*PRE-FORK SYNTHESIS: A MODEL FOR DNA REPLICATION**

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Abstract.—A model of DNA replication is presented in which DNA synthesis is continuously initiated from parental strand nicks and occurs, with conservation of helix winding number, ahead of the so-called replicating fork. The fork in this model is the locus of unwinding of already replicated, but presumably unstable, DNA. The model, involving Okazaki's notion of multiple initiation, is based upon the properties of Kornberg's DNA polymerase and accounts for the presence of single-stranded nascent DNA fragments in cell lysates. In addition to acting as sites of initiation, the parental strand nicks are implicated as sites of free rotation allowing unwinding of the replicated DNA.

Okazaki's finding that nascent DNA is synthesized in a discontinuous pattern[1–3] and the discovery of a joining enzyme, DNA ligase,[4] have led to a spate of models[3, 5–7] of DNA replication which resolve the paradox that both strands of the parental duplex are apparently replicated simultaneously[8] and in the same direction[8, 9] by an enzyme capable of only unidirectional synthesis.[10]

In these models, the Okazaki-type fragments are thought to originate as a consequence of discontinuous synthesis through repeated initiation of replication on the single-stranded templates provided by the unwinding of the parental duplex. However, apart from Guild's and Kornberg's models the others gloss over the problem of initiation and all of them lack an explicit accounting for the observation that some of the Okazaki fragments are present in a single-stranded state in cell lysates prepared under nondenaturing conditions.[3, 11–13] Finally, none of the models have any bearing on, or make provision for, the unwinding of the parental template.[14] For this reason we have exhumed and refurbished the older models[14, 22] of DNA replication that minimize the unwinding problem by the introduction of single-strand nicks in the duplex ahead of the replicating fork.

This updated version of the nicking models incorporates the *in vitro* properties of Kornberg's polymerase[10] and explicitly accounts both for the formation of Okazaki fragments in the process of replication, and for their single-stranded characteristics upon isolation. Furthermore, the model can account for those observations suggesting that DNA ahead of the fork is in an unusual physical state.[12, 13, 15]

The model here described is compatible with the rolling circle-type models of replication[16] and is distinguished from previous models in that replication occurs before unwinding, the "fork" being the locus of the unwinding and separation of the already replicated strands into two daughter double helices.[35]

The Model.—The present model of DNA synthesis incorporates the following features which are continually present throughout the course of replication: (1) the serial introduction in the parental DNA of dual-purpose single-strand nicks which act both as swivels for rotation and as sites for the initiation of synthesis,

(2) the restoration of parental strand integrity, and hence its conservation, after a second nick that separates the nascent DNA from its parent primer, (3) discontinuous synthesis of nascent DNA, (4) the location of these nascent fragments in easily dissociable multistranded regions, (5) the resolution of these multistranded regions by unwinding into daughter duplexes.

Nicking: As pictured in Figure 1, alternating single-strand nicks exposing 3′-OH termini are introduced at predetermined locations on both strands of the parental DNA. In the model this nicking is restricted to a limited part of the parental DNA immediately ahead of the area of replication. Free 3′-OH termini could be produced directly by a pancreatic-type of endonuclease,[17] or indirectly after phosphatase action[18] in a nick formed by an endonuclease II[19]-type enzyme.

Initiation: Once attached to the nick, polymerase molecules initiate synthesis at the free 3′-OH with subsequent synthesis displacing the nicked parental strand (Fig. 2). Soon after, the nascent DNA strand must be severed from its parental primer by a second nick presumably at the same site as the first (Fig. 3).

Reconstitution of the parental strand: To allow ligase mediated reconstitution[20] of the nicked parental strand it may be necessary to have some degradation or displacement of the 5′ end of the nascent strand (Fig. 3), leaving the parental strands in the vicinity of the original nick available for hydrogen bonding with each other once again (Fig. 4), and thus allowing repair of the nick (Fig. 4). When the parental nick is repaired, the winding number of the replication region is conserved during the course of further synthesis, a restriction which results in a three-stranded helical configuration bounded on both sides by unreplicated hydrogen-bonded parental DNA (Fig. 4). If the 5′-exonuclease function[21] of the DNA polymerase is active during the initiation of synthesis, then a patching

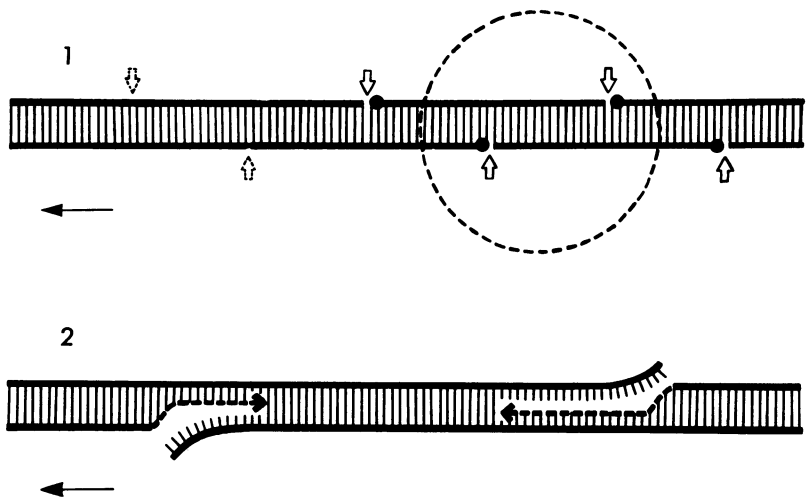

FIG. 1.—Nicking of parental duplex by endonuclease ( ) and formation of 3′-OH termini (●). The direction of advance of the replication region is indicated by →. A potential replication unit is encircled.

FIG. 2.—Unit of replication after DNA synthesis has been initiated, showing displacement of the parental strand and growing nascent strand ( ).

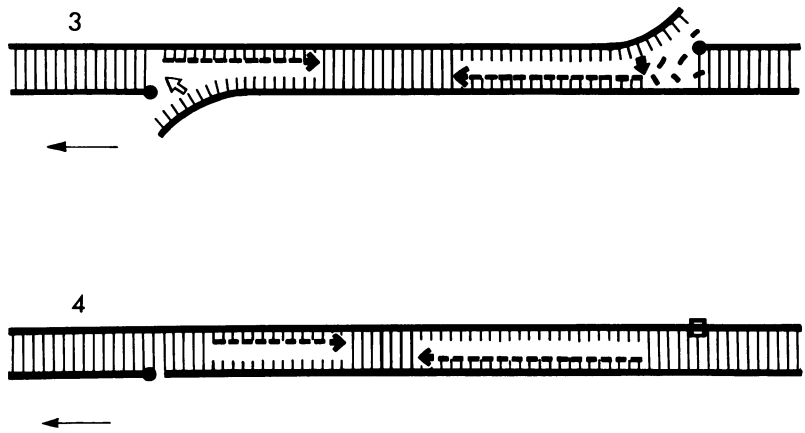

FIG. 3—Separation of nascent strand from parental primer by endonuclease attack ( ) shown on the right, followed by limited 5′-exonuclease degradation ( ) shown on the left.

FIG. 4.—The displaced parental strand re-anneals with its complement (*left*) and then ligase (□) restores the integrity of the parental strand.

synthesis would be necessary before the parental strand could be reconstituted by ligase action.[19]

Termination of local synthesis: As opposing fronts of replication originating from two adjacent nicks on opposite strands (defined here as a unit of replication, see Fig. 2) advance and pass each other, a four-stranded complex will be formed where they overlap (Fig. 5). This four-stranded complex forms with conservation of the winding number of the parental duplex. The nature of hydrogen bonding within this complex is not clear, but for convenience we will refer to this four-strand complex as a double duplex.

Initially a unit of replication is bounded by unreplicated parental DNA (Fig.

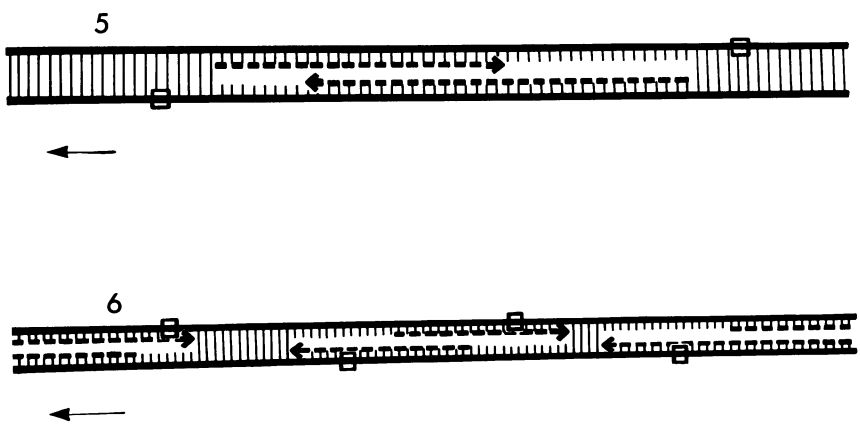

FIG. 5.—With continuing extension of the nascent strands, a four-stranded complex (or double duplex) is formed where they overlap.

FIG. 6.—A number of contiguous replication units still separated from one another by parental hydrogen bonding are shown.

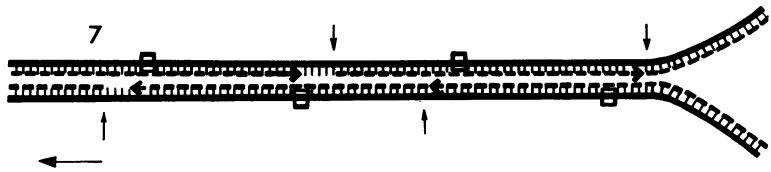

FIG. 7.—Fusion of replication units with break-through to the locus of unwinding (or "fork") is shown. The location of nascent strand nicks is indicated (↑) where the nascent strand of one replication unit has intersected with the stationary 5′ end of the nascent strand of the neighboring replication unit.

6), but as it extends through replication, the nascent strands in that unit overlap with the nascent strands of opposite polarity and meet with the nascent strands of the same polarity which originate from neighboring units. One end of the oldest replication unit thus breaks through to the fork where the double duplex is unwound into its component sister duplexes (Fig. 7). The formation of a covalent linkage between meeting nascent strands may be delayed until the distorted double duplex is resolved by unwinding.

Unwinding: When all parental hydrogen bonds of a unit have been severed, the double duplex is free to separate into its two daughter double helices (Fig. 7). Such separation must be accompanied by the rotation of the entire region of replication from the fork to the nearest parental strand nick in the advancing front of replication, this nick acting as a swivel for rotation.

The energy for this limited rotation could be provided by the transition of the unstable double duplex into its two-member duplexes which, upon attainment of the correct van der Waals distances, immediately form two rigid Watson-Crick double helices thus creating a "fork." If the fusion of the nascent fragments is delayed until the daughter duplexes have been resolved by unwinding, the single-strand nicks in the nascent DNA strand of the daughter duplexes would provide for rotational freedom of the molecule as unwinding proceeds. The entire sequence of events is summarized in Figure 8.

Discussion.—There is at best only indirect evidence for parental strand nicking in association with DNA replication. Hanawalt[22] observed an excess of phosphorus exchange in recently replicated parental DNA corresponding to that expected for nicks spaced at intervals of approximately 1000 nucleotides, a distance roughly equivalent to the length of an Okazaki fragment.[26] Such phosphorus exchange would be expected if an enzyme like endonuclease II[19] was responsible for nicking, as the resulting 3′-P would have to be removed by a phosphatase[18] and the 5′-OH phosphorylated[23] before ligase could act to seal the gap.[20]

FIG. 8.—The overall replication region in which Fig. 1 through 7 would run from left to right.

If replication can continue in the absence of ligase, then parental DNA fragments comparable in size to the Okazaki fragments should accumulate in the course of such replication. The evidence on this point is quite conflicting—some observations recording parental strand nicking.[24, 25] and others not.[26-28] However, the T4 data[24, 27, 28] are complicated by the presence of host ligase, while the ligase mutant[26] of *E. coli* is leaky.

Two kinds of nicking specificity are implicated in our model—a site specificity and a regional specificity. A precedent for the former kind of specificity has been observed for endonuclease II action, wherein DNA is attacked at sites modified by alkylation.[29] Lark[30] comments that the sparse degree of methylation of *E. coli* DNA apparently corresponds to that expected if there were one methyl group per Okazaki fragment equivalent. Indeed, it appears that unmethylated DNA cannot be replicated.[31] A signaling device of this kind for initiation (or termination) has already been suggested by Okazaki *et al.*,[3] to account for discontinuous synthesis. The regional specificity, i.e., the restriction of the nicking to a limited region of the DNA, is not demanded by the model, but such a possibility is implicit in Ganesan's suggestion[25] that endonuclease forms part of a membrane-localized replication complex.[32]

The nascent fragments of DNA have been isolated as single-stranded fragments in the absence of denaturing conditions indicating the possibility that they are either replicated as nonhydrogen-bonded single strands or are present immediately after synthesis in a very easily dissociable form. Oishi[11] has suggested that the presence of these nascent single-strand fragments may be a result of a distorted stretched template which prohibits the formation of the normal double helix.

Both the existence of single-stranded fragments and structural differences apparently ahead of the replicating region are compatible with the three- and four-strand complexes specified by the model since it is possible, as suggested by Okazaki *et al.*,[3] that extraction and isolation procedures could cause a displacement of nascent DNA fragments from these complexes as a result of partial or complete reconstitution of the parental DNA into a normal double helix. It is possible that with careful isolation of the entire DNA molecule, one may preserve this unstable structure—indeed, Cairns[8] observed that nascent pulse label was "tangled" in autoradiographs of *E. coli* DNA and not clearly resolved into post-fork daughter molecules.

Nicking, strand displacement, reconstitution and DNA synthesis are common to current models of repair and recombination.[33, 34] Our model of replication employs all these functions, suggesting to us that repair, recombination, and replication are different derivatives of the same basic process.

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[1] Sakabe, K., and R. Okazaki, *Biochim. Biophys. Acta.*, **129**, 651 (1966).

[2] Okazaki, R., T. Okazaki, K. Sakabe, and K. Sugimoto, *Japan. J. Med. Sci. and Biol.*, **20**,

255 (1967); Okazaki, R., T. Okazaki, K. Sakabe, K. Sugimoto, and A. Sugino, these PROCEEDINGS, **59**, 598 (1968).

[3] Okazaki, R., T. Okazaki, K. Sakabe, K. Sugimoto, R. Kainuma, A. Sugino, and N. Iwatsuki, in *Cold Spring Harbor Symposia on Quantitative Biology*, vol. 33 (1968), p. 129.

[4] Becker, A., G. Lyn, M. Gefter, and J. Hurwitz, these PROCEEDINGS, **58**, 1996 (1967); Cozzarelli, N., N. E. Melecker, T. M. Jovin, and A. Kornberg, *Biochem. Biophys. Res. Commun.*, **28**, 578 (1967); Gefter, M. L., A. Becker, and J. Hurwitz, these PROCEEDINGS, **58**, 240 (1967); Gellert, M., these PROCEEDINGS, **57**, 148 (1967); Olivera, B. M., and I. R. Lehman, these PROCEEDINGS, **57**, 1426 (1967); Weiss, B., and C. C. Richardson, these PROCEEDINGS, **57**, 1021 (1967); Zimmerman, S. B., J. W. Little, C. K. Oshinsky, and M. Gellert, these PROCEEDINGS, **57**, 1841 (1967).

[5] Hurwitz, J., *J. Cell Physiol., Suppl.* 1, **70**, 197 (1967).

[6] Guild, W. R., in *Cold Spring Harbor Symposia on Quantitative Biology*, vol. 33 (1968), p. 142.

[7] Kornberg, A., *Science*, **163**, 1410 (1969).

[8] Cairns, H. J. F., *J. Mol. Biol.*, **6**, 208 (1963).

[9] Cairns, H. J. F., in *Cold Spring Harbor Symposia on Quantitative Biology*, vol. 28 (1963), p. 43; Huberman, J. A., and A. D. Riggs, *J. Mol. Biol.*, **32**, 327 (1968); Meselson, M., and F. W. Stahl, these PROCEEDINGS, **44**, 671 (1958); Nagata, T., these PROCEEDINGS, **49**, 551 (1963); Yoshikawa, H., and N. Sueoka, these PROCEEDINGS, **49**, 559, 806 (1963).

[10] Kornberg, A., *Science*, **131**, 1503 (1960); Englund, P. T., M. P. Deutscher, T. M. Jovin, R. B. Kelley, N. R. Cozzarelli, and A. Kornberg, in *Cold Spring Harbor Symposia on Quantitative Biology*, vol. 33 (1968), p. 1; Mitra, S., and A. Kornberg, *J. Gen. Physiol.*, **49**, 59 (1966).

[11] Oishi, M., these PROCEEDINGS, **60**, 329 (1968).

[12] Kidson, C., *J. Mol. Biol.*, **17**, 1 (1966).

[13] Kidson, C., in *Cold Spring Harbor Symposia on Quantitative Biology*, vol. 33 (1968), p. 179.

[14] Delbruck, M., and G. Stent, in *The Chemical Basis of Heredity*, ed. W. D. McElroy and B. Glass (Baltimore: The Johns Hopkins Press, 1957) p. 699; Watson, J. D. and F. H. C. Crick in *Cold Spring Harbor Symposia on Quantitative Biology*, vol. 18 (1953), p. 123.

[15] Rolfe, R., these PROCEEDINGS, **49**, 386 (1963); Rosenberg, B. H., and L. F. Cavalieri, these PROCEEDINGS, **51**, 826 (1964).

[16] Frankel, F. R., in *Cold Spring Harbor Symposia on Quantitative Biology*, vol. 33 (1968), p. 485; Gilbert, W., and D. Dressler, in *Cold Spring Harbor Symposia on Quantitative Biology*, vol. 33 (1968), p. 473; Werner, R., in *Cold Spring Harbor Symposia on Quantitative Biology*, vol. 33 (1968), p. 501.

[17] Studier, F. W., *J. Mol. Biol.*, **11**, 373 (1965); Vanecko, S., and M. Laskowski, *J. Biol. Chem.*, **236**, 1135 (1961); Young, E. T., and R. L. Sinsheimer, *J. Biol. Chem.*, **240**, 1274 (1965).

[18] Becker, A., and J. Hurwitz, *J. Biol. Chem.*, **242**, 936 (1967).

[19] Hurwitz, J., A. Becker, M. L. Gefter, and M. Gold, *J. Cell. Physiol., Suppl.* 1, **70**, 181 (1967).

[20] Gellert, M., J. W. Little, C. K. Oshinsky, and S. B. Zimmerman, in *Cold Spring Harbor Symposia on Quantitative Biology*, vol. 33 (1968), p. 21; Olivera, B. M., Z. W. Hall, Y. Anraku, J. R. Chien, and I. R. Lehman, in *Cold Spring Harbor Symposia on Quantitative Biology*, vol. 33 (1968), p. 27.

[21] Klett, R. P., A. Cerami, and E. Reich, these PROCEEDINGS, **60**, 943 (1968).

[22] Hanawalt, P., in *Genetical Aspects of Radiosensitivity: Mechanisms of Repair* (International Atomic Energy Agency, 1966), p. 97; Hanawalt, P. C., *J. Cell Physiol., Suppl.* 1, **70**, 70 (1967).

[23] Richardson, C. C., these PROCEEDINGS, **54**, 158 (1965).

[24] Richardson, C. C., Y. Masamune, T. R. Live, A. Jacquemin-Sablon, B. Weiss, and G. C. Fareed, in *Cold Spring Harbor Symposia on Quantitative Biology*, vol. 33 (1968), p. 151.

[25] Ganesan, A. T., in *Cold Spring Harbor Symposia on Quantitative Biology*, vol. 33 (1968), p. 45.

[26] Pauling, E. C., in *Cold Spring Harbor Symposia on Quantitative Biology*, vol. 33 (1968), p. 148.

[27] Newman, J., and P. Hanawalt, in *Cold Spring Harbor Symposia on Quantitative Biology*, vol. 33 (1968) p. 145; Newman, J., and P. Hanawalt, *J. Mol. Biol.*, **35**, 639 (1968).

[28] Sugimoto, K., T. Okazaki, and R. Okazaki, these PROCEEDINGS **60**, 1356 (1968).

[29] Goldthwait, D. A., and E. C. Friedberg, in *Cold Spring Harbor Symposia on Quantitative Biology*, vol. 33 (1968), p. 271.

[30] Lark, K. G., *Ann. Rev. Biochem.*, in press.

[31] Lark, C., *J. Mol. Biol.*, **31**, 401 (1968).

[32] Botstein, D., *J. Mol. Biol.*, **34**, 621 (1968); Earhart, F., G. Y. Tremblay, M. J. Daniels, and M. Schaecter, in *Cold Spring Harbor Symposia on Quantitative Biology*, vol. 33 (1968), p. 707; Frankel, F. R., C. Majumdar, S. Weintraub, and D. M. Frankel, in *Cold Spring Harbor, Symposia on Quantitative Biology*, vol. 33 (1968), p. 495; Ganesan, A. T., and J. Lederberg, *Biochem. Biophys. Res. Commun.*, **18**, 824 (1965); Goldstein, A., and B. J. Brown, *Biochim. Biophys. Acta.*, **53**, 19 (1961); Hanawalt, P. C., and D. S. Ray, these PROCEEDINGS, **52**, 125 (1964); Knippers, R., and R. L. Sinsheimer, *J. Mol. Biol.*, **34**, 17 (1968); Smith, W. D., and P. C. Hanawalt, *Biochim. Biophys. Acta.*, **49**, 519 (1967).

[33] Setlow, R. B., *J. Cell. and Comp. Physiol.*, **64**, Suppl. 1, 51 (1964).

[34] Howard-Flanders, P., *Ann. Rev. Biochem.*, **37**, 175 (1968); Holliday, R., *Genet. Research*, **5**, 282 (1964); Whitehouse, H. L. K., *Nature*, **199**, 1034 (1963).

[35] Jehle, H., these PROCEEDINGS, **53**, 1451 (1965), has presented an incestuous model of DNA replication which results in replication ahead of the fork where a transient daughter-daughter duplex is converted into two orthodox semi-conserved duplexes with the parental strands.