GROWING POINTS OF DNA IN CULTURED MAMMALIAN CELLS

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ABSTRACT A fraction of DNA, known to be replicating, was examined in the electron microscope. Characteristic branch points were identified as sites at which replication had been taking place. No localized strand separations, nor other structural alterations were discernible at these points.

INTRODUCTION

DNA in mammalian cells, as DNA in general, undergoes semiconservative replication (1, 2). Autoradiographic study of bacterial DNA (3) showed that the DNA engaged in replication has the appearance of a "fork." Since there is now a method for fractionating the DNA of mammalian cells and isolating replicated, replicating, and unreplicated DNA (4), we have attempted to examine such fractions of DNA with the electron microscope to look for "forks" at the point of replication and for any other structural features possibly associated with the replication process, such as a localized strand separation.

MATERIALS AND METHODS

Cultured mammalian cells (L5178Y) were labeled with 5-bromo-deoxyuridine (BUdR) during a 3–4 hr incubation period. The cells were washed, grown without BUdR for an hour, and finally pulse-labeled (10 min) with thymidine-^{3}H (TdR-^{3}H). DNA isolated from these cells was separated into "heavy" and "light" peaks by cesium chloride density gradient centrifugation. Most of the radioactivity was found between these two peaks (Fig. 1), indicating that the DNA has been separated into three fractions: (*a*) that which has just completed replication (heavy fraction, BUdR-labeled); (*b*) that which is in the process of replication (middle fraction TdR-^{3}H–labeled); and (*c*) that not yet engaged in replication (light fraction or unlabeled BUdR) (4).

DNA from each fraction was placed on a carbon-coated grid with a wide mouth pipette, and after 20 sec, "streaked" across the grid by touching the edge of the drop with a torn piece of filter paper. A drop of saturated uranyl acetate (pH 4.6) was placed on the grid for 20 sec and removed with filter paper as above. The grids were then transferred to a Petri dish lined with filter paper and allowed to dry.

The grids were examined in a Philips EM 200 electron microscope (Philips Electronics and Pharmaceutical Industries Corp., New York) operating at an accelerating voltage of 60 kv with a 35–50 μa beam current. The microscope was equipped with a 200 μ condenser aperture and a 40 μ objective aperture, and was used with the anticontamination device at a temperature of about −130°C.

Before each set of 12 plates was exposed, astigmatism with the objective aperture in place was compensated for to less than 0.1 μ by examining a holey grid. Study of the magnification, determined by means of a replica grating and polystyrene latex particles, revealed variations of up to 20% during the period of this study. Focus was always approached from over-focus (5).

Random fields from several grids were recorded at nominal magnifications of 43.400 × on Eastman Kodak lantern slides (medium and contrast grades) and printed on Kodabromide

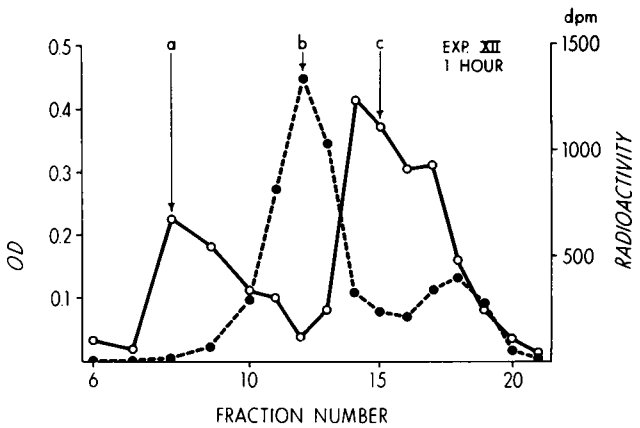

FIGURE 1 DNA fractions (*a*, *b*, and *c*) examined under the electron microscope. Solid line with open circles; optical density at 260 mμ. Dotted line with solid circles; ^{3}H activity in dpm. *a* is the fraction that is newly replicated, *b* the fraction that is replicating, and *c* the fraction that has not engaged in replication yet.

paper (Eastman Kodak Co., Rochester, N. Y.). Thus each micrograph included only parts of very long strands. Strands with a diameter less than 30 A and greater than 15 A in diameter were measured with a Keufel and Esser map measure which had a 3% error for straight lines, and a reproducibility of 0.40% and 0.46% for straight and wavy lines, respectively. The total length of these strands, the number of ends, and the number of sites (termed "forks") at which two 20 A fibers emanated from a single 20 A fiber were recorded.

RESULTS AND DISCUSSION

Electron microscope examination of the DNA revealed very long strands running in one direction. The strands were easily identified as belonging to one of three classes: 10 A wide, 20 A wide, and 40 A or more wide. Precise measurements of these widths were neither practical nor valid because of the graininess of the electron microscope images; but the division by classes was clear and reproducible.

In the three fractions (*a*, *b*, and *c* in Fig. 1), most DNA strands were 20 A in width. Many strands were 40 A or thicker, and occasionally a 20 A strand divided to reveal

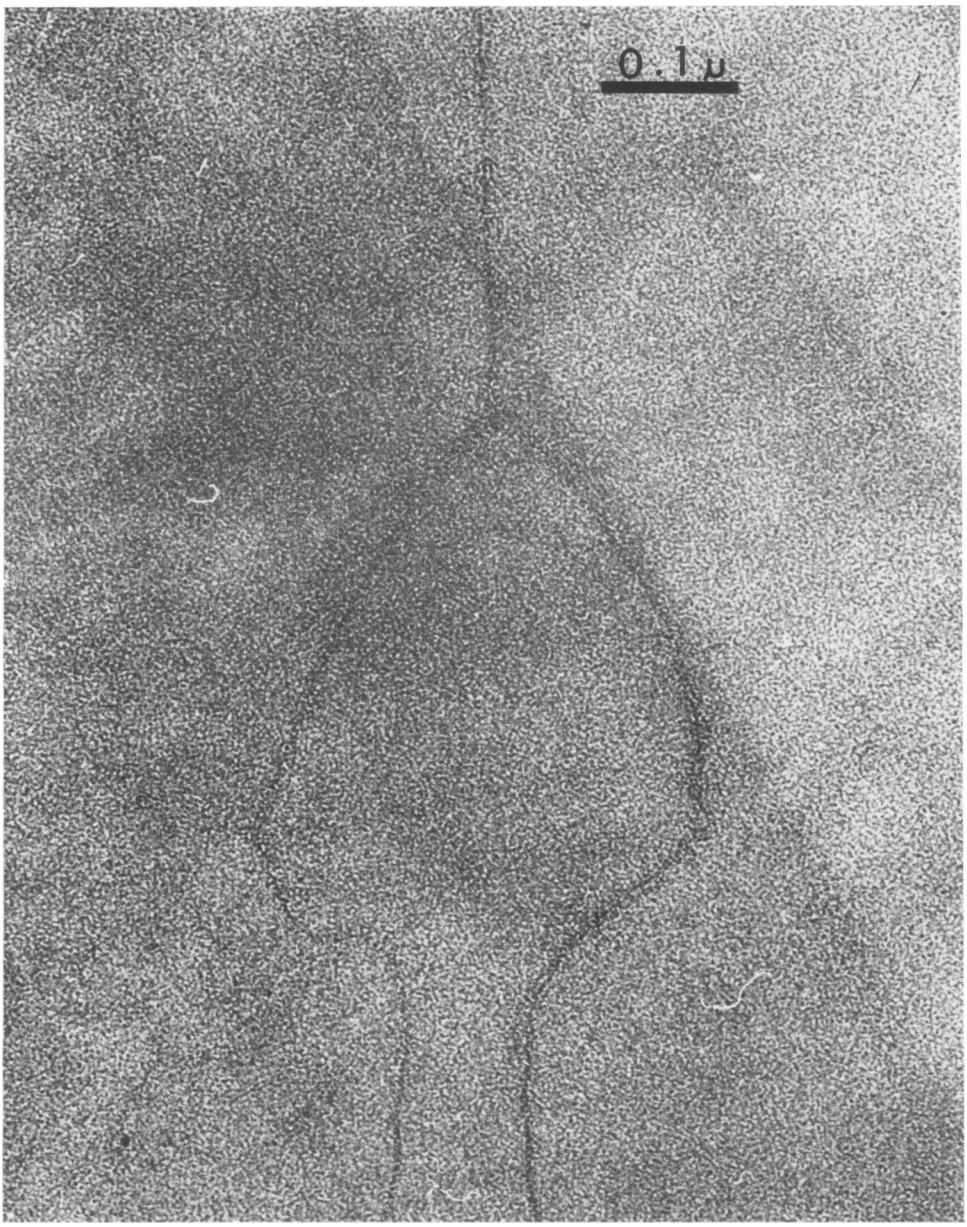

FIGURE 2 Electron micrograph of a portion of a DNA molecule present in the fraction *b*, the fraction containing replicating DNA. One 20 A strand is seen to divide into two 20 A strands and thus this structure represents a "fork." 188,000 ×.

two 10 A strands. The DNA strands of 20 A width were probably double-stranded DNA (6, 7), while thicker strands may represent two or more 20 A strands side by side. The 10 A strand may well be a single polynucleotide strand. Presumably the "streaking" process that orients the DNA fibers maintains the linearity of this single chain. One characteristic feature of the fraction (*b*) of replicating DNA was the presence of "forks;" these structures appear as 20 A strands which divide into two 20 A strands (Fig. 2). No such forks were seen in other fractions (*a* and *c*) or nonreplicating DNA.

We examined random micrographs of the two nonreplicating preparations to determine length, the number of forks, and the number of ends of 20 A strands.

TABLE I
RESULTS OF MEASUREMENTS ON SAMPLES OF REPLICATED DNA AND DNA IN THE PROCESS OF REPLICATING

	Replicated DNA (fraction *a*)	Replicating DNA (fraction *b*)
Length measured	94.2×10^4 A	98.6×10^4 A
Number of ends	15	19
Number of forks	0	8
Molecular weight of DNA pieces/ends	2.4×10^7 daltons*	—
Molecular weight/fork of DNA pieces	—	2.3×10^7 daltons‡

* Molecular weight of DNA pieces was estimated by assuming two ends per piece. Total length $\times$ 1.96 $\times$ 10^6 daltons/μ $\times$ $\frac{1}{\text{number of ends}/2}$.

‡ Molecular weight of DNA pieces was estimated by assuming one fork per one piece. Total length $\times$ 1.96 $\times$ 10^6 daltons/μ $\times \frac{1}{\text{number of forks}}$.

As shown in Table I, no forks were recorded in the nonreplicating DNA fraction (*a*). The average size of DNA was estimated by dividing the total length by one-half of the number of ends and by multiplying this figure by 1.96 daltons per micron (8, 9). This gave 2.4×10^7 daltons. In the replicating fraction (*b*), the DNA strands with forks should have three ends; since we do not know what percentage of DNA in this preparation has three ends, a better estimate of the average size of DNA was obtained by dividing the total length by the number of forks. This gave a value of 2.4×10^7 daltons. It should be added that the size of the DNA in this fraction had been reported previously to be 1.3×10^7 daltons by sedimentation study (4) and we found it to be 1.3×10^7 daltons after preparation by the method of Kleinschmidt et al. (10). The reason for this discrepancy may be that the method of preparation for electron microscopy, the "streaking" process, may select for the longer strands in a sample. Considering the possible errors in the different methods

of estimation of sizes, agreement of this magnitude is reasonably good and acceptable to confirm that the DNA was not grossly altered during preparation.

By eliminating the following three possibilities, it is considered likely that the forks seen in the electron micrographs represent the replicating points of DNA.

(*a*) The fork could be an artifact in which one end of a 20 A strand was attached to a side of another 20 A strand. If this were the case, one would expect to find "forks" in all three fractions; however, they were found only in the fraction containing DNA in the process of replication.

(*b*) The fork may contain partially single-stranded structures (a result of separation of double strands to two single strands during preparation), but such single-stranded portions may have a thicker "coat" of uranyl stain, thus measuring about 20 A. Then, both single and double strands would appear 20 A wide. This possibility, however, is unlikely since in the present experiment we observed the presence of uranyl-stained 10 A strands.

(*c*) The fork could be a "pleat" similar to that described by Mitra and Kornberg (11); each arm of the fork representing a hairpin loop (i.e., one strand doubling back on itself). If this were the case, one should have seen some "puddles" (an accumulation of denatured single-stranded DNA in the end of loops). However, these were not seen in the terminals of the arms of the forks.

Bode and Morowitz (12) demonstrated sites of replication in *Mycoplasma* chromosomes. These were prepared according to a modified Kleinschmidt (10) procedure which "coats" the DNA with basic protein and shadows the resulting fiber with platinum. These authors speculated that if any fine structure alterations, such as localized strand separations of single polynucleotide chains, were present at these sites they were below the 40 A resolution limits of their method. We find that even with resolution sufficient to discern single polynucleotide strands (about 10 A) such alterations are not seen. If, however, only one or two base pairs were separated during replication these would still lie below the resolution limits of the electron microscope. Alternatively, the "streaking" process may bring such separated strands to a close enough apposition so that the separation is not evident (an extremely likely situation if the DNA is still helical) or so that some renaturation can occur.

CONCLUSION

Three fractions of DNA, that which is newly replicated, that which is replicating, and that which has not yet engaged in replication, were examined under the electron microscope. "Forks" appearing as one 20 A wide DNA strand which branches to form two strands each 20 A in diameter, occurred only in the replicating fraction and not in the other two fractions. The size of DNA calculated from the length of 20 A strands divided by the number of forks in the replicating fraction was comparable to the size of the DNA in the preparation as measured by other methods.

It is suggested, therefore, that the forks seen in the replicating fraction are likely to be the replicating points of DNA in mammalian cells. No other special structural feature appeared to be associated with these replicating forks.

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