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Extrachromosomal DNA of pea (*Pisum sativum*) root-tip cells replicates by strand displacement

(DNA replication/single-stranded DNA/electron microscopy)

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ABSTRACT **In cultured pea roots there is extrachromosomal DNA associated with cells that differentiate from the G_2 phase of the cell cycle that is absent from those that differentiate from the G_1 phase. We examined this extrachromosomal DNA by electron microscopy and found that it consisted of three types: (*i*) double-stranded linear molecules with single-stranded branches (74%), (*ii*) double-stranded molecules without branches (26%), and (*iii*) free single-stranded molecules. The double-stranded molecules with or without branches were similar in length, having a modal length of 10–15 μm. The free single-stranded molecules were shorter and had a mean length of 3.8 μm. The length of the branches attached to the duplex molecules was only slightly less than that of the free form. The duplex molecules with branches were interpreted as configurations reflecting an ongoing strand-displacement process that results in free single-stranded molecules. Finally, measurements on duplex molecules with multiple branches suggested that the extrachromosomal DNA may exist in the form of tandemly repeated sequences.**

Recent experiments by Van't Hof and Bjerknes (1) showed that cells of the pea-root meristem have extrachromosomal DNA (exDNA). In an individual root the exDNA is produced in cells located in the 2-mm tip but not in those of the adjacent elongation zone, even though nuclei in both tissues are replicating DNA. The presence of newly formed radioactive exDNA is detectable either by sedimentation in an alkaline sucrose gradient after 24 hr of labeling with [^{3}H]thymidine or by selective extraction by the Hirt (2) procedure. The exDNA is a linear double-stranded molecule, which bands at the same buoyant density as nuclear DNA but has a shoulder on the higher density side of the profile. Cytological analyses showed that the exDNA is associated with root-tip cells that stopped dividing and differentiated from G_2 phase but not with those that differentiated from G_1 phase.

Sedimentation experiments indicated that exDNA molecules had a modal molecular weight of about 26×10^6 (1) but these analyses were insufficient to give needed details about the structure of the molecules. Consequently, the exDNA was examined by electron microscopy and we present the results of this examination in this paper. We were surprised to find that exDNA had three forms: there were double-stranded linear molecules, double-stranded duplexes with single-stranded branches (RF DNA), and free single-stranded molecules (ss DNA) corresponding in length to that of the branches. The presence of RF DNA and free ss DNA molecules was interpreted as an indication that the single-stranded forms were produced by strand displacement.

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MATERIAL AND METHODS

Culture conditions and nuclear isolation of root tips from *Pisum sativum* (var. Alaska) have been described (1). exDNA samples extracted by the procedure of Hirt (2) were prepared from 50 2-mm root tips. After isolation, nuclei were sedimented by centrifugation at 300 × *g* for 5 min at 0°C, the supernatant was removed, eliminating possible contamination by cytoplasmic organelles, and the pellet was lysed by addition of 100 μl of 0.6% $NaDodSO_4$ solution at pH 7.5 containing 0.01 M EDTA. After 20 min at room temperature, 5 M NaCl was added to make a final concentration of 1 M. Samples were stored at 0°C overnight and then were centrifuged at 17,000 × *g* for 30 min. The supernatant was extracted and prepared for electron microscopy.

Electron Microscopy. One hundred-microliter samples containing exDNA were dialyzed on Millipore filters (type VS) at room temperature against 10 mM Tris·HCl/1 mM EDTA, pH 7.4, for 30 min and were digested with pancreatic RNase (50 mg/ml) and T1 RNase (500 units/ml) for 1 hr at 37°C. DNA was prepared for electron microscopy according to the method of Davis *et al.* (3). A solution containing 15 μl of DNA in Tris·HCl/EDTA, pH 7.4, 10 μl of cytochrome *c* (1 mg/ml in Tris·HCl/EDTA, pH 7.4), and 25 μl of formamide was spread onto a hypophase of 10% formamide in distilled water. The grids were stained with 50 mM uranyl acetate in 90% ethanol for 30 sec and were rotary-shadowed with platinum/paladium, 80:20 (wt/wt), at an angle of 8°. Micrographs were taken on a Phillips 300 electron microscope at magnifications of 5,000–8,000. Contour lengths were measured from enlarged projections of the negatives with a Numonic electronic graphics calculator. Identification and calibration of double-stranded DNA were performed by using pBR325 plasmid DNA, which was a gift from B. Burr (Brookhaven National Laboratory).

RESULTS AND DISCUSSION

A survey of the population of molecules in the Hirt extract (2) showed that none of the molecules were circular duplexes, indicating an absence of both mitochondrial and chloroplast DNA, none had replication bubbles indicative of bidirectional replication, and all were free of proteinaceous contamination. There were no double-branched, Y-shaped duplex molecules in the preparations that could result from breakage of replication bubbles. Likewise, examination of restriction fragments of exDNA after *Bam*HI digestion failed to produce Y-shaped duplex molecules.

The molecules extracted were only of three types: duplex molecules, RF DNA molecules, and free ss DNA molecules. Consequently, all of the molecules on a given grid preparation

Abbreviations: exDNA, extrachromosomal DNA; ss DNA, single-stranded DNA; RF DNA, double-stranded DNA with single-stranded branches.

were scored. Of the double-stranded molecules, those with branches (Figs. 1 and 2) were most numerous (74%), whereas those without branches were in the minority (26%). Because the lengths of duplex molecules, with or without branches, were similar, their measurements were combined in a single histogram, presented in Fig. 3. The histogram shows that, although the double-stranded molecules ranged from a few microns up to 115 μm, most were longer than 5 and shorter than 70 μm. Their modal length was 10–15 μm, a length in agreement with that determined by velocity sedimentation (1), and 56% were between 2 and 45 μm long. Of molecules longer than 25 μm, 28% were between 25 and 45 μm and only 15% were longer than 45 μm. The modal length of these longer molecules was 30–40 μm.

The most numerous molecules seen in the preparations were single-stranded (Fig. 4). These ss DNA molecules ranged in length from a few microns up to 13 μm and had a modal length of 2–3 μm, another (lower) peak at 4–5 μm, and a nearly normal distribution (Fig. 5). The mean length was 3.8 μm and only 13% exceeded 6 μm. These statistics were clearly different from those of the double-stranded molecules but were similar to those of the single-stranded branches associated with the double-stranded DNA (Fig. 6). Although most of the ss DNA showed a linear configuration, 8% of this population ap-

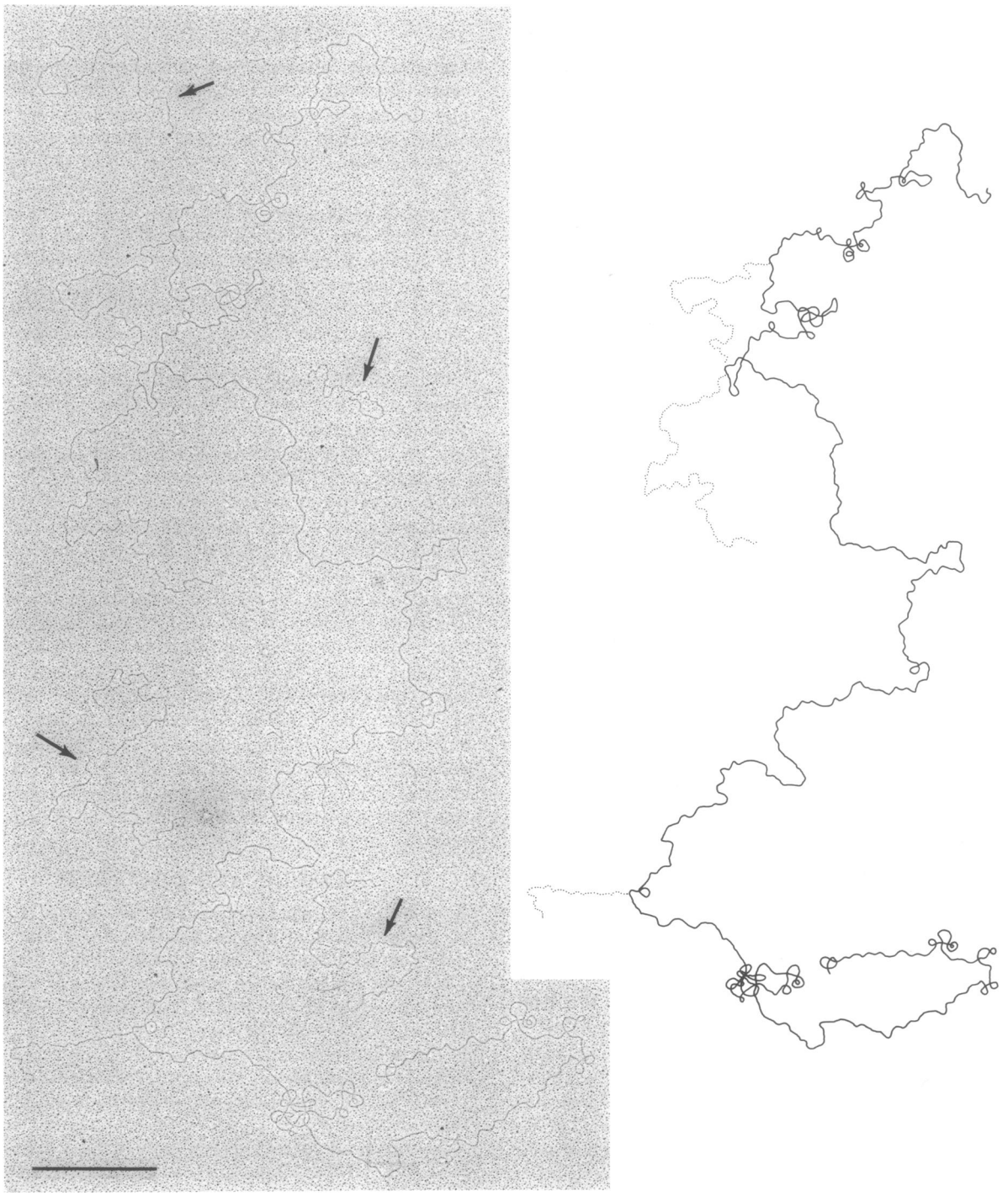

FIG. 1. Electron micrograph and interpretative tracing of a double-stranded exDNA molecule mounted by the formamide technique (3). In the diagram, double-stranded DNA is represented by solid lines and ss DNA, by dotted lines. Three single branches of 3.1, 4.2, and 1.5 μm in length are associated with the double-stranded molecule. The total length of the double-stranded DNA is 39.2 μm. Free single-stranded molecules, indicated by arrows, also are observed. (Bar = 1 μm.)

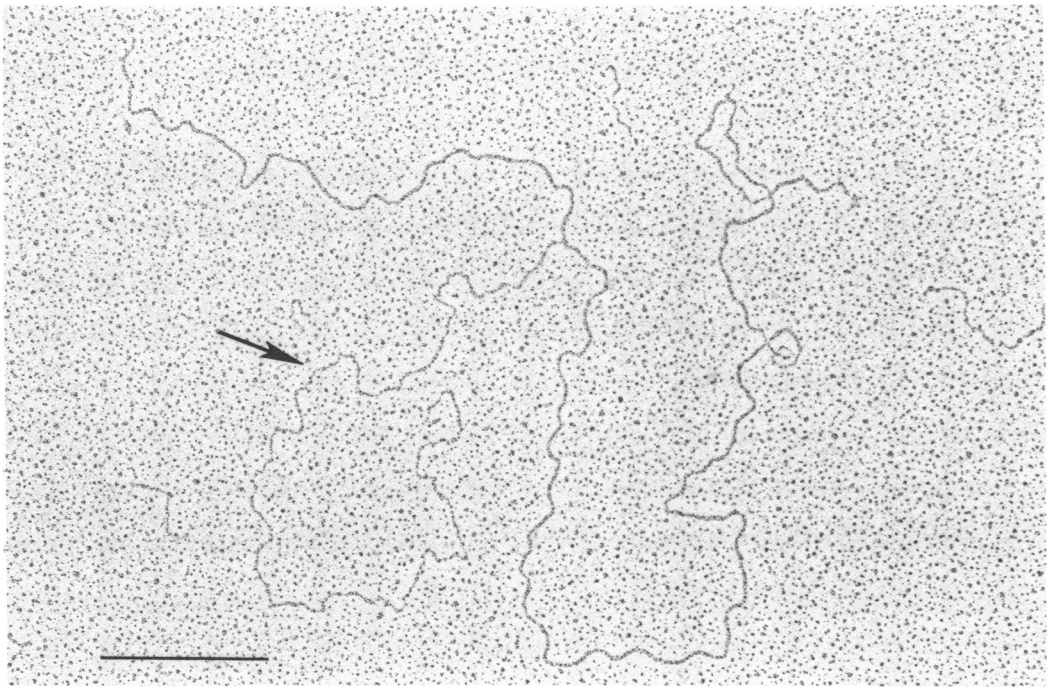

FIG. 2. Electron micrograph of a linear double-stranded DNA molecule, 7.2 μm in length, with a single-stranded branch (arrow), 3.4 μm in length. (Bar = 0.5 μm.)

peared as relaxed circles (Fig. 4), with contour lengths similar to the linear form (Fig. 7). Occasionally, these circular forms had small double-stranded projections, suggesting that they were not covalently closed (data not shown).

The double-stranded molecules with single-stranded branches were of particular interest because they resemble the replicative intermediate molecules of certain animal viruses (4, 5). For simplicity, we called these branched duplex molecules RF DNA, after replicative form, the assumption being that the single-stranded branches eventually would be released as free molecules upon completion of the displacement process. If this assumption is correct, it would be expected that the length of the branches, measured on the RF DNA, would be slightly less than the fully replicated free ss DNA molecules. Evidence supporting this assumption is given in Fig. 6, in which a histogram of the length of single-stranded branches is presented. The branches ranged from a few microns to 9 μm and had a mode at 2.0–2.5 μm and a distribution weighted toward smaller sizes. That the branches were shorter and more heterogeneous in length than free ss DNA provided evidence consistent with the idea that they were displaced from the RF DNA by the movement of a replicating fork moving along the template. The branches would be shorter because a portion of their length remained attached to the RF DNA and they would be of various sizes because the length awaiting displacement would differ depending on how close the process was to completion.

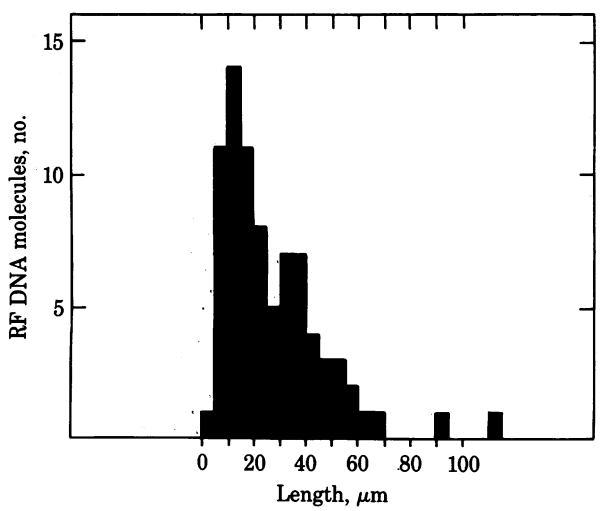

FIG. 3. Size distribution of RF DNA molecules. Measurements correspond to both double-stranded forms with (60 molecules) and without (21 molecules) associated single-stranded branches. Total number of molecules = 81.

The number of branches on a given RF DNA molecule varied from one to six and in cases in which there were two or more branches on one molecule, the lengths of the various branches often were unequal. The presence of more than one single-stranded branch on the same RF DNA molecule indicated that the longer duplex molecules had multiple initiation sites. The mean distance between the points of attachment of tandem branches on these longer duplex molecules was 7.9 μm. The difference between this value and the mean length of the ss DNA (3.8 μm) is ≈4 μm. Thus, the initiation sites were separated by about 8 μm of DNA, 4 μm of which is not displaced from the duplex molecule. Recent data from velocity sedimentation of exDNA in alkaline sucrose gradients agree with this interpretation, in that the radioactive chains of replicating exDNA show a stepwise increase in molecular weight from about 3×10^6 (equivalent to 3 μm of DNA) to $7–8 \times 10^6$ (7–8 μm) (6). This stepwise increase in molecular weight suggests that the chain displacing the ss DNA is eventually ligated to the 4 μm of DNA located between the point of release of one single-stranded branch and the initiation site of its tandem neighbor.

In addition to branched molecules, such as those shown in Figs. 1 and 2, there were others that we called "cross-branched." An example of a cross-branched molecule is shown in Fig. 8. The origin of these molecules is unknown. They may represent ss DNA molecules that were either laying across the RF DNA or wrapped around it. In either case, the ratio of the lengths of the two free ends would not be 1 because

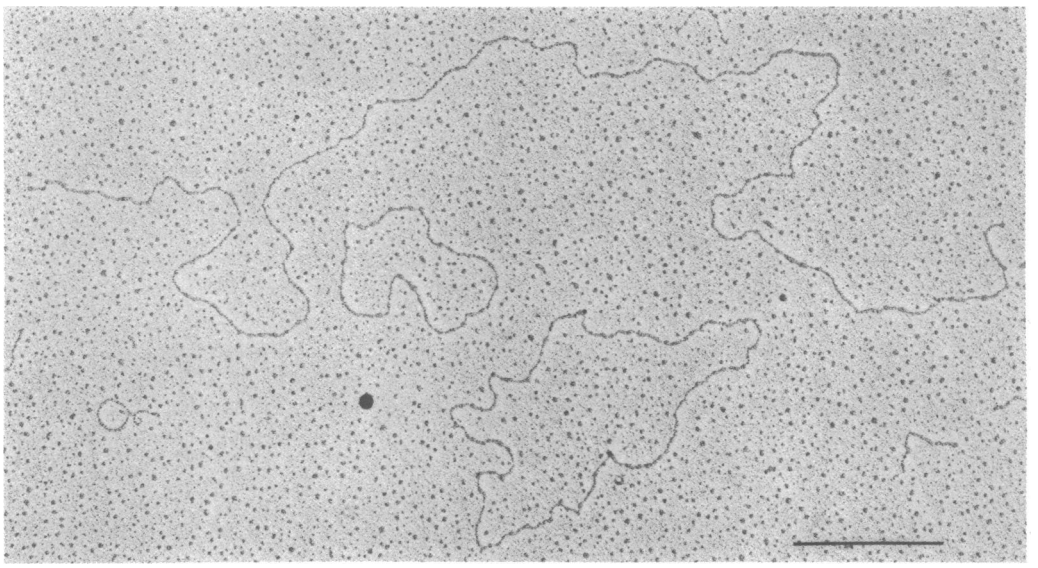

FIG. 4. Electron micrograph of free ss DNA molecules extracted by the Hirt procedure (2) and mounted for electron microscopy by the formamide technique (3). A linear molecule, 6.6 μm in length, and two relaxed circles (1.7 and 3.5 μm in length), all single-stranded, are observed. (Bar = 0.5 μm.)

the probability of the RF DNA bisecting the ss DNA molecule to give axial symmetry would be low. The cross-branched molecules also could result from two tandem-displaced strands that began at the same site on the RF DNA at different times but stopped at nearly the same position before being rendered as free ss DNA molecules. In this instance, the ratio of the two displaced segments would be ≈1. Finally, the cross-branched DNA may have been produced by two converging replicating forks that met and stopped before releasing the ss DNA. In this case, initiation sites would occur in both parental strands and replication would be performed in opposite directions. Here again, if (*i*) the sites were equal distances apart, (*ii*) replication started simultaneously at both sites, and (*iii*) the forks traveled at the same rate, the ratio of the lengths of the two displaced strands would be near 1. Conversely, if one of these three contingencies were not met, the ratio would be far from unity.

Measurements of the displaced strands on the cross-branched molecules showed that the ratio (±SD) of their lengths was 0.5 ± 0.2. This observation eliminated the possibility that the cross-branches were produced by two tandem replication forks that began and ended displacement at similar sites, but it failed to provide unequivocal support for either of the two remaining possibilities. However, the frequency of the cross-branched molecules was high (42%), and their origin requires further scrutiny.

The ss DNA molecules themselves apparently were not capable of further replication because single-stranded replicative forms such as rolling circles or hairpins (7, 8) were not observed. Moreover, double-stranded DNA comparable in size to ss DNA molecules was infrequent or absent in the preparations.

The absence of double-stranded molecules of the size of ss

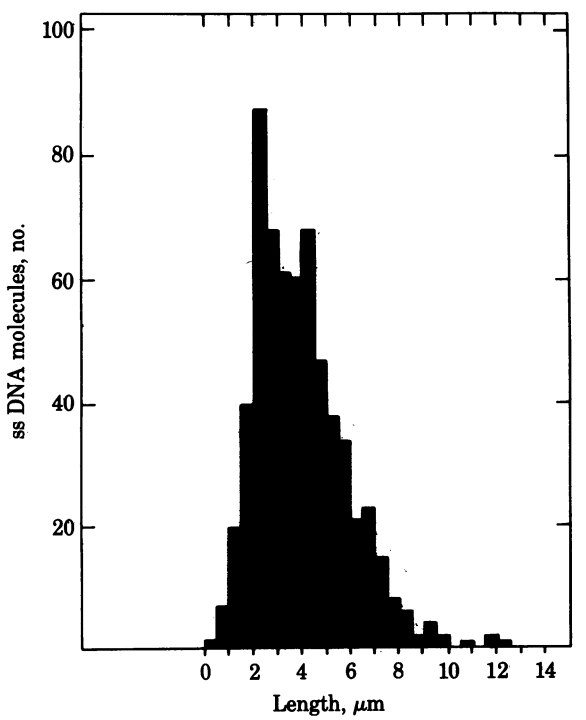

FIG. 5. Contour lengths of free ss DNA molecules. Both linear and circular forms are included. Total number of molecules measured = 617; linear molecules = 566 and circles = 51.

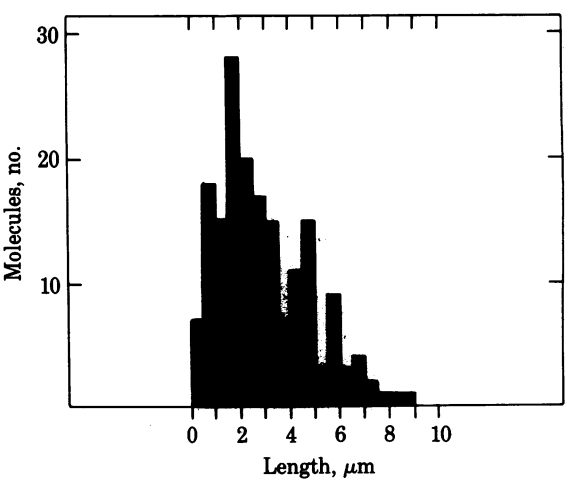

FIG. 6. Size distribution of branches on double-stranded molecules. Single-stranded forms as well as cross-branches are included. In the case of cross-branches, each arm of the cross was considered as a single value. Total number of branches = 180; single branches = 104 and cross-branches = 76.

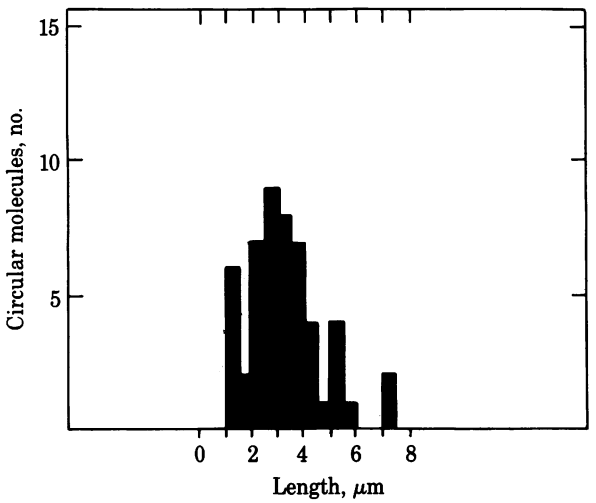

FIG. 7. Size distribution of single-stranded circular molecules. Total number = 51.

DNA also argues against a high degree of homology in the ss DNA population. A partial or total association between ss DNA molecules would be expected if different segments of the RF DNA would share homologous sequences. It is possible, of course, that once produced within the cell the ss DNA is associated with RNA or proteins preventing DNA–DNA associations. But, because the isolation procedure of exDNA involves $NaDodSO_4$ and high salt concentrations, the DNA–protein complex would be disassociated and if there were a DNA–RNA association *in vivo*, this too would be eliminated by the RNase treatment. Consequently, the notion of homology of the ss DNA awaits the completion of reassociation experiments.

The finding that there were three forms of exDNA proved to be useful in interpreting the previously published (1) banding pattern of the radioactive exDNA molecules in CsCl. Though 80% of the exDNA cobanded with genomic pea DNA, the remaining portion formed a shoulder at a higher density. We now believe that this shoulder was produced by the effect of single-stranded branches attached to duplex molecules, because the molecules were not sheared before banding. Also, the CsCl gradient did not have a large peak of radioactivity at the density expected for ss DNA. The absence of such a peak suggests that the single-stranded exDNA was not labeled under the conditions at which the experiment was performed. This observation also is supported by the absence of a peak with a molecular weight of 3.8×10^6 when labeled exDNA was sedimented in an alkaline sucrose gradient (1). These two observations in conjunction with other new data (6) suggest that the free ss DNA molecules and those displaced on the duplex form are sequences of chromosomal DNA—i.e., portions of the chromosomal duplex as it exists in G_1 phase prior to replication.

In summary, we propose that after being excised out of the chromosomes, the exDNA is capable of autonomous replication. However, its replication is not a regular replication process but occurs via a strand-displacement mechanism, resulting in the release of ss DNA molecules. If the base sequence of the displaced ss DNA were identical to that of its replacement on the duplex RF DNA, than a kind of amplification would result. On the other hand, if the base sequence were different—for example, if the displaced ss DNA had modified bases and its replacement did not—then the information on the duplex RF DNA could be altered by the process. The net result in either case would provide cells, in which exDNA strand displacement occurred, with a change in genetic information, in kind or degree, that may be useful to the differentiation mechanism.

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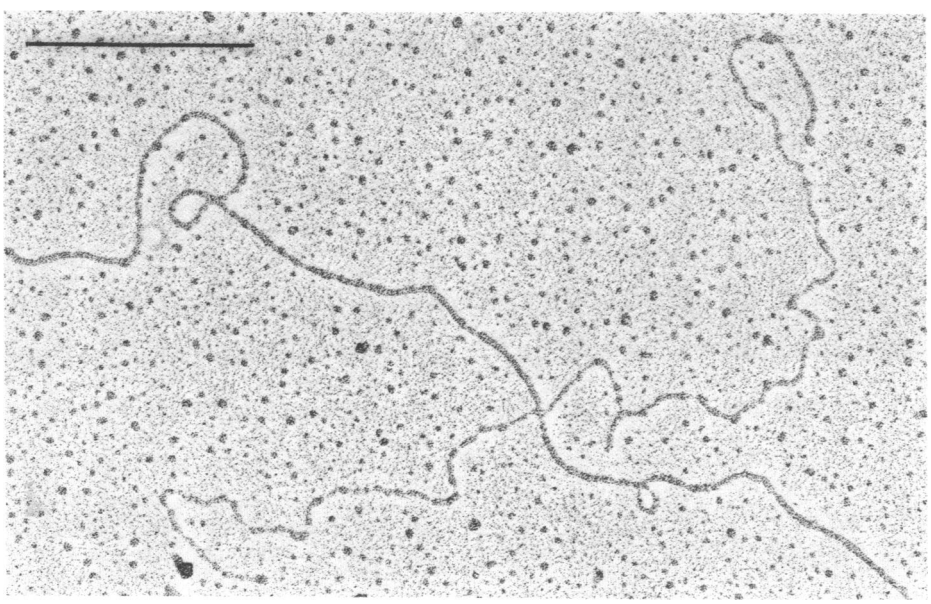

FIG. 8. Electron micrograph showing a detail of a double-stranded exDNA molecule. Two single-stranded branches in a cross-branch configuration are observed. (Bar = 0.5 μm.)