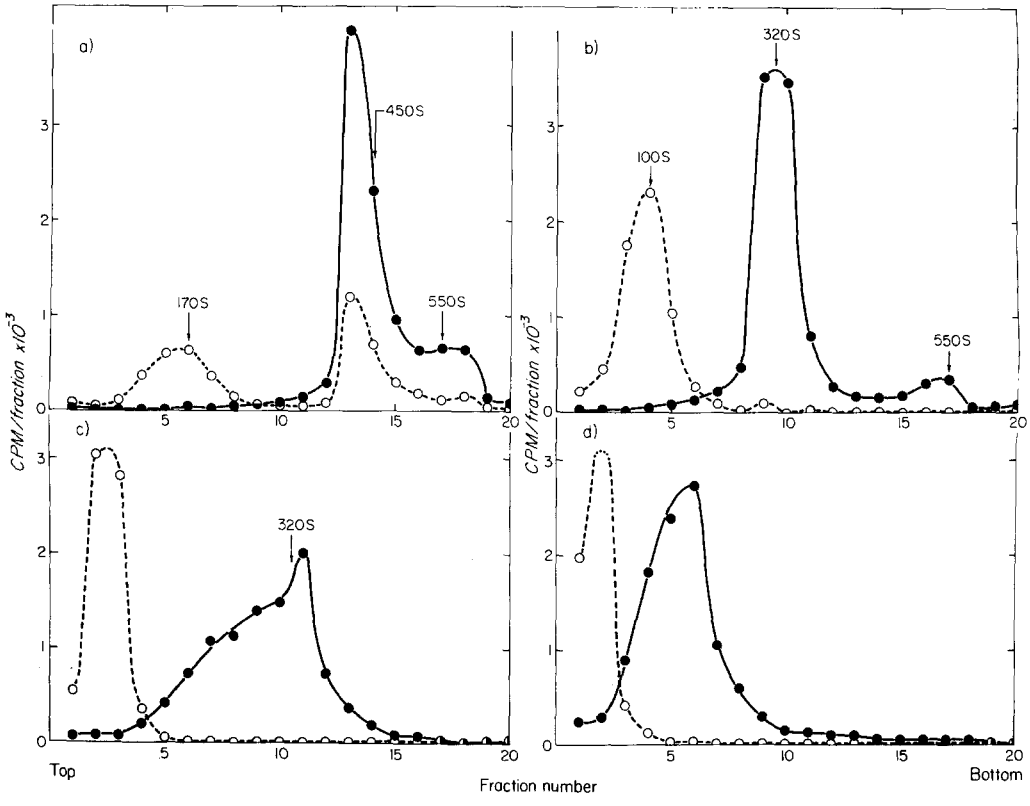

Figure 1.

Sedimentation profiles in alkaline sucrose density gradients of ^{3}H-BU-DNA (o----o) and ^{14}C-DNA (●——●) released from L-cells labelled as described in Materials and Methods. Prior to layering on the gradients, cells were exposed for various intervals to a source of fluorescent light: panel a) 0 min, b) 2 min, c) 10 min, and d) 60 min. The direction of sedimentation is from left to right.

RESULTS

The ^{3}H-BU-DNA and ^{14}C-DNA profiles shown in figure 1a were obtained from labelled cells placed directly onto the gradient with no deliberate

exposure to light. Approximately half of the ^{3}H-BU-DNA cosediments with the ^{14}C-DNA at about 450 S, while the remaining ^{3}H-BU-DNA sediments at about 170 S. This 170 S ^{3}H-BU-DNA appears to have been broken, probably due to accidental exposure to light. In order to introduce more single strand breaks into the BU-substituted strand, cells were exposed to fluorescent light prior to being placed on the gradient. The data is shown in panels b to d of figure 1. After as little as 2 minutes exposure (panel b), there appears to be complete separation of ^{3}H-BU-DNA (100 S) from ^{14}C-DNA. This ^{14}C-DNA must now be single stranded and sediments in a narrow peak at about 320 S. Much longer exposures to light (panels c and d) apparently give rise to significant breakage of the ^{14}C-labelled strand (12) in addition to causing further fragmentation of the BU-substituted strand.

In an attempt to confirm the single stranded nature of the DNA in the 320 S peak of figure 1b, DNA sedimentation patterns were obtained from cells which were x-irradiated to randomly fragment both DNA strands. Figure 2 shows DNA profiles obtained from cells exposed to various x-ray doses prior to being exposed to fluorescent light for 2 minutes. The ^{14}C-DNA sedimentation profile in panel a, consists of a narrow spike which sediments at approximately 320 S. As the x-ray dose is increased (panels b, c and d), a tail appears on the trailing edge of the spike and the proportion of DNA sedimenting in the spike decreases while the amount in the tail increases proportionally. This flow of ^{14}C-DNA from the spike into the tail with increasing x-ray doses is analogous to results obtained previously for single stranded DNA (11).

DISCUSSION

We have demonstrated that it is possible to isolate single stranded DNA molecules with sedimentation coefficients of about 320 S by combining the use of the modified alkaline sucrose gradient technique for DNA sedimentation with BUdR labelling of one strand of this DNA. Single stranded DNA with a sedimentation coefficient of 320 S would be expected to have a molecular weight close to 2×10^{9} (13); however, we shall explain later that the value

of 2 x 10^9 is a minimum estimate for the molecular weight of the DNA molecules in the 320 S ^{14}C-DNA peak.

In discussing the DNA profiles of figures 1a and 1b, we shall refer to figure 3 which is our schematic representation of DNA molecules in the alkaline sucrose. The profile shown in figure 1a was obtained from cells which were labelled with BUdR for slightly less than one generation time, and hence some ^{14}C-DNA was probably not replicated during the interval of exposure to BUdR. This unreplicated DNA is represented in figure 3a as incompletely separated ^{14}C-labelled sister strands and this DNA constitutes the 550 S ^{14}C-DNA peak present in both figures 1a and 1b. Figure 3b illustrates the effect of a low dose of light on DNA with one strand BU-substituted. The single strand breaks in the BU strand allow some of the fragments of this strand to separate from the ^{14}C strand. In figure 1a the separated ^{3}H-BU-DNA fragments resulting from inadvertent exposure to light sediment in a broad peak at 170 S while the ^{14}C strand with the attached ^{3}H-BU strands sediment at 450 S. Figure 3c represents the situation in figure 1b where the ^{3}H-BU strand has been extensively broken so that all ^{3}H-BU strand fragments separate from the ^{14}C strand. The ^{3}H-BU-DNA sediments at about 100 S while the ^{14}C-DNA is found in a narrow 320 S peak. The narrow distribution of ^{14}C-DNA at 320 S indicates neither molecular homogeneity nor DNA aggregation. Rather, it is probably a result of a previously described speed-dependent sedimentation phenomenon for large single stranded DNA (11,12) which would suggest that the 320 S peak is composed not only of DNA molecules which are 2 x 10^9 daltons, but of all molecules which are larger. The value of 2 x 10^9 is, then, a minimum estimate for the DNA molecular weight in this gradient.

The data of figure 2, as well as providing additional evidence for the single stranded nature of the 320 S DNA, also indicate that single strand breaks in this DNA may be detected following doses as low as 220 rads. This is a dose which is well within the biologically significant range, hence the system provides a sensitive assay for single strand breaks in DNA.

It has been previously postulated that the DNA in mammalian chromosomes

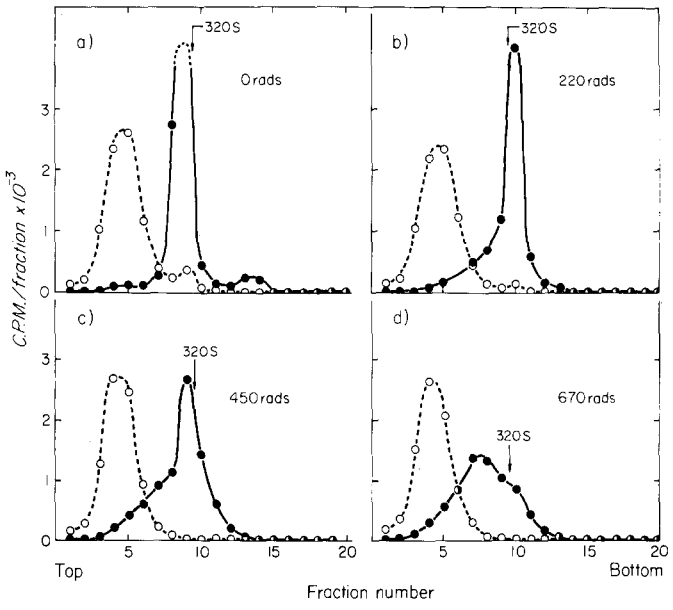

Figure 2.

The effect of x-irradiation of cells on the sedimentation properties of DNA labelled as in figure 1 with ^{14}C-TdR (●——●) and ^{3}H-BUdR (o----o). Control and x-irradiated cells were exposed to fluorescent light for 2 minutes prior to being layered onto the gradients. X-ray doses given to cells are shown in each panel.

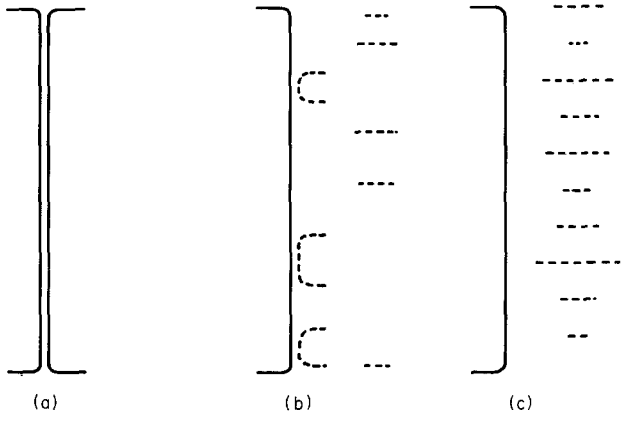

Figure 3.

A schematic model for the form of DNA in the alkaline sucrose gradients of figures 1a and 1b. Solid lines represent ^{14}C-labelled DNA and dashed lines are ^{3}H-BU-labelled strands. Panel a represents ^{14}C-DNA homoduplex which is single stranded only at the molecule ends. The regions of parallel lines represents regions of interwound sister strands. Panel b represents DNA with one BU-substituted strand. This strand contains few single strand breaks. Panel c represents BU-substituted DNA where the BU strand has been extensively fragmented.

consists of structural subunits each having a single stranded molecular weight of approximately 5 x 10^8 (6) or less (2). The experiments described above indicate that the majority of DNA molecules in the mammalian nucleus have molecular weights in excess of 4 x 10^9. Since the technique of velocity sedimentation in alkaline gradients appears to be unsuitable for determination of sedimentation coefficients of greater than 320 S, it seems possible that the DNA sedimenting at 320 S is much larger than 2 x 10^9 daltons and may, in fact, be as large as the entire DNA compliment of a chromosome.

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