INTERRUPTIONS IN SINGLE STRANDS OF THE DNA IN SLIME MOLD AND OTHER ORGANISMS

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ABSTRACT The molecular weight of single-stranded DNA from the slime mold *Physarum polycephalum* has been determined by alkaline gradient centrifugation. The average molecular weight during DNA synthesis ($\sim 1.5 \times 10^7$ D) is less than that observed in nonsynthetic periods ($\sim 4 \times 10^7$ D). On the basis of a chromosome number of 50 per nucleus and a DNA content of 1 $\mu\mu$g per nucleus, we are led to conclude that at pH 12 each chromosome dissociates into 300 (single-stranded) pieces of DNA. We have also compared the sedimentation profiles of single-stranded DNA from *Escherichia coli*, PPLO, and T2 bacteriophage. These data support the conjecture that each bacterial chromosome can be dissociated into 10 or 12 single-stranded pieces of DNA. Dissociation of DNA into multiple pieces under our experimental conditions is best interpreted in terms of interruptions in the continuity of the DNA either by naturally occurring gaps or at alkali-labile bonds.

INTRODUCTION

It is possible to use alkaline sucrose gradient centrifugation to study large pieces of single-stranded DNA[1] from *Escherichia coli*. These pieces have an average molecular weight of about 2×10^8 daltons (D) (1, 2). Based on radioautographic measurements of chromosomal length (3), this molecular weight represents approximately $\frac{1}{6}$ the length of the *E. coli* chromosome. It seemed reasonable to conclude that shearing and mechanical breakage precluded isolation of the intact chromosomal length (if such existed). During preliminary experiments with the PPLO *Microplasmodium laidlaw B*, however, we discovered that single strands of DNA larger than 2×10^8 D could be obtained by the methods we were using. Moreover, on alkaline sucrose gradients the DNA from PPLO was distributed symmetrically, whereas that from *E. coli* was not. Abelson and Thomas (4), by similar alkaline gradient methods, had observed that the single-stranded DNA of bacteriophage T5 was composed of multiple pieces, whereas that of T2 was continuous. The latter

[1] "Single-stranded DNA" should be interpreted as DNA that dissociates into single strands during alkaline treatment.

finding, combined with the observations of PPLO, suggested to us that the *E. coli* chromosome might actually be composed of several (perhaps five or six) pieces of DNA in each single strand. If so, higher organisms might also be expected to contain interruptions in the DNA of each chromosome.

To test this hypothesis we used synchronously growing cultures of the slime mold *Physarum polycephalum* as a DNA source and devised methods for measuring the molecular weight of its nuclear DNA. The purpose of this paper is to describe these experiments and to reexamine our *E. coli* data related to this question. Results of our investigation lead to the conclusion that in interphase (G_2) slime mold the average single-stranded molecular weight of DNA is about 4×10^7 D. We interpret our data as evidence that single-stranded DNA of both *Physarum* and *E. coli* chromosomes is structurally interrupted instead of being continuous in length.

MATERIALS AND METHODS

Slime mold (*Physarum polycephalum*) was grown on a semidefined culture medium that contained hemin (5). A general discussion of laboratory methods for this organism is contained in a review by Daniel and Baldwin (10). A description of our laboratory conditions for maintaining shake cultures and growing cultures that divided synchronously is given in a previous report (6). Deoxyribonucleic acid, RNA, and protein were labeled respectively with ^{3}H-thymine (6.7 c/mM, 36 mμg/ml), ^{3}H-uridine (5 c/mM, 26 mμg/ml), and a ^{14}C amino acid mixture (average values 0.1 mc/ml, 670 mμg/ml of culture medium). Medium containing the radioisotope was added to cultures at the time of fusion, and growth in the presence of labeled precursor was continued for two or three cycles of division and DNA synthesis. Cultures were examined microscopically to determine the cell cycle stage. Nuclei were isolated from the slime mold essentially by the technique of Mohberg (7), resuspended in 0.1 M Tris buffer at pH 8.1, and pipetted into a layer of 0.5 N NaOH that was floating on top of a 5–20% alkaline (pH 12.0) sucrose gradient. The volume of nuclear suspension used was between 20 and 50 μl (approximately 2×10^6 nuclei). Preparations were then allowed to remain at room temperature (22°C) for 1 hr in order to disrupt nuclei and denature the DNA before centrifugation at 30,000 rpm for 2.5 hr at 20°C in an SW-39 rotor of a Spinco model L centrifuge (Spinco Div., Beckman Instruments, Inc., Palo Alto, Calif.).

PPLO was grown in typtose medium[2] and labeled by adding ^{3}H-thymidine (same concentration and specific activity as given above) to the medium and allowing growth to continue overnight. Cells were centrifuged, washed in medium without ^{3}H-thymidine, resuspended, and applied directly to 0.5 N NaOH that was floating on top of alkaline sucrose gradients. Gradient centrifugation conditions were 90 min at 30,000 rpm and 20°C. After centrifugation the tubes were punctured and two drop-fractions were collected on filter paper discs. Discs were processed to remove acid-soluble radioactive material and were counted in a liquid scintillation counter. Additional details of this procedure may be found in a previous report in which handling procedures for *E. coli* and T2 bacteriophage have been given (2). Other technical details are given in the text as necessary.

[2] Tryptose (Difco Laboratories, Detroit, Mich.), 20 g; NaCl, 5 g; Tris, 3.75 g; and H_2O, 1000 ml. Adjusted to pH 8.0; glucose, 1%; penicillin, 100 units/ml; phenol red, 0.018 g/l. Recipe supplied by M. Tourtelotte.

RESULTS AND CONCLUSIONS

Single-stranded DNA obtained from slime mold nuclei by alkaline sucrose gradient centrifugation has a sedimentation constant ($s_{20,w}$) of about 59. The corresponding molecular weight (mol wt), calculated from Studier's values for alkaline conditions (8), is approximately 4×10^7 D (Fig. 1; also, see note at end of Table I). A comparison of $s_{20,w}$ and mol wt of single-stranded DNA from *E. coli*, T2 bacteriophage, the PPLO *M. laidlaw B*, and the slime mold *P. polycephalum* is given in Table I.

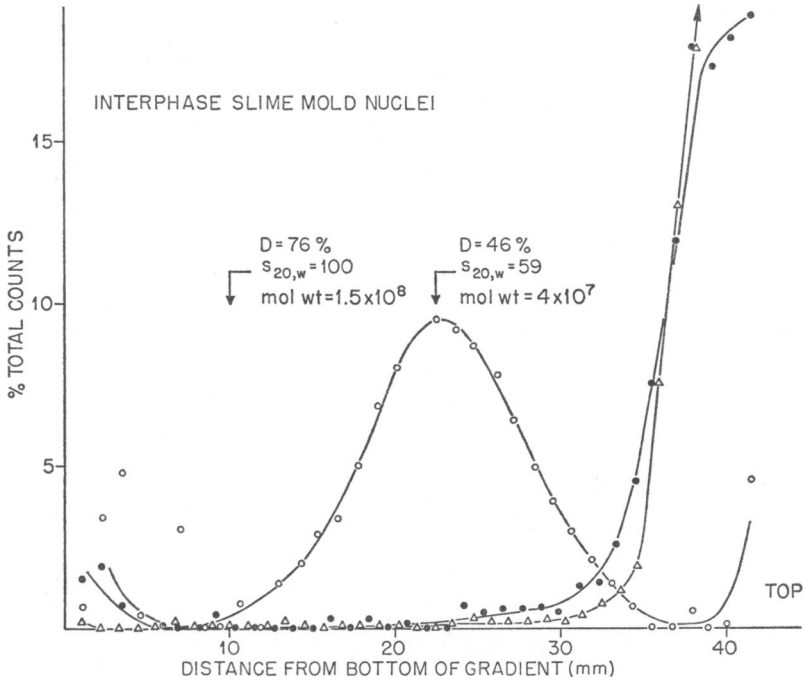

FIGURE 1 Profiles of radioactivity from labeled interphase (G_2) slime mold nuclei on alkaline sucrose gradients. ○, labeled with ^{3}H-thymidine; △, labeled with ^{3}H-uridine; ●, labeled with a mixture of 13 ^{14}C amino acids. In all cases slime mold was grown in the presence of radioisotope for two or three cycles of division and DNA synthesis. Centrifugation time: 150 min at 30,000 rpm. 10,000–20,000 cpm were placed on the gradient. Arrows show the position of various $s_{20,w}$ and mol wt values related to the distance sedimented (indicated as a percentage of gradient length). Calculation procedures are given in notes at end of Table I.

Several approaches were used to establish that the relatively low molecular weights given for slime mold DNA do not represent experimental artifacts but are probably maximum values for uninterrupted lengths of DNA.

1. Since our handling methods, which were similar with all organisms tested, allowed detection of pieces of DNA as large as 2–4 $\times$ 10^8 (for PPLO), it is clear that the small pieces of DNA isolated from slime mold do not result from uncontrolled shearing or mechanical breakage of larger molecules during handling.

2. To test whether incubation in 0.5 N NaOH caused degradation of denatured DNA, preparations of *E. coli* protoplasts, slime mold nuclei, and intact T2 bacteriophage were allowed to remain separately in 0.5 N NaOH that was floating on top of alkaline gradients for several hours at room temperature before centrifugation. Subsequent centrifugation and analysis of data showed no significant differences in the sedimentation constant of the DNA pieces isolated after 1 and 16 hr (slime mold), 10 min and 3 hr (T2 bacteriophage), or 10 min and 5 hr (*E. coli*). On the basis of these experiments we were satisfied that the alkaline treatment used in the course of the experiments had not degraded large molecules of DNA into smaller pieces.

TABLE I

COMPARISON OF SOME PHYSICAL CHARACTERISTICS OF SINGLE-STRANDED DNA ISOLATED BY ALKALINE GRADIENT CENTRIFUGATION

Organism	$s_{20,w}$*	Molecular weight‡ daltons	Fraction of chromosome length
E. coli§	115	2.2×10^8	1/6
T2 bacteriophage	70	6×10^7	1/1
PPLO *M. laidlaw B*	110–145	$2\text{–}4 \times 10^8$	1/2–1/1
Slime mold (non-S)‖	59	4×10^7	1/300
" " (S)	40	1.5×10^7	uncertain

* Based on modified Burgi-Hershey relationship where $s_{20,w} = \frac{\beta \times 10^{10} D}{(\text{rpm})^2 T}$ (9). D = distance sedimented in centimeters; T = time in hrs; $\beta = 7.1$ (see reference 2).

‡ Based on Studier's relationship for DNA in alkaline conditions where $s_{20,w} = 0.0528\ M^{0.400}$ (8).

§ Values are the same for the following strains tested by us: B/r, B_{s-1}, 15 T-, B 94; and the same for strains WP_2 Hcr-Try-, AB1884, AB1885, AB1886, and B_{s-3}, tested by S. Roland in our laboratory.

‖ Non-S = nuclei labeled for several cell generations then isolated and centrifuged when not in DNA synthesis. S = nuclei from cultures exposed to ^{3}H-thymidine for a 30 min pulse during the DNA synthesis period then lysed and centrifuged. The S period lasts about 3 hr.

Note: $s_{20,w}$ and mol wt are calculated on the basis of the position of maximum radioactivity for all distributions other than *E. coli*. Since *E. coli* has a very assymmetrical distribution, the weight average molecular weight was calculated and is given in the table. The corresponding peak values for *E. coli* are $s_{20,w} = 129$, mol wt $= 3 \times 10^8$, and fraction of chromosome length = 1/5.

3. To test whether nucleases had degraded DNA during the nuclear isolation procedure but prior to alkaline gradient centrifugation, we proceeded as follows: A labeled, synchronously growing culture of slime mold was frozen in liquid nitrogen then ground to a fine powder at liquid nitrogen temperature (Intact nuclei are microscopically visible after this treatment.). A small amount of frozen powder was sprinkled directly on top of a layer of 0.5 N NaOH that was floating on an alkaline sucrose gradient. After incubation at room temperature for 1 hr, the preparation was centrifuged. The sedimentation constant of labeled DNA isolated in this manner was

not larger than that obtained from isolated nuclei. These results show that DNA is not reduced in molecular weight during the nuclear isolation procedure.

To determine whether the material we have been calling DNA was in fact this nucleic acid alone or a linked DNA-RNA molecule, isolated nuclei were incubated in the presence of 50 μg/ml of RNase for 45 min at 37°C. These nuclei were centrifuged on alkaline sucrose gradients and the position of radioactive DNA determined and compared to that of nuclei which had been incubated in buffer without RNase for 45 min at either 37°C or at ice bath temperature. The peaks of radioactive material were at identical positions in each of the three samples, thus showing that (*a*) no degradation of DNA had occurred during this treatment and (*b*) no RNase-sensitive material could be dissociated from the DNA in amounts sufficient to change the sedimentation constant of the DNA. In separate experiments slime mold cultures were labeled with ^{3}H-uridine, after which the nuclei were isolated and subjected to alkaline gradient centrifugation. Fig. 1 shows that within the limits of detection of this method most of the radioactivity remains on top of the gradient. 95% of the ^{3}H-uridine radioactivity is found in regions of the gradient other than the DNA-sedimenting position. The lack of RNA associated with the sedimenting DNA agrees with a similar result from studies with *E. coli* (see Fig. 2).

To determine the level of protein contamination in the case of slime mold DNA, cultures were exposed to a mixture of 13 ^{14}C-labeled amino acids for several cell generations. Nuclei were isolated and subjected to alkaline gradient centrifugation. 99% of the radioactivity remained on top of the gradient separated from the region in which DNA sediments (see Fig. 1). As an additional test, nuclei from slime mold cultures labeled with ^{3}H-thymidine were incubated for 2 hr in the presence of 100 μg/ml pronase at 37°C. That pronase worked under our conditions of incubation was obvious since the nuclear suspension was partially lysed during this time. A sample of lysate-suspension was placed on top of an alkaline sucrose gradient and centrifuged. The peak of radioactivity associated with labeled DNA was in the same position as that normally found with nuclei which had not been treated with pronase. Since DNA from nuclei treated with this general proteolytic enzyme had a sedimentation constant similar to that found with untreated cells, we conclude that any protein present with DNA at the time of centrifugation is insufficient to change the observed molecular weight within the limits of sensitivity of the method used.

DISCUSSION

As the alkaline sucrose centrifugation technique comes into general use for measuring the mol wt of single-stranded DNA (11), it is important to caution that treatment with alkali before centrifugation must be continued for a sufficient time to yield a constant molecular weight of DNA. Otherwise, the material observed may be an incompletely dissociated nucleoprotein or RNA-DNA complex. The necessary time in 0.5 N NaOH seems to vary from one organism to the next, ranging from

a few minutes for T2 bacteriophage to at least 30 or 45 min for slime mold nuclei. Other factors being equal, we recommend somewhat longer, rather than minimum, digestion times.

We conclude that in the slime mold *Physarum polycephalum*, pieces of DNA are found with an average single-stranded molecular weight of 4×10^7 D. Within the limitations of our detection and assay techniques, this DNA is essentially free of contaminating protein or RNA. If, however, contamination existed, an impression

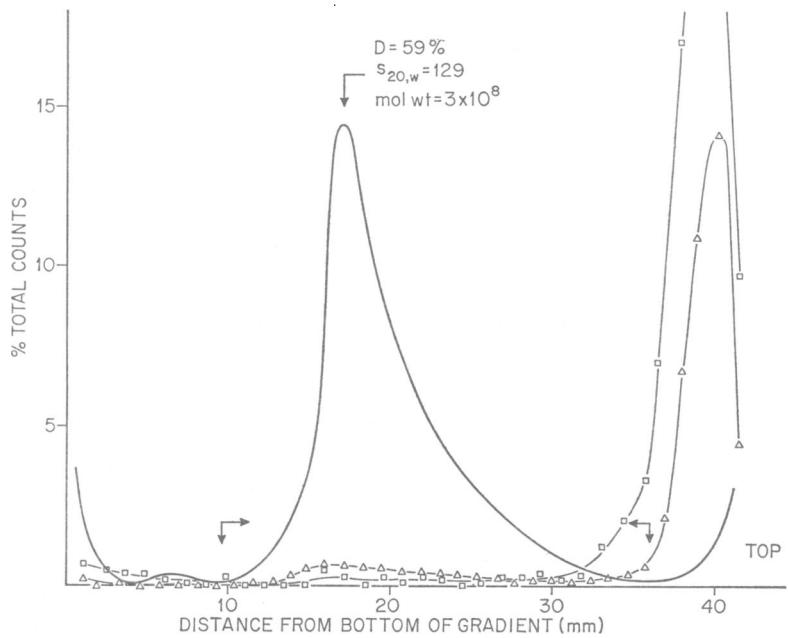

FIGURE 2 Solid curve, profile of radioactivity from ^{3}H-thymidine-labeled *E. coli* B/r grown in the presence of radioisotope for three to four cell generations. Centrifugation conditions were 90 min at 30,000 rpm. The curve represents an average of several trials and was redrawn from already published data (2). Double-barred arrows indicate region used to determine weight average mol wt (2.2×10^8 D). Single arrow at peak of curve indicates information as in legend for Fig. 1. Typical profiles of radioactivity from cells that were labeled with ^{3}H-uridine △, or ^{14}C-algal protein hydrolysate □, for several cell generations. Of the 13% labeled material within the DNA sedimenting region after ^{3}H-uridine labeling, only about 5% was shown (after hydrolysis and chromatography) to be uridine.

of larger, not smaller, pieces of DNA would be given. The molecular weights we report, then, are probably maximum values. Nuclei prepared by the methods used contain about 1 $\mu\mu$g DNA per interphase nucleus (7); 1 $\mu\mu$g represents approximately 6×10^{11} D per nucleus. If we assume a chromosome number of 50 [estimates range from 20 to 80 (J. W. Daniel. Personal communication)], each chromosome should contain approximately 12×10^9 D of (double-stranded) DNA. Since we observed experimentally an average mol wt of 4×10^7 D of single-stranded DNA, we are led to

conclude that each chromosome contains about 300 (single-stranded) pieces of DNA.[8] The distribution for *Physarum* DNA is nearly symmetrical but very broad. The value given must be considered as an average, and it is clear that both smaller and larger pieces of DNA are also present. In contrast, the shape of distributions from T2 bacteriophage particles is extremely sharp and reasonably symmetrical (see Fig. 3), indicating that nearly all molecules of DNA isolated from such preparations are of the same molecular weight (approximately 6×10^7 D).

It is of interest to reconsider the shape of the distribution found for DNA of *E. coli* in light of the observations reported here. This distribution is not symmetrical.

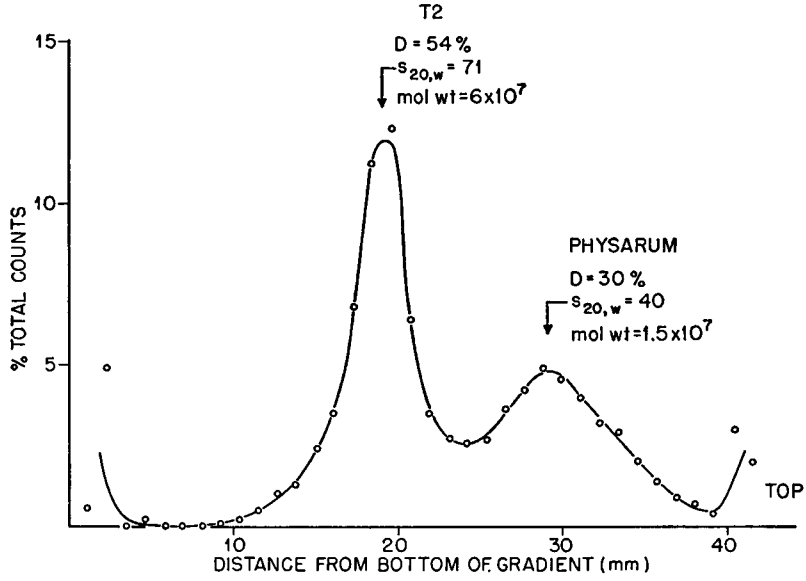

FIGURE 3 Cosedimentation profiles of ^{3}H-thymidine-labeled T-2 bacteriophage and slime mold nuclei in middle DNA synthesis. Slime mold was exposed to ^{3}H-thymidine for 30 min, starting 1 hr after an observed mitosis in the culture. Centrifugation conditions and indicating arrows are the same as those in Fig. 1. Single symmetrical peaks were observed for either T2 or *Physarum* when run independently.

An extremely sharp leading edge (high molecular weight) occurs at approximately 3×10^8 D, and this material is followed by a trailing distribution of smaller pieces (see Fig. 2). If sense can be made from such comparisons of shape, it is reasonable to conclude that in *E. coli* the majority of single-stranded DNA occurs in pieces that are approximately $\frac{1}{5}$ the expected value of the single-stranded chromosomal length. The trailing of smaller molecular weight pieces may well be a reflection of pieces of DNA in the process of synthesis and therefore shorter than the "finished" length.

[8] If material not susceptible to RNase or pronase is bound to sedimenting DNA, the apparent mol wt should be increased. An average of 300 single-stranded pieces per *Physarum* chromosome must therefore be considered as a minimum value.

In synchronous cultures of slime mold we compared the mol wt of DNA isolated from molds in non-DNA synthetic periods to that found after pulse labeling and isolation during the S period. Smaller labeled pieces can indeed be seen in DNA isolated during the S period of slime mold after such pulse-labeling experiments (see Fig. 3). These observations tend to support the interpretation given for the asymmetrical shape of the *E. coli* DNA distribution, but we admit that the experiments are not strictly comparable.

A consideration of PPLO is complicated by the fact that material of about 4×10^8 D seems near the limit that can be handled by the method used, but since the curves seem to be symmetrical we are inclined to argue that in this organism, the average length of unit pieces of single-stranded DNA is near $2\text{–}4 \times 10^8$ D. On the basis of radioautographic measurements by K. Wepsic of Yale University it is known that some circular pieces of DNA in *M. laidlaw B* have a length corresponding to this value (Personal communication). Thus it would seem that the size of DNA in PPLO (such as *M. laidlaw B*, at least) is either the whole or ½ of the chromosome length. It is clear, since a number of people have made essentially identical measurements, that in T2 bacteriophage the piece of DNA with a molecular weight of 6×10^7 D represents the entire single-stranded chromosomal length.

The question of how a large number of short pieces of DNA are assembled in chromosomes enters an area of sheer speculation. We shall spend little time considering this question other than to observe that (*a*) the pieces of DNA may be joined by some sort of "linker," or (*b*) gaps (single- or double-stranded) may exist between adjoining pieces, the integrity of the chromosomes being maintained by either hydrogen bonding of complementary regions or by nucleoprotein overcoating. It is alternatively possible that an interruption at any time in single-stranded DNA may reflect maintenance, i.e. correction of everyday wear and tear, rather than a discrete locus at which the DNA remains interrupted in a permanent way.

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