CONFORMATION OF DNA IN ALKALINE SUCROSE: THE SUBUNIT HYPOTHESIS IN MAMMALIAN CELLS

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Received May 6,1974

SUMMARY

Alkaline lysis and sucrose gradient sedimentation of mammalian cells produces DNA molecules with sedimentation coefficients up to 400 S. Prolonged lysis can produce molecules that are relatively stable to further exposure to alkaline conditions and have a peak sedimentation coefficient of approximately 140 S. The size of these alkali-stable molecules appears to represent an upper limit for single-stranded DNA obtained by alkaline lysis. Faster sedimenting molecules are predominantly double stranded and may be DNA molecules that have not denatured fully during exposure to alkali.

Mammalian cell DNA, when studied by alkaline sucrose gradient techniques, exhibits a variety of possible sedimentation properties dependent on the precise conditions adopted for cell lysis on the top of the gradients (1-5). The terminal DNA product obtained from unirradiated cells after long lytic times (several hours) consists of a population of molecules with a peak sedimentation coefficient (S) between 120 and 165 S (1,2,6-10). These molecules have been regarded as representing some fundamental "subunit" in the organization of DNA (1). This term will be used here, but without implications of biological significance.

Lytic times shorter than those required to produce the subunit-sized molecules or addition of sucrose or salt to the lytic layer enables DNA-containing structures to be obtained with sedimentation coefficients as high as 300-400 S (1,4,5,8,9) or DNA-membrane complexes exhibiting anomalous sedimentation behavior (2). There is increasing evidence that DNA with high sedimentation coefficients (5) and the complex (11,12) are not necessarily fully denatured single-stranded molecules. Consequently I attempted to

measure the amount of single- and double-stranded DNA present in neutralized fractions of sucrose gradients over a wide range of sedimentation coefficients. The results indicate that the subunit may represent the upper limit of size for single-stranded molecules.

MATERIALS AND METHODS

Culture Techniques and Radioactive Labeling

Chinese hamster (V79) cells were grown in Eagle's minimum essential medium with 10% fetal calf serum. DNA was labeled by growing cells for several genetations (24-48 hr) in 0.05 - 0.1 μCi/ml, 33 mCi/mmole ^{14}C-dThd. Some ^{14}C-labeled cultures were frozen at -196°C in medium plus 10% DMSO and stored for periods up to 1.5 yr. These frozen samples were used for other experiments to observe DNA strand breaks resulting from radioactive decay (7,13) but were also used as a source of ^{14}C-labeled DNA with known amounts of radioisotope damage and sedimentation coefficients less than that of the subunit.

Alkaline Sucrose Gradient Sedimentation

Sucrose gradients were done as described previously (1,5,7). After centrifugation at 25,000 revs/min in SW25.1 rotors, 1-ml fractions were collected into tubes that contained 1 ml of 0.5 M KH_2PO_4. The final pH of the collected fractions was between 6 and 7 so that neutralization was rapid and would preserve as far as possible the double-stranded or single-stranded character of the DNA in the gradients. Radioactivity was determined in 50 μl of each fraction as previously described (7). The sucrose gradients were calibrated with T_4 phage DNA (1). Standards consisting of ^{3}H-labeled double-stranded DNA and heat-denatured DNA were prepared as described previously (5). Fractions from sucrose gradients representing DNA that had sedimentation coefficients between 30 and 400 S were selected, and alkali-denatured single-stranded or double-stranded ^{3}H-labeled DNA

standards were added and dialyzed against 0.3 M NaCl, 0.03 M sodium acetate, 0.003 M $ZnCl_2$, pH 4.5, for 2 days before further assay by enzyme digestion.

Determination of Proportions of Single-stranded DNA

Quantitative estimates of the proportion of single-stranded DNA in a mixture of single- and double-stranded DNA were made using a crude extract of a single-strand-specific nuclease S_1 from *Aspergillus oryzae* prepared as described by Sutton (14). 10 µl of this enzyme solution was added to an incubation mixture consisting of the labeled DNA from alkaline sucrose gradients in 1 ml of 0.3 M NaCl, 0.03 M sodium acetate, 0.003 M $ZnCl_2$, pH 4.5, containing 10 µg/ml nonradioactive heat-denatured calf thymus DNA. Incubation was continued for up to 2 hr at 50°C, after which samples were chilled on ice, 100 µg of calf thymus DNA was added as carrier, and 50 µl of 70% PCA was added to precipitate macromolecular DNA. The percentage of DNA rendered soluble by S_1 nuclease, i.e., the percentage of single-stranded DNA, was calculated from the radioactivity in supernatants and precipitates as previously described (5).

RESULTS

S_1 nuclease degrades single-stranded DNA to various extents depending on the precise incubation conditions, the method of DNA preparation, and the DNA source (15-17). In preliminary experiments it was found that 30-min incubation at 50°C was sufficient for S_1 nuclease to degrade 91-98% of heat-denatured DNA, 86% of alkali-denatured DNA, and 1.5% of double-stranded DNA. In dialyzed gradient fractions only 80% of alkali-denatured ^3H-labeled DNA standards was degraded even after 2-hr incubation at 50°C, at which time 7-8% of double-stranded DNA was rendered acid soluble. This reduced amount of degradation may have been due to the presence of some interfering material from gradients or secondary structure to DNA introduced during dialysis. The precise reason for the interference was not resolved, but since discrimina-

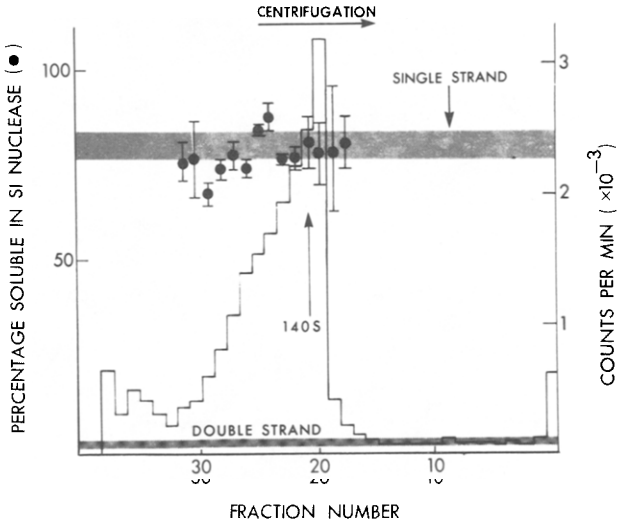

Fig. 1. Alkaline sucrose gradient profile (cpm, histogram) of subunit-sized molecules obtained by lysing V79 cells for approximately 4 hr in alkaline EDTA before centrifugation and percentage of DNA degraded by S_1 nuclease (●). Each fraction assayed in triplicate by S_1 and mean and standard errors indicated for percentage degradation. Shaded areas indicate extremes of standard error for nuclease digestion of DNA standards.

tion between standards known to be initially single or double stranded was sufficiently large (80% versus 8%, respectively), analysis of DNA from sucrose gradients was performed under these conditions of 2-hr incubation at 50°C.

In the first series of experiments, two gradient profiles obtained by different lytic procedures were analyzed to determine whether there was any variation in the properties of DNA across the profiles. A subunit profile (Fig. 1) contained single-stranded DNA throughout, but one obtained by lysing for 2.5 hr (185 S peak) (Fig. 2) contained a variable amount of single- and double-stranded DNA, the leading edge being more double-stranded than the trailing edge.

In a second set of experiments a number of gradients was done by varying the lytic time before centrifugation for unirradiated cells or by using cells that had been frozen for long periods and had accumulated high doses of ^{14}C-beta irradiation. These gradients provided sources of DNA with sedimentation coefficients between 30 and 400 S that were analyzed to see whether

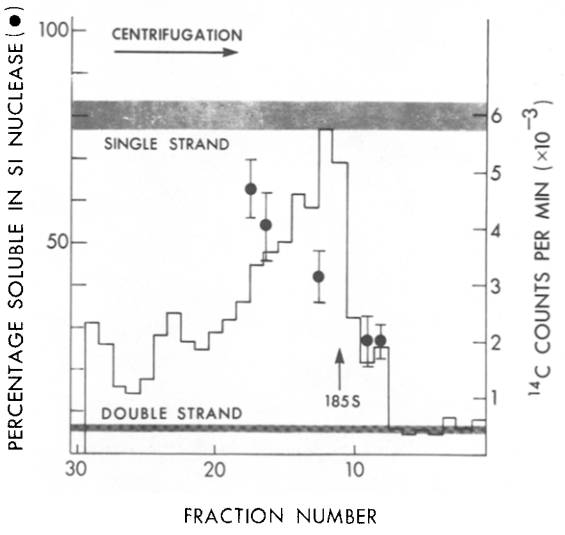

Fig. 2. Alkaline sucrose gradient profile (cpm, histogram) of DNA from V79 cells lysed for 2.5 hr in alkaline EDTA before centrifugation and percentage of DNA degraded by S_1 nuclease (●) from various portions of the gradient profile. Each region assayed in triplicate by S_1 and mean and standard errors indicated for percentage degradation. Shaded areas indicate extremes of standard error for nuclease digestion of DNA standards.

there was any general relationship between the sedimentation coefficient and the degree of nuclease susceptibility, irrespective of the manner in which the DNA was produced. The sedimentation coefficient of the subunit appeared to represent an upper limit for single-stranded molecules (Fig. 3). Below the subunit S value, DNA from the gradients was degraded to the same extent as single-stranded DNA standards; above this value, DNA from the gradients appeared to be predominantly double-stranded, although it was degraded to a greater extent than double-stranded DNA standards (Fig. 3). The presence of detergent during lysis of cells, although accelerating the rate at which cells lysed to produce subunit-sized DNA, did not affect the proportions of single- and double-stranded DNA at various S values. DNA with sedimentation coefficients greater than that of the subunit will be referred to as "high S" DNA.

DISCUSSION

These results suggest that the DNA molecules described as subunits in alkaline sucrose gradients are the fastest sedimenting single-stranded mole-

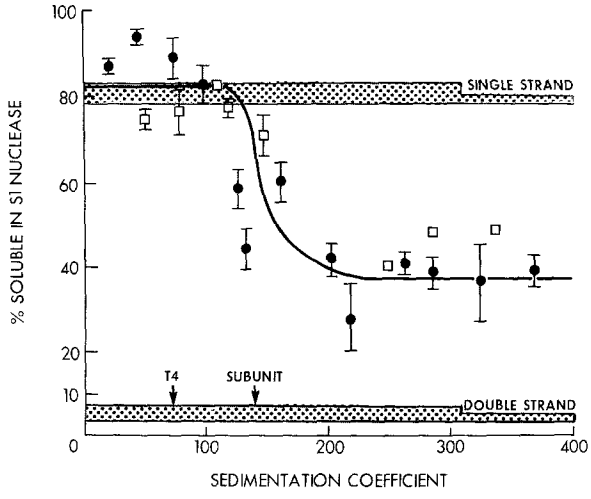

Fig. 3. Percentage of DNA, neutralized from alkaline sucrose gradients, that is degraded by S_1 nuclease as a function of the sedimentation coefficient. ●, DNA from cells lysed in alkaline EDTA before centrifugation; □, DNA from cells lysed in alkaline EDTA containing 0.1% Nonidet NP40. Standard error bars indicated for DNA from three or more sucrose gradient profiles covering a range no wider than ± 10 S; other data from single sucrose gradients. Shaded areas indicate extremes of standard error for nuclease digestion of DNA standards.

cules observed under conditions of alkaline lysis and high-speed (25,000 revs/min) centrifugation (Figs. 1-3). DNA with higher S values appears to be predominantly double-stranded. Previous experiments using isopycnic analysis have also shown that high-S DNA is a mixture of molecules with double-stranded and single-stranded densities (5).

At least three interpretations of these results can be suggested:

(a) A differential speed effect on single- and double-stranded molecules during centrifugation results in all single-stranded molecules exhibiting an S value no larger than 140 S (10,18).

(b) Aggregation to form gels or networks (19) that have double-stranded, S_1-resistant regions may increase sharply above a critical size of DNA.

(c) Denaturation and strand separation in alkali may involve breaks from shear forces or hydrolysis at random or specific linkage

sites. This could set an upper limit to the size of single-stranded DNA that is stable at high pH.

Speed and molecular weight dependence of sedimentation coefficients appears an unlikely interpretation because this would require a greater speed effect on single- than on double-stranded DNA. Zimm's (18) analysis of sedimentation coefficient versus molecular weight and centrifugation speed indicates that such effects would be in the opposite direction (see also review, 20).

Explanations such as (b) and (c) seem to be the most reasonable interpretations of these results. The consistent value of about 30-40% single-strandedness obtained in these experiments for high-S DNA may be determined by some structural feature such as the proportion of repetitive DNA (21) that would renature most rapidly. The data and these interpretations indicate that the subunit has a unique status among DNA molecules of various sizes. It represents the largest single-stranded molecule obtained by alkaline lysis in these experiments either because of a high degree of aggregation of larger molecules or because of a physical limitation to size set by denaturation forces. Whether or not the subunit has biological significance in chromosomal structure, for example by being the basic unit in construction of larger molecules by alkaline labile linkages (1), cannot be decided on the basis of the present experiments.

ACKNOWLEDGMENTS

I am grateful to J.M. Bishop for introducing me to the use of S_1 nuclease, to G.H. Thomas and W. Charles for technical assistance, and to J.T. Lett and K. Wheeler for their stimulating role in the development of this work. Work performed under the auspices of the U.S. Atomic Energy Commission.

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