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Mechanism of DNA Replication in *Drosophila* Chromosomes: Structure of Replication Forks and Evidence for Bidirectionality

(electron microscopy/Okazaki fragments/cleavage nuclei/branch migration/fork rate)

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ABSTRACT **The replicating chromosomal DNA in *Drosophila melanogaster* cleavage nuclei has been visualized in the electron microscope as a serial array of closely spaced replicated regions created by pairs of diverging replication forks. The fine structure of the forks is very similar to that observed for the replication forks of bidirectionally replicating bacteriophage DNAs. However, the mean length of the single-stranded gaps in *Drosophila* forks is less than 200 nucleotide residues, much shorter than the gaps in phage forks. This difference in gap length corresponds to the observed difference in the size of Okazaki fragments from *Drosophila* and phage.**

The pleasing concept that the genetic information in a eukaryotic chromosome is contained in a single molecule of double-stranded DNA is supported by recent experiments with *Drosophila* (1) and yeast (2, 3). Given such a molecular continuity, the problem of reproducing the genetic order in a chromosome is reduced to the problem of replicating a single long DNA molecule which, for the largest chromosome in the fruit fly, *Drosophila melanogaster*, has a length of about 2.1 cm., or 62,000 kb [ref. 1; kb (kilo bases) is a unit of length equal to 1000 bases or base pairs in single-stranded or double-stranded nucleic acids].

We have studied this replication problem in *D. melanogaster* by electron microscopic examination of the DNA from rapidly dividing cleavage nuclei. At 24°, the cleavage nuclei divide every 9.6 min and exhibit an interphase of only 3.4 min (4), during which each chromosomal DNA molecule must be replicated. The molecular replication rate for the DNA in the largest chromosomes should therefore be equal to or greater than 18,000 kb min^{-1} $molecule^{-1}$. Since the upper estimate for the rate of movement of a DNA replication fork in animal chromosomes is about 3 kb min^{-1} $fork^{-1}$ (5, 6), we expect that this rapid molecular replication will require the cooperative action of 6000 or more forks per molecule, or at least one fork per 10 kb of DNA. It was this expectation that provided the hope that we could determine the structure and distribution of replication forks in the DNA of a eukaryotic chromosome by electron microscopic observation. In this article, we show that this hope has been realized. The DNA from the cleavage nuclei of *Drosophila* exhibits a serial array of replicated regions, or "eye forms" (ref. 7 and Fig. 1), created by pairs of diverging replication forks which display a fine structure very similar to that observed in prokaryotic chromosomes (8, 9).

Abbreviations: kb (kilo bases), a unit of length equal to one thousand bases or base pairs in single-stranded or double-stranded nucleic acids, respectively; CAPS, cyclohexylaminopropane sulfonic acid.

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MATERIALS AND METHODS

Media. C medium is Lewis's medium (10) modified by substitution of corn flour for corn meal and by increasing the agar concentration to 0.9%. G medium is derived from C medium by increasing the agar to 1.9%, eliminating the corn flour, adding grape juice (Welch's) to 39% (v/v), and titrating to pH 5.25 with 1.25 N NaOH; after autoclaving, acid mix A (10) is added until the final pH is 4.25. Yeast paste is 40% (w/w) active dry baker's yeast and 0.36% (w/w) propionic acid in water.

Collection of Eggs Containing Cleavage Nuclei. Adult flies (*D. melanogaster*, Oregon R) were maintained at 25° and 60% relative humidity on C medium partially overlaid with yeast paste. Eggs were collected for 40 min on G medium sprayed with 0.05 M ethyl acetate, starting at the time of the light-to-dark transition of a 10 hr light–14 hr dark cycle, when there is a burst of egg laying. After incubating the collected eggs for 15 min at 25°, they were washed with 0.12 M NaCl, dechorionated by suspension in 2.25% sodium hypochlorite for 90 sec (20–25°), and washed again. DNA was isolated from the cleavage nuclei of these eggs as described in the legend for Fig. 1.

Digestion of "Whiskers" with E. coli Exonuclease I. Reactions were carried out at 37° in 33.5-μl volumes at the following concentrations: 60 mM glycine (pH 9.5), 6 mM $MgCl_2$, 1.5 mM β-mercaptoethanol, 1.9 μg/ml of DNA, and 580 units/ml of *Escherichia coli* exonuclease I (11). The exonuclease I (specific activity, 55,000 units/mg) was a gift of Sidney Kushner.

Contour Lengths of DNA. Electron micrographs were projected onto a Hewlett-Packard 9864A Digitizer and contour lengths measured to an accuracy of $\pm 0.5\%$ using a 9810A Calculator with a fully smoothed program. Phage PM-2 DNA (9.9 kb) and phage M13 DNA (6.6 kb; ref. 12) were used as internal standards for double-stranded and single-stranded lengths, respectively. The PM-2 DNA length is based on a PM-2:λ length ratio of 0.213 and a λ DNA length of 46.5 kb (13).

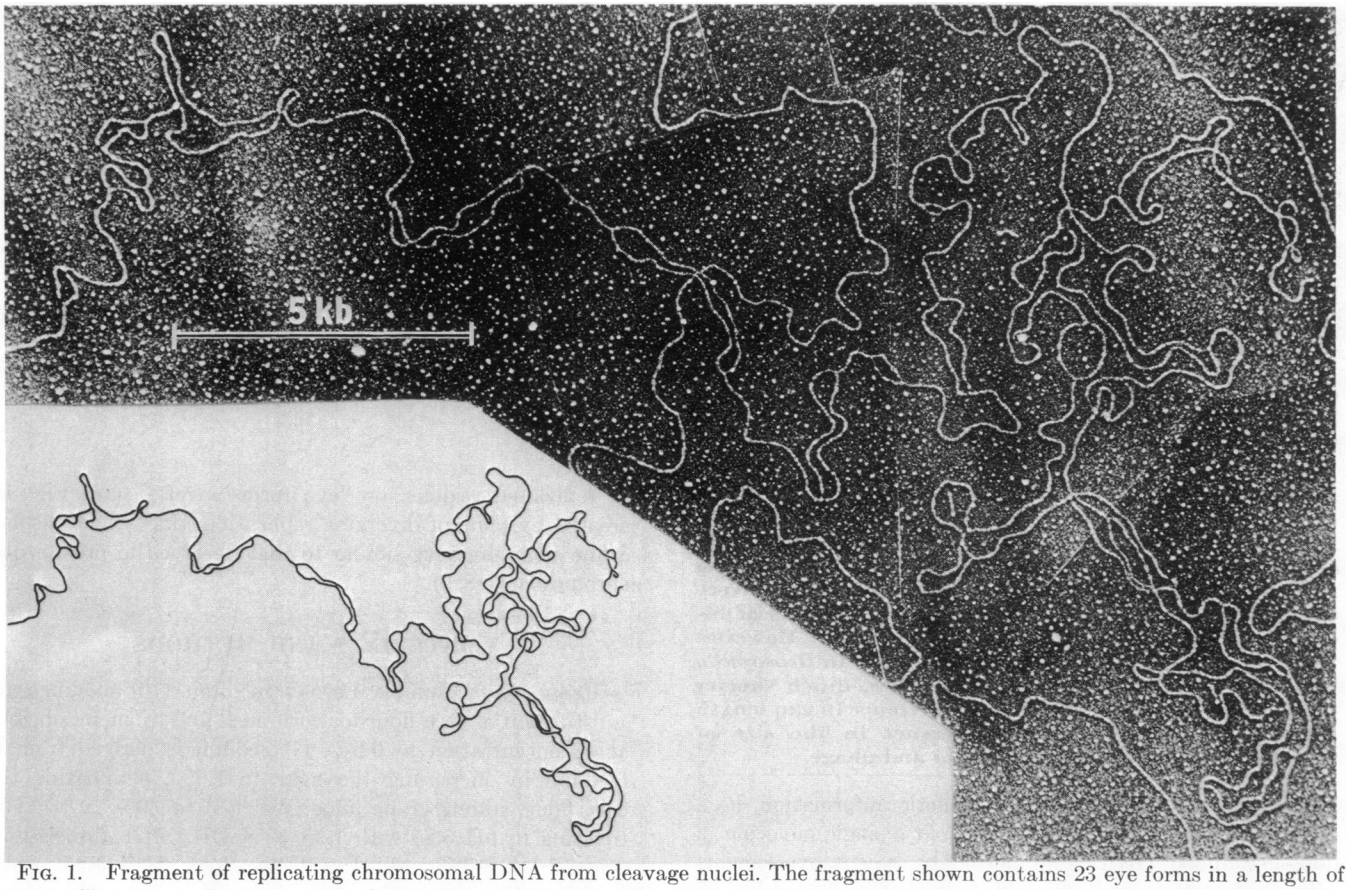

FIG. 1. Fragment of replicating chromosomal DNA from cleavage nuclei. The fragment shown contains 23 eye forms in a length of 119 kb. Cleavage nuclei were prepared at 0–4° by pressing 1 g of dechorionated *D. melanogaster* eggs (*Materials and Methods*) through a fine stainless-steel screen into 1.0 M 2-methyl,2,4-pentanediol, 0.002 M $CaCl_2$, 0.01 M CAPS buffer, pH 10.4; filtering the suspension through a nitex ASTM 400-37 screen (Ernst Tobler); and centrifuging the 10 ml of filtrate at 2400 rpm in a Sorvall centrifuge with a SS-34 rotor for 10 min. The nuclei were resuspended in 0.75 ml of 10 mM EDTA in 10 mM CAPS buffer, pH 10.4, and lysed by the addition of 0.75 ml of the same buffer containing 2% sarkosyl. After 5 min of gentle agitation at 20–25°, the lysate was floated on top of a solution of 8.85 g of CsCl in 5.5 ml of 10 mM EDTA in 10 mM CAPS buffer, pH 10.4, and centrifuged at 20° for 72 hr at 40,000 rpm in a Spinco ultracentrifuge with a 50 rotor. Fractions were collected slowly through a 16-gauge needle, and the fractions containing DNA were pooled and dialyzed against 10 mM EDTA in 100 mM Tris·HCl, pH 8.5. The dialyzed DNA was spread for electron microscopy by the formamide technique (14), using 40 and 10% formamide in the hyperphase and hypophase, respectively.

RESULTS

The DNA from Cleavage Nuclei Contains Multiple Eye-Forms. The molecules of DNA isolated from cleavage nuclei exhibited a mean length of 90 kb, and about one-tenth contained eye forms such as those shown in Fig. 1. The mean length of 279 randomly selected eye forms is 5.6 kb, and the mean distance between centers of 316 pairs of adjacent eye forms is 9.7 kb.† These distributions are described in more detail in another article (15).

Eye Forms Result from DNA Replication. If the eye forms result from replication, then the two segments which form an eye should exhibit the same length and sequence of base pairs. The length ratio of the two segments was determined for each of 44 eye forms. The mean of these ratios was 1.00, and the standard deviation was 0.02.

The criterion of equivalent base-pair sequences in the segments was tested by electron micrographic mapping of the denatured regions that result when the concentration of formamide in the spreading solution is increased to 80% (16). The denatured regions are seen as "blisters" formed by the unwound single strands (Fig. 2). The blister pattern is determined by the sequence of base pairs in the DNA segment (17), presumably because of the preferential melting of regions with lower frequencies of GC base pairs (18). The apparent correlation between the blister patterns in the two eye segments seen in Fig. 2 was confirmed by scoring 50 partially denatured eyes in the following manner. The distance from a fork to each terminus of a blister in either of the presumed daughter segments was measured, and the equivalent interval in the other segment was then examined for the presence of a blister. If a part or all of a blister was found in this interval, it was scored as a "success." Successes occurred in 101 out of 126 trials (80%). When the equivalent interval in the third member of the fork (the presumed parental segment) was examined as a control, only 37 successes were scored in 126 trials (29%). There is therefore a highly significant, positive correlation between the blister patterns in the eye segments, which indicates that they exhibit related sequences of base pairs.

† Subsequent to the preparation of this manuscript, a paper has been published which also describes the isolation of *Drosophila* DNA molecules which contain about the same distribution of eye forms as we have observed (31).

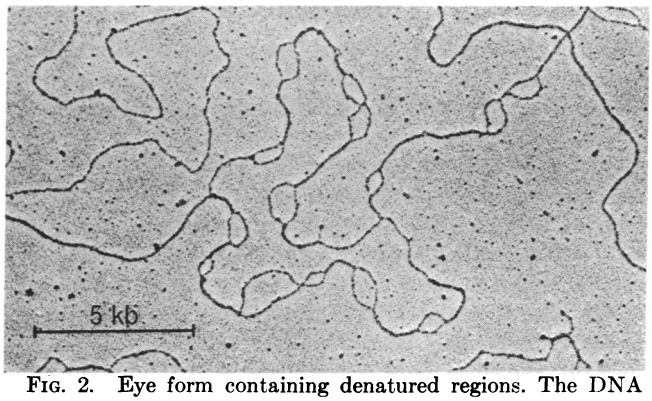

FIG. 2. Eye form containing denatured regions. The DNA was prepared and spread as described in Fig. 1 except that the concentrations of formamide in the hyperphase and hypophase were increased to 80 and 50%, respectively.

Additional evidence that eye forms result from DNA replication is provided by the observation that the frequency of eye forms in DNA isolated from the slowly dividing nuclei of *D. melanogaster* cell cultures (S phase = 600 min at 25°) is about two orders of magnitude less than in DNA from cleavage nuclei (15).

The Forks in the Eye Forms Look Like the Replication Forks in Phage Chromosomes. A total of 363 forks in eye forms were photographed; three forks were eliminated because of ambiguities in the photographs. The remaining 360 forks are distributed into five classes as indicated in Fig. 3. As shown in Fig. 3*A*, 45% of the forks contain one single-stranded gap in one daughter segment where it joins the fork, and are called SSG_1 forks. This is the most common single-stranded configuration observed in the replication forks of λ phage DNA (8) and T7 phage DNA (9).

Single strands which extend from a fork to a free end are called whiskers (Fig. 3*B*) and were previously observed in most of the replication forks of T4 phage DNA (19). It has been suggested (19) that whiskers are derived from SSG_1 forks by branch migration (20) during preparation of the sample, as indicated in the diagram in Fig. 4. The fact that the length distributions of the single-stranded regions in the SSG_1 and whisker forks do not differ significantly (Fig. 4) is consistent with this explanation, which we therefore adopt. Forks that contain both a whisker and an SSG_1-type region (whisker-SSG_1, Fig. 3*C*) exhibit the configuration expected for intermediate states in the branch migration process (Fig. 4), and we suppose that they are also derived from SSG_1 forks.

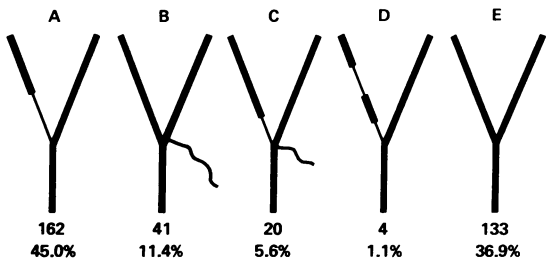

FIG. 3. The five classes of replication forks and their observed frequencies. *A*, SSG_1 fork; *B*, whisker fork; *C*, whisker-SSG_1 fork; *D*, SSG_2 fork; *E*, all-duplex fork. The values given under each fork indicate the number of observed forks in each class (*first row*) and the corresponding frequencies (*second row*).

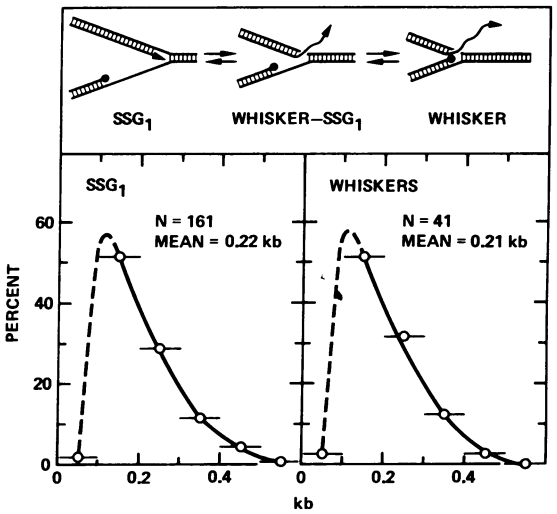

FIG. 4. The length distributions of the single-stranded segments in SSG_1 and whisker forks. The arrow and dot at the ends of daughter strands indicate 3′-hydroxyl and 5′-phosphoryl termini, respectively. The frequency for the first length interval (0–0.1 kb) is certainly too low because the shortest single-stranded region that can be convincingly identified is only slightly less than its upper boundary. Frequencies of 1.2 and 0.6% at 0.65 (±0.05) kb and 0.75 (±0.35) kb, respectively, were not included in the SSG_1 distribution.

The frequency of SSG_1 forks *in vivo* is therefore presumed to be equal to or larger than the sum of the frequencies of the preceding three observed classes, or 62%. The only other forks containing single-stranded regions belong to the SSG_2 class (Fig. 3*D*). The fork configurations that are relevant to replication can therefore be reduced to three classes: SSG_1, SSG_2, and all-duplex. The replication forks in λ phage DNA and T7 phage DNA were observed to fall into these same three classes (8, 9).

All SSG_1 Forks Exhibit the Orientation Shown in Fig. 4. Of 128 eye forms in which each daughter segment could be unambiguously traced from one fork to the other, 37 contained SSG_1 forks at both ends. In each case, the single-stranded gaps at the two forks were in opposite daughter segments,

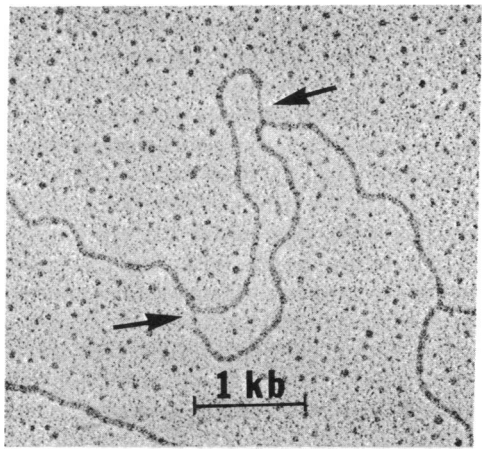

FIG. 5. *Trans* configuration of two SSG_1 forks in an eye form. Arrows indicate the single-stranded regions in each fork. DNA was prepared and spread as described in Fig. 1.

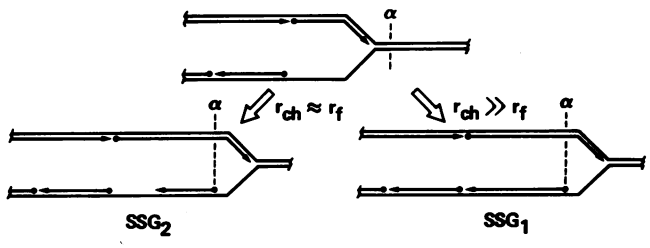

FIG. 6. A two-strand discontinuous model for a replication fork (26). See Fig. 4 for the meaning of the arrows and dots on the daughter strands. The position of initiation of a new chain is indicated by α. Initiation of chains may occur in either daughter strand but is assumed here to occur less frequently in the "leading" strand (5′ → 3′ direction = fork direction) because of competition with chain elongation (30). This model can be transformed into the one-strand discontinuous model by decreasing the frequency of initiation in the leading strand to zero.

i.e., they exhibited the *trans* configuration shown in Fig. 5. This result indicates that all SSG_1 forks have the same orientation.

The diagram in Fig. 4 indicates one of two possible orientations for SSG_1 forks. Whiskers derived from such forks by branch migration will have 3′ termini. This orientation was confirmed for whiskers in T4 forks by observing their disappearance upon exposure to the 3′ → 5′ specific *E. coli* exonuclease I (19). We have performed this same experiment with DNA from cleavage nuclei. The frequency of whisker-containing forks after exposure of the DNA to exonuclease I (*Materials and Methods*) for 0, 20, and 100 min was 22, 10, and 6%, respectively. By contrast, this frequency was 23% after a 100-min incubation of a control sample in which the enzyme was omitted. The number of forks counted in each sample was 145 ± 33, and essentially the same results were obtained in two different experiments. We conclude that the SSG_1 forks have the orientation shown in Fig. 4.

DNA Replication in Eye Forms is Bidirectional. The *trans* configuration of SSG_1 forks observed in *Drosophila* eye forms is also a characteristic of the eye and θ forms of the replicating DNAs in phages T7 and λ, respectively (8, 9), both of which replicate bidirectionally (7, 17). This correlation and the fact that the orientation of SSG_1 forks is that expected from models for replication forks (see ref. 21 for review) suggest that the eyes exhibiting the *trans* configuration replicate bidirectionally. However, the forks that can be identified in the electron microscope as replication forks also include the whisker-containing forks (because they derive from SSG_1 forks) and the rare SSG_2 forks (see *Discussion*), or a total of 63.1% of all forks (Fig. 3). If all forks in all eye forms are replication forks and the chance of detecting a replication fork by our assay is 0.631, then of the 128 eye forms examined $[(0.631)^2(128)]$, or 51, should exhibit single-stranded forks at both ends; the observed number was 54. The number containing all-duplex forks (i.e., those forks *not* identifiable as replication forks) at both ends should be $[(0.369)^2(128)]$, or 17.4; the observed number was 21. The number of eye forms containing one all-duplex and one single-stranded fork should be $[(2)(0.369)(0.631)\ (128)]$, or 59.6; the observed number was 53. We infer from this agreement between the expected and observed numbers that essentially all eye forms replicate bidirectionally.

DISCUSSION

When the information that eye forms expand bidirectionally is included in one's view of the replicating chromosomal DNA seen in Fig. 1, one obtains a good illustration of the model proposed by Huberman and Riggs (5) to account for the autoradiographic patterns generated by replicating DNA from the chromosomes of mammalian cells. Thus, the activation of each of many origins generates two diverging replication forks which create a serial array of bidirectionally expanding eye forms that merge to form the two daughter DNA molecules. Since the same kind of autoradiographic patterns detected for mammals have been observed for amphibia and fowl (6), and since multiple eye forms have been observed in the replicating chromosomal DNA of yeast (22), the topography of replication seen in Fig. 1 can reasonably be generalized to all eukaryotic chromosomes.

The similarity between the structure of the replication forks in *Drosophila* chromosomes and in the bidirectionally replicating forms of phage chromosomes is striking, and suggests that a single basic mechanism of DNA replication may suffice for both eukaryotes and prokaryotes. There are, however, some significant differences which we wish to discuss in the context of the two-strand discontinuous model given in Fig. 6. The frequency of SSG_2 forks in *Drosophila* DNA is about an order of magnitude less than that observed for λ phage DNA and T7 phage DNA (8, 9). The ratio of SSG_2 to SSG_1 forks is 0.02 in *Drosophila* DNA and 0.34 and 0.13 in λ phage DNA and T7 phage DNA, respectively. This ratio should depend on the relative rates of chain elongation, r_{ch}, and fork movement, r_f, as is illustrated in Fig. 6. If r_{ch} and r_f are about equal, SSG_2 forks will be formed which exhibit lifetimes of the order of those for SSG_1 forks; if $r_{ch} \gg r_f$, an SSG_2 fork will immediately be transformed into an SSG_1 fork and will be infrequently observed. We therefore suggest that the ratio of r_{ch} to r_f is substantially greater in *Drosophila* forks than in phage forks.

The value of r_f for *Drosophila* cleavage nuclei is 2.6 kb min^{-1} $fork^{-1}$ at 25° (15), about 6-fold less than the values of r_f estimated for T4 phage (23) and *E. coli* (24) at this temperature. Since the r_f values reported for other eukaryotes are about the same as that for *Drosophila* (5, 6), we suppose that there is a rate-limiting process necessary to fork movement that is common to eukaryotes and not present in prokaryotes. An obvious candidate for this process is the redistribution of histones which must occur in eukaryotic, but not in prokaryotic, replication forks. Since the ratio $r_{ch}:r_f$ is about one in *E. coli* (24), and only somewhat greater in T4 phage (25) our contention that the $r_{ch}:r_f$ ratio is much greater than one in *Drosophila* would then require that chain elongation is less affected by the histone redistribution than is fork movement. We refer here, of course, only to the daughter strand in which the direction of chain elongation is opposite to fork movement.

A second difference between *Drosophila* and phage replication forks is the size of the single-stranded gaps in SSG_1 forks. The mean length for *Drosophila* forks is probably less than 0.2 kb (Fig. 4), whereas it is 0.5 and 1.5 kb in λ and T7 forks, respectively (8, 9). One might therefore expect the chains created by discontinuous elongation (Okazaki fragments, ref. 26) to be shorter in *Drosophila* than in λ and T7 phages. In experiments performed with the collaboration of Alan Blumenthal, we have obtained Okazaki fragments from cell

cultures of *D. melanogaster* that exhibit a sedimentation coefficient in alkaline sucrose gradients of 4S, whereas Ginsberg and Hurwitz (27) find that replicating λ phage DNA yields Okazaki fragments of 7S. The corresponding fragment lengths are about 0.15 and 0.6 kb (28), in rough agreement with expectation. The sedimentation coefficient of Okazaki fragments for T7 phage has not been reported but, according to the preceding argument, should be about 10S, in the range of values reported for other phage and for bacteria (8–11S, ref. 26). These observations would indicate that the initiation of new chains on one or both strands occurs more frequently per unit length of DNA replicated in *Drosophila* than in the prokaryotes. This also appears to be the case for mammalian cells, where Okazaki fragments of about 0.1 kb have recently been reported (29).

The characteristics of replication forks visible in the electron microscope that we have discussed thus far can be related to either the one-strand or the two-strand discontinuous models of replication (21) with equal ease. There is, however, one peculiar aspect of the observed fork frequencies for which the two-strand model offers an explanation that the one-strand model does not—and this concerns the high frequencies of all-duplex forks observed for Drosophila (37%, Fig. 3), λ phage (49%, ref. 8), T4 phage (47%, ref. 19), and, to a lesser extent, T7 phage (15%, ref 9). All-duplex forks can be created by displacement of a chain from the "leading" daughter strand (i.e., the strand with 5′ → 3′ direction equal to the direction of fork movement) if initiation of chains on both daughters occurs at approximately the same position; this is the expected result of branch migration in the upper fork shown in Fig. 6. In those cases where the initiation positions in the two daughters are displaced from each other, the result of branch migration would be a whisker (e.g., the lower right fork in Fig. 6). This explanation would also account for the Okazaki fragments that have been observed without prior denaturation of the DNA (26), an observation that offers an obvious means for testing this proposition.

In conclusion, we note that among the more than 1000 *Drosophila* eye-forms examined, we have never observed an eye form within an eye form, i.e., the reactivation of an origin within a daughter segment. Evidently the activation of origins is restricted to the unreplicated chromosomal DNA in each S phase. Although tacitly assumed in previous considerations of chromosomal replication in eukaryotes (5, 6), our observations provide the first direct evidence for this restriction. It is an important point because the reactivation of origins prior to completion of chromosomal replication is a common observation among prokaryotes (7, 19, 21) and, *a priori*, could occur in eukaryotes. Perhaps this restriction is necessary for the proper packing of the chromatin required for the separation of daughter chromosomes during mitosis. Or, the restriction may be related to the mechanisms that regulate the different arrangements of active origins in the chromosomal DNA of nuclei with different S phases, a subject we have reported in another article (15).

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1. Kavenoff, R. & Zimm, B. H. (1973) *Chromosoma* **41**, 1–27.
2. Petes, T. D. & Fangman, W. L. (1972) *Proc. Nat. Acad. Sci. USA* **69**, 1188–1191.
3. Blamire, J., Cryer, D. R., Finkelstein, D. B. & Marmur, J. (1972) *J. Mol. Biol.* **67**, 11–24.
4. Rabinowitz, M. (1941) *J. Morphol.* **69**, 1–49.
5. Huberman, J. A. & Riggs, A. D. (1968) *J. Mol. Biol.* **32**, 327–341.
6. Callan, H. G. (1973) *Cold Spring Harbor Symp. Quant. Biol.* **38**, in press.
7. Dressler, D., Wolfson, J. & Magazin, M. (1972) *Proc. Nat. Acad. Sci. USA* **69**, 998–1002.
8. Inman, R. B. & Schnös, M. (1971) *J. Mol. Biol.* **56**, 319–325.
9. Wolfson, J. & Dressler, D. (1972) *Proc. Nat. Acad. Sci. USA* **69**, 2682–2686.
10. Lewis, E. B. (1960) *Drosophila Inform. Serv.* **34**, 117–118.
11. Lehman, I. R. & Nussbaum, A. L. (1964) *J. Biol. Chem.* **239**, 2628–2636.
12. Marvin, D. A. & Hohn, B. (1969) *Bacteriol. Rev.* **33**, 172.
13. Davidson, N. & Szybalski, W. (1971) in *The Bacteriophage Lambda*, ed. Hershey, A. D. (Cold Spring Harbor Lab., Cold Spring Harbor, N.Y.), pp. 45–82.
14. Davis, R. W., Simon, M. & Davidson, R. (1971) in *Methods in Enzymology XXI, part D*, eds. Grossman, L. & Moldave, K. (Academic Press, New York), pp. 413–428.
15. Blumenthal, A. B., Kriegstein, H. J. & Hogness, D. S. (1973) *Cold Spring Harbor Symp. Quant. Biol.* **38**, in press.
16. Wolfson, J., Dressler, D. & Magazin, M. (1972) *Proc. Nat. Acad. Sci. USA* **69**, 499–504.
17. Schnös, M. & Inman, R. B. (1970) *J. Mol. Biol.* **51**, 61–73.
18. Champoux, J. J. & Hogness, D. S. (1972) *J. Mol. Biol.* **71**, 383–405.
19. Delius, H., Howe, C. & Kozinski, A. W. (1971) *Proc. Nat. Acad. Sci. USA* **68**, 3049–3053.
20. Lee, C. S., Davis, R. W. & Davidson, N. (1970) *J. Mol. Biol.* **48**, 1–22.
21. Klein, A. & Bonhoeffer, F. (1972) *Ann. Rev. Biochem.* **41**, 301–332.
22. Petes, T. D., Newlon, C. S., Byers, B. & Fangman, W. L. (1973) *Cold Spring Harbor Symp. Quant. Biol.* **38**, in press.
23. Werner, R. (1968) *J. Mol. Biol.* **33**, 679–692.
24. Manor, H., Deutscher, M. P. & Littauer, U. Z. (1971) *J. Mol. Biol.* **61**, 503–524.
25. Sugino, A. & Okazaki, R. (1972) *J. Mol. Biol.* **64**, 61–85.
26. Okazaki, R., Okazaki, T., Sakabe, K., Sugimoto, K., Kainuma, R., Sugino, A. & Iwatsuki, N. (1968) *Cold Spring Harbor Symp. Quant. Biol.* **33**, 129–143.
27. Ginsberg, B. & Hurwitz, J. (1970) *J. Mol. Biol.* **52**, 265–280.
28. Studier, F. W. (1965) *J. Mol. Biol.* **11**, 373–390.
29. Huberman, J. A. & Horwitz, H. (1973) *Cold Spring Harbor Symp. Quant. Biol.* **38**, in press.
30. Olivera, B. M. & Bonhoeffer, F. (1972) *Nature New Biol.* **240**, 233–235.
31. Wolstenholme, D. K. (1973) *Chromosoma* **43**, 1–18.