Review

Days weaving the lagging strand synthesis of DNA — A personal recollection of the discovery of Okazaki fragments and studies on discontinuous replication mechanism —

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(Communicated by Kunihiko SUZUKI, M.J.A.)

Abstract: At DNA replication forks, the overall growth of the antiparallel two daughter DNA chains appears to occur 5′-to-3′ direction in the leading-strand and 3′-to-5′ direction in the lagging-strand using enzyme system only able to elongate 5′-to-3′ direction, and I describe in this review how we have analyzed and proved the lagging strand multistep synthesis reactions, called Discontinuous Replication Mechanism, which involve short RNA primer synthesis, primer-dependent short DNA chains (Okazaki fragments) synthesis, primer removal from the Okazaki fragments and gap filling between Okazaki fragments by RNase H and DNA polymerase I, and long lagging strand formation by joining between Okazaki fragments with DNA ligase.

Keywords: lagging strand synthesis, Okazaki fragments, DNA ligase, primer RNA dependent synthesis of Okazaki fragments, processing of Okazaki fragments before ligation, function of RNase H and DNA polymerase 1

Prologue: Days before the research on DNA replication

In 1945, when I was in the sixth grade of elementary school, Japan was defeated in the World War II, and few years later new Japanese Constitution declared equal rights for women and men under the law. Thus, I became the first generation Japanese women who received coeducation with man in high schools and universities. I was admitted to study at Nagoya University, School of Science in 1952 and majored Biology. The Hershey-Chase experiment was reported when I was a freshman[1)] and the next year double helical model of DNA was proposed.[2)] Thus, my research career overlaps with the history of Molecular Biology. Japanese universities as well as society in general were still suffering the damages received during the war time in those days. In 1956, when I entered graduate school of Nagoya University, Institute Molecular Biology, I got married with Reiji Okazaki (1930–1975). I remember the surprise when I read Watson and Crick's paper on the structure of DNA, which even explained the semi-conservative DNA replication and genetic phenomena solely by the principles of physical chemistry, although no biochemical mechanisms were there. In 1956, discovery of DNA polymerase of *E. coli* (now called DNA polymerase I) was reported by Arthur Kornberg and the basic concepts and technologies to study DNA biosynthesis seemed to be established by his colleagues. They demonstrated that polynucleotide chains complementary to the template DNA were synthesized with deoxyribonucleoside-5′triphosphates (dNTPs) in reaction mixtures.[3)] Since buildings of Nagoya University were burned down during the war, laboratories were in a barrack and libraries equipped not enough journals from abroad so that we had to visit American Cultural Center to read them. We could expect little financial support, if any, for research from the government. Reiji and I decided to analyze nucleotides in sea urchin and frog eggs. We thought that unfertilized eggs must store high level of nucleotides required for rapid syntheses of DNA and RNA for the cleavage stage after fertilization. Eggs of sea urchins and frogs were popular materials in the Developmental Biology Laboratory we belonged to then. We extracted nucleotides from those eggs with ice cold TCA solution, purified by charcoal

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doi: 10.2183/pjab.93.020

treatments and then separated by column chromatography. A fraction collector driven by balancing mechanism was our important instrument which we bought with our pocket money. We could recover various nucleotides from the column and fortunately enough discovered a novel sugar-linked nucleotide which was identified to be thymidine-diphosphate rhamnose.[4] Later on, we began to conduct experiments with bacteria and *Escherichia coli* became the favorite experimental materials. When [^{3}H]-thymidine became available for us, we traced the fate of [^{3}H]-thymidine added to the culture medium of *Escherichia coli* cells. Tritium radioactivity in *E. coli* cells detected first in nucleosides, then in TMP, TDP, and TTP in this order, and about 20 seconds after the administration to the culture medium it began to be detected in DNA fraction and accumulated with time in it. In retrospect, such kinetic analyses of conversion from [^{3}H]-thymidine to nucleotide and DNA *in vivo* by pulse labeling experiment later served our strong background for the analyses of synthetic reactions at replication forks and lead to the discovery of the discontinuous mechanism of DNA synthesis. It was impossible for us to study mechanisms of DNA synthesis *in vitro*, since even radioactive substrates were not commercially available then and should be prepared by ourselves and we didn't have enough technologies for that. Only a few privileged laboratories such as Dr. Kornberg's laboratory at Stanford University could afford such research environment. Luckily, our discovery of the novel nucleotide sugar compound provided us with an opportunity to work in Dr. Strominger's laboratory and subsequently in Dr. Kornberg's laboratory in U.S.A. I remember vividly that we were shocked to find the Meselson-Stahl' paper in PNAS which elegantly proved the semi-conservative replication of DNA with *E. coli*[5] on a night train to Tokyo to challenge the examination for Fulbright travel grant to U.S.A.

In September 1960, all the Fulbright Grantee from Japan sailed across the North Pacific Ocean for 11 days on the ship, Hikawa-maru, and landed Seattle. We spent some days in Seattle for orientation, and then got on the train, Northern Pacific Pullman coach, got to Chicago after two days, from where two of us reached St. Louis by ordinary train.

We stayed J. L. Strominger's Lab. in Washington University, St. Louis 15 months (September 1960 to November 1961) and investigated novel nucleotide linked sugar compounds.[6)–9)] In addition, there we learned micro-technology which could handle ul volume reagents and samples utilizing home-made glass micro pipettes. The micro-technology is now widely used and manufactured-plastic micropipettes are available but then was utilized in few limited laboratories.

In winter 1961, after spending 15 months at Washington University, moved to Arthur Kornberg's Laboratory at Stanford University to study biochemistry of DNA synthesis. We drove across the continent on the route 66 to California, and in the way, visited Grand Canyon and Petrified field and had opportunity to stay at many small towns in country side.

We stayed in Stanford only for 15 months (December 1961–Early March 1963). Although this was a great opportunity to learn details of the biochemical reactions and technologies of DNA polymerase, we simultaneously learned that *in vivo* DNA replication could not be explained solely by the *in vitro* reactions of the DNA polymerase.[10] DNA polymerase cannot unwind the DNA double helix, and the intact double-stranded DNA, which must be the genuine replication template *in vivo*, does not serve as template for the DNA polymerase reaction *in vitro*. With the double-stranded DNA template, synthesis of new DNA is observed only at the nicks or gaps of the template. Besides, DNA polymerase cannot *initiate* synthesis of a new DNA chain — namely, this enzyme requires a *primer* polynucleotide and is only able to *elongate* the primer chain — and the DNA synthesis occurs only at the 3′-OH termini. Therefore, DNA synthesis by DNA polymerase occurs only in the 5′-to-3′ direction (*i.e.*, tail growth); the 3′-to-5′ chain elongation, or head growth, is never observed. Prolonged DNA polymerase reaction produces branched DNA because of template-switching, a phenomenon in which DNA polymerase suddenly switches its template strand from one antiparallel DNA chain to the other. The products of such replication reactions are abnormal DNA molecules that cannot be denatured.

A challenge to the paradox of the directions of DNA chain elongation

In 1963, a number of publications reported the sequential replication of bacterial chromosomes. Once a DNA polymerase has begun adding nucleotides to growing DNA chains, it remains on the chain, continuing to add new nucleotides until a signal is reached that tells it to detach. All reports, including the famous autoradiography work reported by Cairns, indicated that the chromosomal DNA was replicated in such a sequential manner from the replication origin.[11] Importantly, these reports also

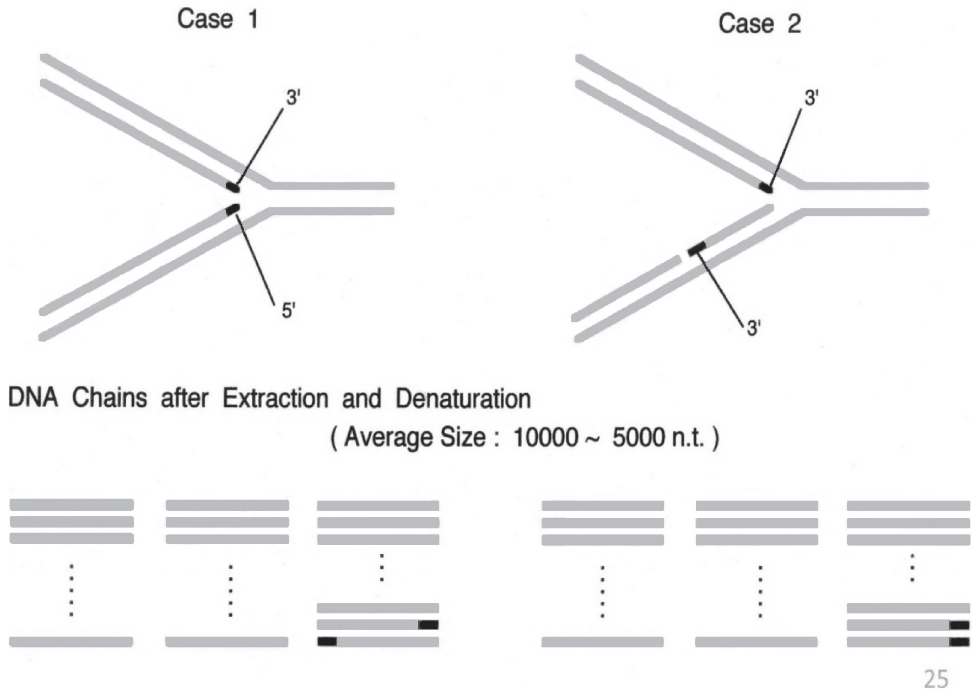


Fig. 1. Models of elongation reaction of daughter DNA chains. (Case 1) One daughter chain is synthesized in the 5′-to-3′ direction while the other chain is synthesized in the 3′-to-5′ direction. Both daughter chains are synthesized continuously. This model requires a new enzyme that catalyzes the 3′-to-5′ DNA polymerization. (Case 2) Both daughter chains are synthesized in the 5′-to-3′ direction in the microscopic analytical level. The leading strand (which appears to elongate in the 5′-to-3′ direction when observed by a macroscopic analytical method) is synthesized continuously whereas the lagging strand (which appears to elongate in the 3′-to-5′ direction) is assembled from the discontinuously synthesized small DNA fragments.

indicated that the replication process synthesized the two complementary daughter chains *simultaneously*. Because all known DNA polymerases could elongate the DNA chain only in the 5′-to-3′ direction, the mechanism of the apparent 3′-to-5′ elongation of one of the two daughter chains was an enigma. During the spring of that year, Reiji accepted an associate professor position in Nagoya University, Department of Chemistry, School of Science, and we returned to Japan. There, we chose the "paradox of the directions of DNA chain elongation" as one of the research themes of our new laboratory. Although many previous studies reported that the two daughter DNA chains were synthesized simultaneously at the replication fork, all such studies employed only analytical methods that exclusively detected long polynucleotide chains (for example a grain of autoradiography represent greater than 1,000 nucleotides), thus providing only the macroscopic view of the replication reaction. We attempted to analyze the *in vivo* replication reaction by employing the microscopic analytical techniques of biochemistry that could evaluate chain elongation even at the single nucleotide level. There seemed two possible explanations. In one model, the two daughter chains are synthesized continuously even at the microscopic nucleotide level (Fig. 1 Case 1). But, this model requires the existence of a still unidentified enzyme that catalyzes the 3′-to-5′ DNA chain elongation. In the other model, both of the two daughter chains are synthesized in the 5′-to-3′ direction, in the same manner as the known DNA polymerase reactions (Fig. 1 Case 2). In this latter model, the apparent 3′-to-5′ synthesis observed with the macroscopic methods actually consists of repeated ligation reactions of small DNA fragments, each of which is synthesized by the 5′-to-3′ polymerase reaction but shorter than the detection limit of the macroscopic methods of analysis (*i.e.*, less than several thousand nucleotides). The short DNA fragments are synthesized in the direction opposite to the replication fork processing. This *discontinuous replication mechanism* explains why the microscopic level of 5′-to-3′ synthesis of DNA fragments is observed as an overall 3′-to-5′ chain elongation by the macroscopic analyses.

Several preliminary approaches did not support the existence of the 3′-to-5′ polymerization at the microscopic level. We therefore attempted to directly

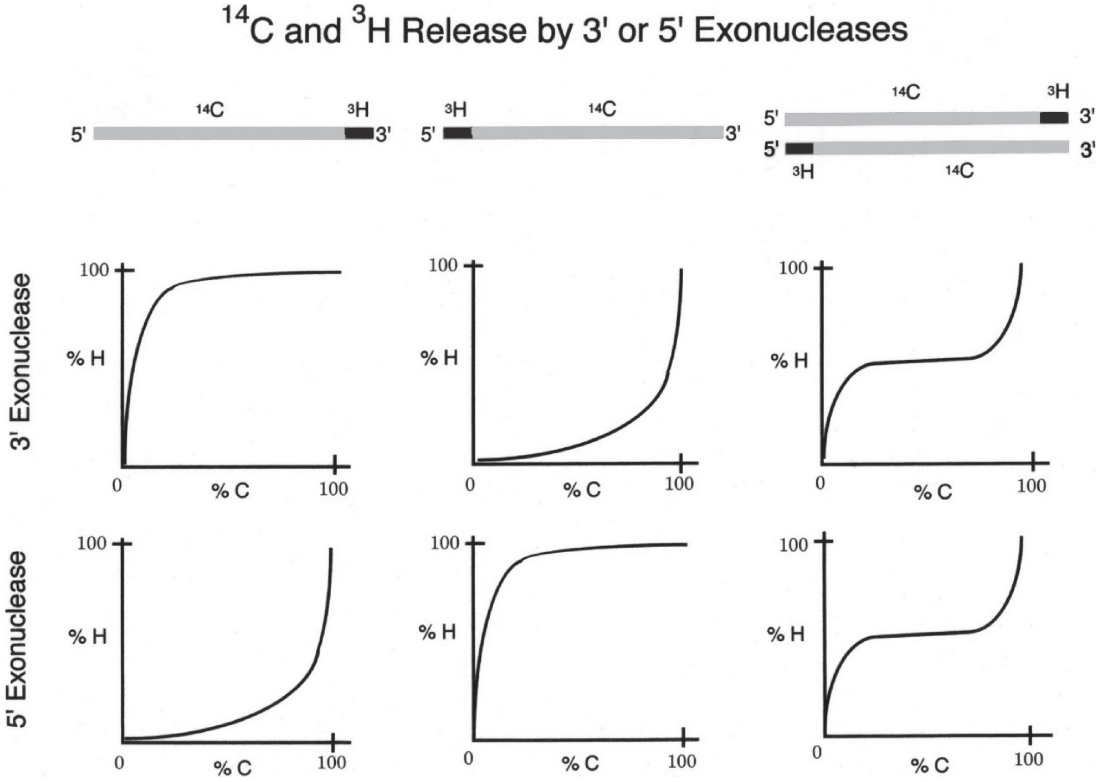


Fig. 2. Strategy to determine the direction of DNA synthesis by exonuclease digestion analysis. T4 phage-infected bacteria are cultured at 20 °C until the peak of DNA synthesis. Bacteria are then cooled to 8 °C and pulse-labeled with [^{3}H]-thymidine for 6 seconds to radiolabel the growing end of DNA and with [^{14}C]-thymidine for 2.5 minutes to radiolabel the entire length of Okazaki fragments. The 9S short-chain DNA fraction enriched with Okazaki fragments is isolated, and the Watson-strand and Crick-strand are separated further. Each strand is subjected to digestion with *E. coli* exonuclease I (5′-to-3′ digestion) and *Bacillus subtilis* exonuclease (5′-to-3′ digestion), and the ^{3}H and ^{14}C radioactivity released from the DNA is counted at varying periods of digestion time. Data points are plotted for the percent release of ^{3}H and ^{14}C. The profiles of the ^{3}H release indicate that the growing point locates at the 3′-end of DNA. Real results obtained are shown in Fig. 6.

determine whether the DNA elongation occurs precisely at the 3′ end or the 5′ end of the growing DNA strand. Our approach to this question was the following (Fig. 2). *E. coli* was grown in an appropriate medium, and [^{3}H]-thymidine was added to the medium to metabolically label the DNA. By limiting the time of [^{3}H]-thymidine incorporation, only the growing end(s) of the DNA was labeled by the radioactive nucleotides derived from [^{3}H]-thymidine. DNA was then isolated from the bacterial cells and subjected to digestion by exonucleases whose processing directions were specific and well-defined. For this purpose, we prepared *E. coli* exonuclease I enzyme, which released the nucleotides only from the 3′ end of a DNA strand,[12] and a new *Bacillus subtilis* exonuclease enzyme that released the nucleotides only from the 5′ end.[13] Through preliminary experiments using a 3′-labeled T7 bacteriophage DNA preparation, we learned that, in order to obtain clear data, it was necessary to limit the length of the radiolabeled DNA ends to a small percent of the total length of the genomic DNA fragments prepared from the bacterial cells. Assuming that the size of the extracted cellular genomic DNA fragments including the growing termini would be about 10,000 nucleotides, we calculated the [^{3}H]-thymidine exposure time to be less than 0.1 seconds at 37 °C (too short!). We then used the pulse-labeling method and exposed the bacteria to [^{3}H]-thymidine for several seconds at a low temperature (20 °C or lower). This methodological improvement later led us to the discovery of Okazaki fragments.

Discovery of Okazaki fragments: Evidence of the discontinuous replication model

Ms. Sakabe, Reiji's first graduate student, performed the low-temperature pulse-labeling experiment using *E. coli*. Unexpectedly, the [^{3}H]-thymidine

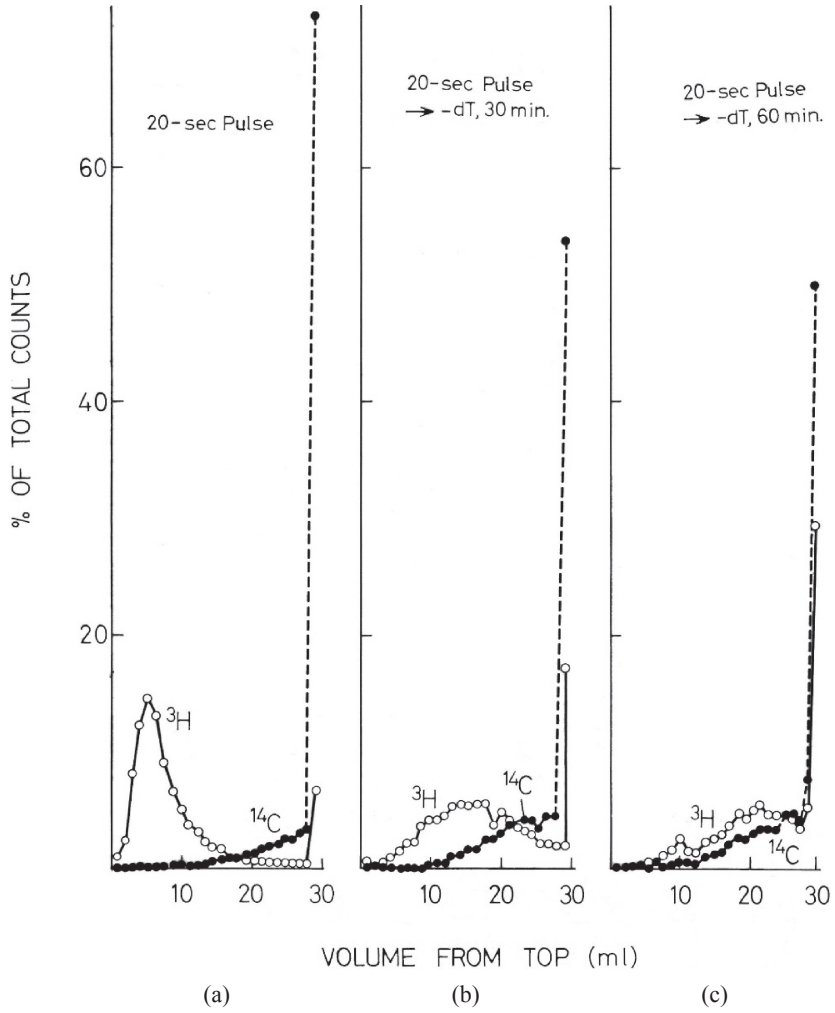


Fig. 3. Okazaki fragments in *E. coli*. *E. coli* was pulse-labeled with [^{3}H]-thymidine at 20 °C. (a) Pulse-labeling for 10 seconds. (b) Pulse-labeling for 10 seconds followed by 2 minutes chase (*i.e.*, an excess amount of non-radioactive thymidine was added to the culture medium). (c) Pulse-labeling for 10 seconds followed by 20 minutes chase. Cellular DNA was then denatured and extracted, and the DNA fragments were separated by length using 5–20% sucrose-gradient centrifuge in an alkaline condition. The ^{3}H peak observed in the fractions 5–7 was the Okazaki fragments.

radioactivity was incorporated into short DNA fragments that were only 1,000–2,000 nucleotides in length (Fig. 3).[14] These newly synthesized short DNA fragments are now known as Okazaki fragments. When the pulse-labeling time was extended or the radiolabeling was chased by non-radioactive thymidine, the tritium radioactivity was transferred from the short DNA fragments to longer DNA chains that showed physical characteristics identical to the overall genomic DNA. These results suggested that the short DNA fragments were synthesized at the very early stage of DNA replication reaction and, only after completion of their synthesis, these DNA fragments were incorporated into the long and continuous chains of genomic DNA — *i.e.*, the *discontinuous replication mechanism*. We obtained these results in 1966, after three years of efforts. When we presented these data in a domestic meeting, we received a comment that the observed short DNA fragments could be artifacts derived from the fragile DNA strands near the replication fork. To address this, we repeated the pulse-labeling experiments using a variety of systems and tested various protocols of cell lysis and DNA extraction. Still, all results suggested the existence of the short DNA fragments. Next year (1967), at the International Congress of Biochemistry in Tokyo, we presented the discontinuous model of DNA replication.

Accumulation of Okazaki fragments in a DNA ligase-deficient bacteriophage strain

Dr. Lark, Kansas State University, attended the International Congress of Biochemistry. He offered us a research opportunity in his laboratory, so we stayed in the U.S. for about six months in the autumn of 1967 as a visiting professor and a visiting associate professor of Kansas State. On the way to Kansas, we stopped by the Kornberg laboratory and were notified of the discovery of DNA ligase, the enzyme that forms a covalent phosphodiester bond between the 3′- and 5′-termini of DNA chains. This enzyme had characteristics that were expected of an enzyme that forms links between Okazaki fragments. Dr. Richardson of Harvard University, who had been our friend since our stay in Stanford, discovered that gene *30* of bacteriophage T4 (a virus that infects bacterial cells) encoded DNA ligase, and he kindly provided us with a temperature-sensitive mutant T4 phage strain whose gene *30* product became dysfunctional at high temperatures.[15] We started experiments using this mutant phage strain immediately after we had arrived Kansas.

When T4 bacteriophage infects *E. coli* cells, the virus is amplified to a huge number in the bacterial cells. This virus amplification process involves very active replication of the phage genomic DNA, which certainly involves synthesis of large amounts of Okazaki fragments to form the daughter strands of the phage DNA. While the viral genome replication is taking place, replication of the genomic DNA of the host bacteria is suppressed. Therefore, practically speaking, all the events involved in the DNA replication processes observed in the phage-infected bacterial cells reflect only the replication procedure of the phage genomic DNA, which is entirely dependent on the DNA polymerases and DNA ligase encoded within the phage's own genomic DNA. The replication procedure of the mutant T4 phage whose DNA ligase is defective would halt at the step immediately before the DNA ligase is required. Our experiments demonstrated accumulation of Okazaki fragments associated with the phage DNA in *E. coli* cells infected with the temperature-sensitive mutant T4 phage strain at 43 °C, a non-permissive temperature in which the enzyme activity of the mutant phage DNA ligase was suppressed.[17] These Okazaki fragments were quickly incorporated into the phage DNA chains when the infected bacteria were transferred to a lower temperature (30 °C), in which the phage DNA ligase regained its enzyme activity that connected the DNA fragments in the tail-to-head fashion to form long DNA chains (Fig. 4). These experimental results convinced us that, during DNA replication, DNA ligase is necessary in the process that assembles the short-length Okazaki fragments into a long and continuous DNA chain. Interestingly, when the ligase activity was suppressed, all of the tritium radioactivity was recovered in the short DNA fragments, implying the possible double-strand discontinuous replication in which the discontinuous DNA synthesis occurs not only for the lagging strand (of which overall elongation occurred in the 3′-to-5′ direction) but also the leading strand (which appeared to elongate in the 5′-to-3′ direction). This was an unexpected result, but based on the subsequent progress in this field made after our experiments (including the discovery of the DNA repair-associated short DNA fragments), the concept that the discontinuous replication occurs only in the lagging strand synthesis was again accepted widely. At the end of this year (1967), we submitted a full paper on the discontinuous replication mechanism to the *Proceedings of the National Academy of Sciences U.S.A.* (also known as PNAS) through the communication by Dr. Hotchikiss of Rockefeller University.[16] Our paper was accepted, and it was published in February of the next year.

In 1968, Reiji was invited to the Cold Spring Harbor Symposium, where he presented the discontinuous replication model (Fig. 5). At that time, the DNA synthesis reaction at the replication fork was considered a major biological mystery. The chair of the symposium even included in his keynote address a slide showing a picture of the fork partly hidden by a fig leaf. Our discontinuous replication model was accepted as a major clue to the solution of this problem and became one of the highlights of the symposium.[18] In this meeting, the term *Okazaki fragment* was given to the short DNA fragments that appear during the lagging strand synthesis, and this name has remained generally accepted even in today's textbooks.

Direction of the elongation of Okazaki fragments

In 1968, we restarted experiments to determine the direction of the DNA synthesis at the microscopic level — the experiments that we had initially planned. It was the era when political conflicts between angry college students and Government escalated quite violently. We labeled the full-length Okazaki fragments with [^{14}C]-thymidine, and a very short region at the growing end of the fragment was

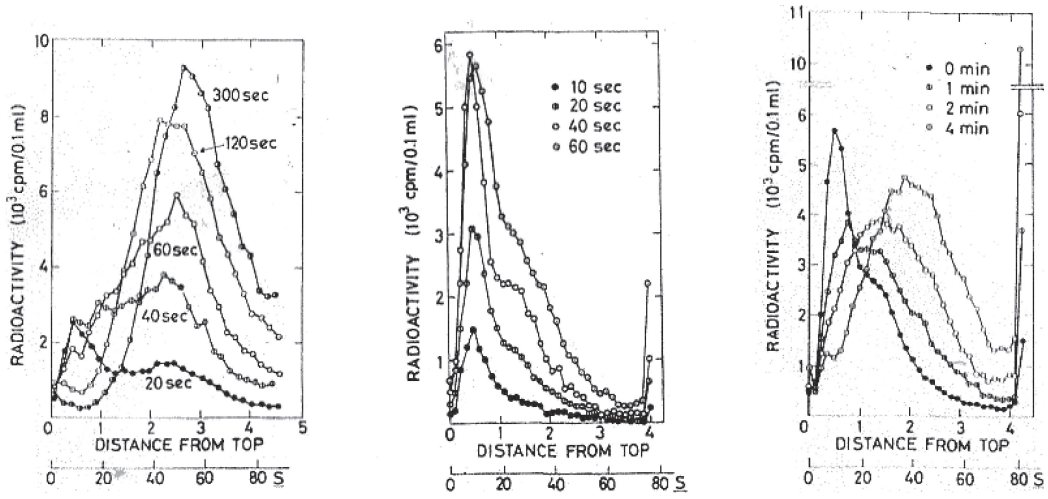


Fig. 4. Okazaki fragments observed in *E. coli* infected with DNA ligase temperature-sensitive mutant strain of T4 bacteriophage. (a) Pulse-labeling experiment at a low temperature. *E. coli* was infected with a gene *30* (encoding DNA ligase) temperature-sensitive T4 phage strain at 20 °C for 70 minutes. Bacteria were then transferred to 30 °C and incubated for 1 minute followed by [^{3}H]-thymidine pulse-labeling for varying periods of time. DNA was then extracted from the bacterial cells, and the length of the DNA fragments were analyzed by sucrose-gradient centrifugation in the alkaline condition. (b) Pulse-labeling experiment at a high temperature. T4 phage-infected bacteria were prepared as the panel (a) experiment and then pulse-labeled at 43 °C. The DNA sedimentation pattern was analyzed by the alkaline sucrose-gradient centrifugation. (c) Pulse-labeling at a high temperature followed by low-temperature incubation. *E. coli* cells were infected with the gene *30* temperature-sensitive T4 phage mutant strain and incubated at 20 °C for 70 minutes. Bacteria were then transferred to 43 °C and incubated for 2 minutes followed by [^{3}H]-thymidine pulse-labeling for one minute. After the labeling, cells were transferred to 30 °C and incubated for varying periods of time. The DNA was then extracted and analyzed by the alkaline sucrose-gradient centrifugation.

labeled with [^{3}H]-thymidine. The labeled Okazaki fragments were purified, and whether the location of the tritium ([^{3}H]) is at the 5′-end or the 3′-end was determined by a kinetic analysis in which the DNA fragments were digested with the direction-specific exonucleases, and the time-dependent release of the ^{3}H and ^{14}C tracers from the DNA was examined (Fig. 6). The ^{3}H radiotracer was released from the substrate DNA immediately after the initiation of the 3′-to-5′ exonuclease digestion whereas it was not released by the 5′-to-3′ digestion until the entire DNA fragment was decomposed. Supporting our expectation, these results indicated that the DNA synthesis occurs exclusively at the 3′-end and that the DNA chain elongates only in the 5′-to-3′ direction.[19]

Functions of DNA polymerase I: Primer degradation and gap filling

When the discontinuous replication model was proposed, DNA polymerase I was the only DNA polymerase enzyme identified in *E. coli*. However, it was soon recognized that the DNA polymerization reaction catalyzed by this enzyme required a primer, a pre-existing short polynucleotide chain. In other words, DNA polymerase I is only capable of adding a nucleotide to the end of a pre-existing polynucleotide chain. For the true initiation of the DNA replication reaction, the existence of another DNA polymerase enzyme that is capable of the *de novo* synthesis of the polynucleotide chain was anticipated. That is, DNA chain synthesis that can be initiated without requiring a pre-existing polynucleotide precursor. When the *E. coli* cell components were separated into the soluble or membrane fractions under mild conditions, most of the DNA polymerase I activity was recovered in the soluble fraction, but the membrane fraction still contained the discontinuous replication activity.

In 1969, Cairns *et al.* isolated an *E. coli* mutant strain *polA1*, which harbored an aberrant DNA polymerase I.[20] This was a turning point in research on the replication mechanism. The strength of this strain's DNA polymerase I activity was so dramatically suppressed that only less than 1% of that found in the wild type *E. coli* strain was detected, and the strain was UV-sensitive (*i.e.*, the mutant strain could not survive irradiation of ultraviolet light — a sign that the strain was unable to repair DNA damages). Nonetheless, the *polA1* strain was viable under a

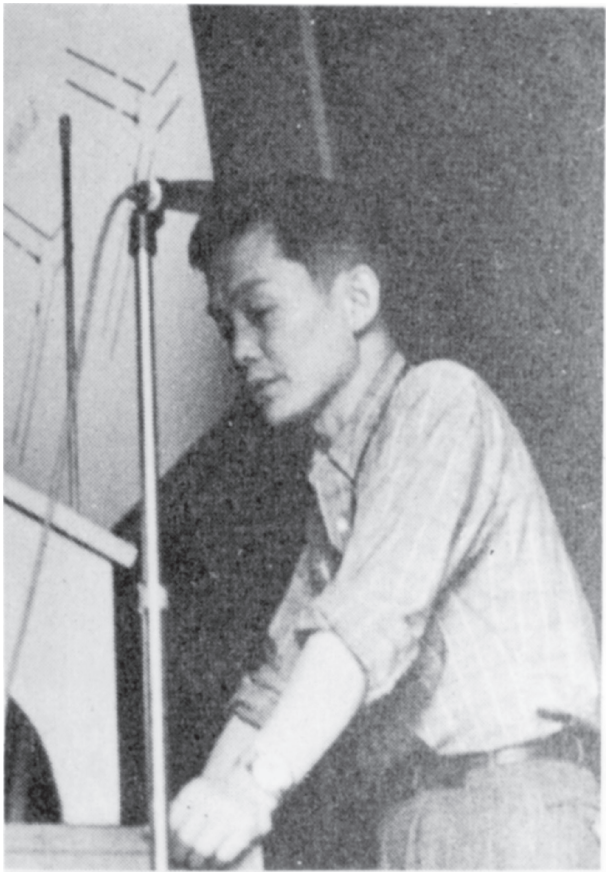


Fig. 5. Reiji Okazaki presenting the discontinuous replication mechanism at the Cold Spring Harbor Symposium in June 1968.

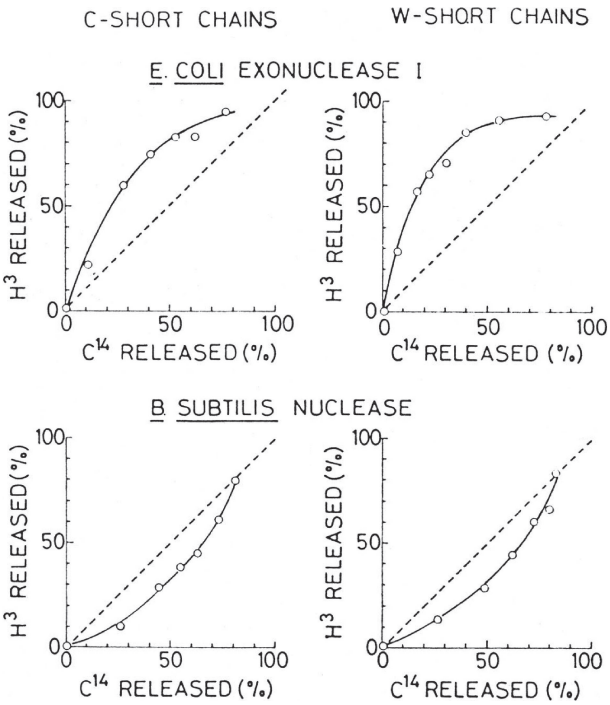


Fig. 6. Determination of the direction of T4 phage Okazaki fragment synthesis by exonuclease digestion analysis. T4 phage-infected bacteria were cultured at 20 °C until the peak of DNA synthesis. Bacteria were then cooled to 8 °C and pulse-labeled with [^{3}H]-thymidine for 6 seconds (to radiolabel the growing end of DNA) and with [^{14}C]-thymidine for 2.5 minutes (to radiolabel the entire length of Okazaki fragments). The 9S short-chain DNA fraction enriched with Okazaki fragments was isolated, and the Watson-strand and Crick-strand were separated further. Each strand was subjected to digestion with *E. coli* exonuclease I (5′-to-3′ digestion) and *Bacillus subtilis* exonuclease (5′-to-3′ digestion), and the ^{3}H and ^{14}C radioactivities released from the DNA was counted at varying periods of time of digestion. Data points are plotted for the percent release of ^{3}H and ^{14}C. The profiles of the ^{3}H release indicate that the growing point locates at the 3′-end of DNA.

standard culture condition. Like the wild type strain, the membrane fraction of the *polA1* strain contained the discontinuous replication activity, and it was soon elucidated that this activity was derived from DNA polymerase III holoenzyme, a multiprotein complex assembled around the *polC*/*dnaE* gene product.[21] Interestingly, within the *polA1* strain cells, a large amount of Okazaki fragments were accumulated (Fig. 7).[22] The purified DNA polymerase I enzyme preparation possesses three activities — namely, the 5′-to-3′ polymerase activity, the 5′-to-3′ exonuclease activity (specific to double strand DNA or RNA-DNA hybrid molecules), and the 3′-to-5′ exonuclease activity (specific to single-stranded DNA substrate; providing the proofreading function).[21] When the former two activities function in a coordinated manner, a nick on the double strand DNA migrates towards the 3′ direction and is eventually filled; this reaction is also known as the *nick translation* reaction. DNA polymerase I of the *polA1* strain was defective in the polymerase activity, but its 5′-to-3′ exonuclease activity was conserved. Consequently, this mutant DNA polymerase I could not catalyze the nick translation reaction and was unable to fill the gaps in the DNA strand. Therefore, the observed accumulation of Okazaki fragments in the *polA1* strain was explained by the inability of the mutant DNA polymerase I to fill the gaps between Okazaki fragments synthesized in the lagging strand. In the presence of such gaps, DNA ligases could not link Okazaki fragments to form a continuous daughter strand, resulting in the observed accumulation of Okazaki fragments in the *polA1* strain. The 5′-to-3′ exonuclease activity of DNA polymerase I

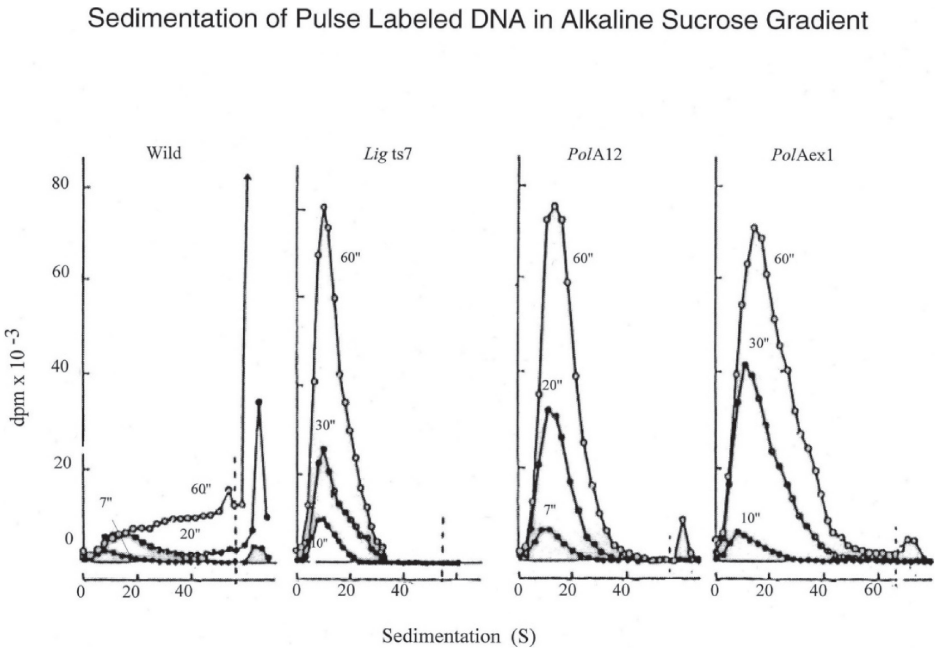


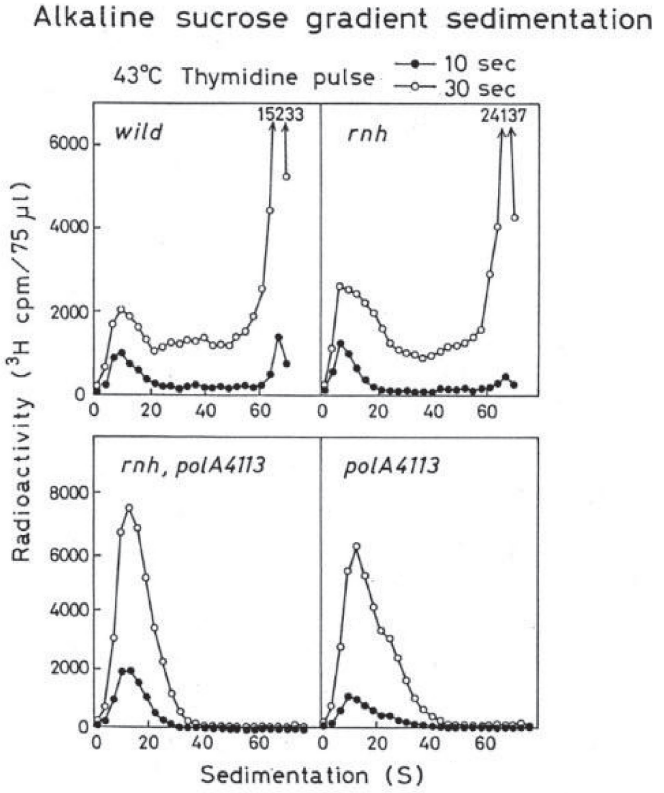
Fig. 7. Short fragments are accumulated in the DNA ligase mutant as well as DNA polymerase mutants of *E. coli* in non-permissive condition. *E. coli* cells were cultured at 30 °C and then the temperature was shifted to 43 °C, added to [^{3}H]-thymidine for the indicated periods of time to radiolabel the newly synthesized DNA. After the indicated time, DNA was extracted from the cells and in a denaturing condition separated by length using the 5–20% alkaline sucrose-gradient centrifugation. Okazaki fragments were observed as short DNA fragments with about 10S sedimentation coefficient. Results with wild type strain; the ligts7 strain harboring a temperature sensitive DNA ligase activity; the *polA12* strain harboring a temperature-sensitive polymerase activity of DNA polymerase I; the *polAex1* strain harboring a temperature-sensitive 5′-to-3′ exonuclease activity of DNA polymerase I, were shown.

was involved in degradation of the special *5′-end structure*, which was required for initiation of Okazaki fragment synthesis and later identified as RNA primer. The nick translation activity of DNA polymerase I simultaneously processed the degradation of RNA primers and the gap-filling between Okazaki fragments. Soon, another temperature-sensitive *E. coli* mutant strain of which DNA polymerase I was defective of the 5′-to-3′ exonuclease activity was isolated. It was demonstrated that this strain also accumulated Okazaki fragments when cultured at a non-permissive temperature (Fig. 8) and that this mutation was lethal.[21] It was also unveiled that most of the DNA polymerase I and DNA ligase enzymes present in the *E. coli* cells were engaged in the DNA repair reaction and that only a small fraction (several percent) of these enzymes were sufficient for the DNA replication reaction at replication fork.[22]

The mechanism of initiation of Okazaki fragment synthesis — Primer RNA

The greatest mystery of discontinuous replication was the mechanism of initiation of Okazaki fragment synthesis. In the 1970s, it became increasingly clear that all DNA polymerases always require primers for initiation of their polymerase reaction and that none of them can initiate DNA polynucleotide chain synthesis from only two nucleotides. As synthesis of Okazaki fragments must be initiated frequently during the process of DNA replication, we had no clues as to how to explain the biochemical basis of such events.

Widely accepted among the investigators specialized in the *in vitro* biochemical reactions was the following idea. As described earlier in this essay, prolonged *in vitro* DNA replication reaction catalyzed by DNA polymerase I produces branched-form DNA because of the template-switching phenomenon. They assumed that the same template-switching was taking place at the replication fork. That is, a DNA polymerase enzyme that has been synthesizing the leading-strand daughter chain in a continuous fashion switches the template strand spontaneously at a certain frequency. As a consequence of the template switching, the same DNA polymerase I is now synthesizing the lagging strand by simply adding nucleotides, still in a continuous fashion, to the end of the same DNA strand that it was synthesizing



Number of RNA-linked DNA fragments in four isogenic strains of E. coli

Strain	DNA fragments with 5′-Ribonucleotide termini† (molecules/cell)	DNA fragments with Intact primer RNA‡ (molecules/cell)
ON112 (*rnh*⁺, *polA*⁺)	11	0·1
ON152 (*rnh-91*)	21	0·9
KN72 (*polA4113*)	57	0·2
ON2104 (*rnh-91*, *polA4113*)	55	2·7

Fig. 8. Okazaki fragments accumulation in the *pol A* and *rnh* mutant *E. coli* strains. Wild type, *rnh* mutant, *rnh* and *polA* 5′ to 3′ exonuclease mutant of *E. coli* strains were grown at 30 °C and then transferred to 43 °C. At this non-permissive temperature, cells were incubated in the presence of [^{3}H]-thymidine for the indicated periods of time to radiolabel the newly synthesized DNA. After the incubation, DNA was extracted in a denaturing condition and separated by length by the 5–20% alkaline sucrose-gradient centrifugation. Okazaki fragments were observed as short DNA fragments with about 10S sedimentation coefficient. Upper left, wild type strain: upper right, the RNase H strain harboring temperature sensitive RNase H activity. Lower left, the double mutant strain harboring a temperature-sensitive 5′ to 3′ exonuclease activity of DNA polymerase 1 and RNase H activities; lower right, the *polA4113* strain, which harbored a temperature-sensitive DNA polymerase I whose 5′-to-3′ exonuclease activity was suppressed under high temperature.

moments before as the leading strand. This forms a hairpin-like structure of the single-stranded daughter DNA, of which 5′-half is the leading strand and the 3′-half is the lagging strand, at the replication fork. The hairpin-shaped, single-stranded daughter DNA will then be cut at the junction between the leading and lagging strands, thus leaving an Okazaki fragment as a precursor of the lagging strand, and the DNA polymerase I goes back to the task of synthesizing the leading strand, again by the spontaneous template switching. By repeating the above processes, both the leading and lagging strands of daughter DNA appear to be synthesized simultaneously. Importantly, this hypothetical model (which is considered incorrect today) did not require frequent initiation of DNA synthesis, and it even explained the origin of Okazaki fragments.

Important insights came from reports that initiation of DNA synthesis in retroviruses and M13 bacteriophage involved RNA.[23),24)] These discoveries

prompted us to presume that the events initiating Okazaki fragment synthesis may also involve RNA. It was already known that all RNA polymerases can initiate polynucleotide chain synthesis without requiring a primer. Moreover, DNA polymerase can utilize an RNA polynucleotide chain as a primer, as long as the RNA forms a heteroduplex structure with the complementary DNA chain. However, rifampicin, an inhibitor of bacterial RNA polymerase that specifically binds to the β-subunit of the enzyme, did not inhibit DNA chain elongation occurring in the *E. coli* chromosome. Therefore, our hypothesis required a new RNA polymerase that was resistant to rifampicin. As a matter of fact, the primase enzyme, which synthesizes very short RNA primer chains on the single-stranded DNA template, was discovered later, and it was indeed a new type of RNA polymerase that was resistant to rifampicin.[25)]

We assumed that the primer RNA segment may attach to the 5′-end of Okazaki fragments and attempted to detect this RNA. However, soon we encountered a great difficulty. The number of Okazaki fragments associated with the primer RNA segment turned out to be extremely low (only about 10 molecules in a wild type *E. coli* cell), and the length of the primer RNA was far shorter than it had initially been predicted (less than 10 nucleotides). Moreover, enormous numbers of RNA fragments with various lengths, which we called free RNA, were found in the bacterial cells. Only a trace amount of contamination of such intracellular free RNA would result in a serious experimental artifact.

While we were struggling to overcome this obstacle, another grave situation emerged. The claws of chronic myeroblastic leukemia seized upon Reiji. He was in Hiroshima City when the Atomic Bomb was ruined the city. His reaction when he knew about his physical condition was that he was lucky to have lived 30 more years since the bombing. Thus, we were forced to compete with the calendar — can we prove the existence of the RNA primer of Okazaki fragments before the date he has to leave?

One of the differences between RNA and DNA is their resistance to alkali. DNA is fairly resistant to alkali whereas RNA is readily degraded in high pH conditions. If the head (5′ end) of a DNA fragment is joined to the tail (3′ end) of an RNA fragment, alkaline treatment of such a composite polynucleotide chain would selectively degrade the RNA portion whereas the DNA portion would survive. Whereas the normal head of a DNA chain has a phosphate, the RNA-linked 5′ end of the DNA portion remaining after the alkaline treatment would lose the phosphate, leaving the tell-tale hydroxyl group [5′-OH] at the DNA head. We anticipated that detection of such a 5′-OH structure at the DNA head would serve as indirect evidence of the existence of the RNA primer. When we analyzed Okazaki fragments of prokaryotic cells based on this strategy, we were able to detect the 5′-OH structure on some of Okazaki fragments. This observation convinced us of the existence of the primer RNA.[26)–29)]

In March, 1975, Reiji and I attended a scientific meeting on DNA replication in Montebello, Canada, but by this time his leukemia turned to acute condition and was already desperate. After we had returned to Japan, Reiji was hospitalized, and on August 1st he passed away at the age of 44 without knowing the nature of the RNA primer. Not enough time was given to him.

Challenges to pseudo-Okazaki fragments and the double-strand discontinuous replication model

Immediately after I had lost Reiji, I received news about a serious challenge to the existence of Okazaki fragments. *Pseudo*-Okazaki fragments: these are short DNA chains newly synthesized during one of the DNA repair reactions known as uracil excision repair. Deamination of cytosine in DNA occurs spontaneously at a low rate to yield uracil base on DNA. The uracil base present in the DNA chain is recognized by an enzyme known as *uracil-DNA glycosilase*, which cuts the bond between the uracil base and sugar in the DNA backbone. The site lacking the base (known as the *AP site*) is then recognized by an endonuclease known as *AP endonuclease*, which cuts the backbone phosphodiester bond to induce a nick in the DNA. This nick initiates the nick translation reaction catalyzed by DNA polymerase I, which repairs the DNA damage using its 5′-to-3′ exonuclease activity and 5′-to-3′ polymerase activity. This final reaction of the uracil excision repair involves synthesis of a new, short DNA fragment, which will be incorporated into the continuous DNA chain by DNA ligase. The news was that an *E. coli* mutant strain *sof* contained a large amount of short DNA fragments that resembled Okazaki fragments and that this mutant lacked dUTPase. In this cell, dUTP (deoxyuridine triphosphate, a uracil-containing nucleotide) was accumulated because of the deficiency of dUTPase, an enzyme that degrades dUTP. The high concentration of cellular dUTP pool resulted in an increased

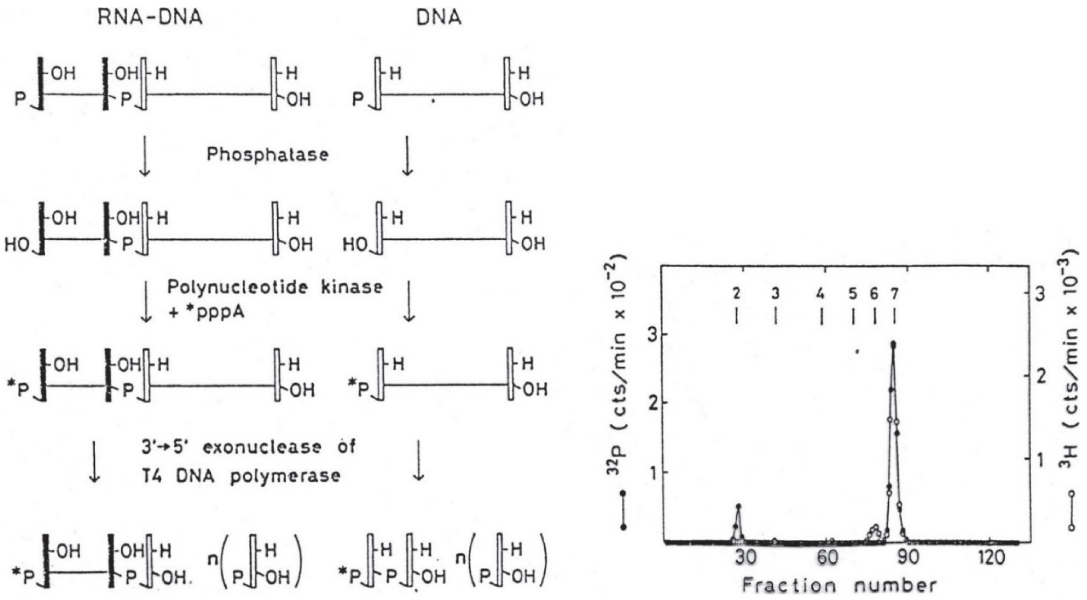


Fig. 9. Method of isolation of primer RNA from Okazaki fragments and determination of chain length.

frequency of misincorporation of dUMP, instead of TMP, into DNA. This error was immediately recognized by uracil-DNA glycosylase, and the uracil excision repair reaction was initiated. Pulse-labeling experiments with ^{3}H-thymidine demonstrated that the *sof* strain frequently produced radioactive short DNA fragments, which resembled Okazaki fragments in size and hence were later called *pseudo-Okazaki fragments*, from the AP site.[30] The intracellular dUTP/TTP ratio was not less than 1/1,200 even in the wild type *E. coli* cells. Based on these observations, it was proposed erroneously that Okazaki fragments could actually be short DNA fragments produced by repair reaction in newly replicated region — but not the replication units in lagging-strands. It was reported by the media that, even though there was no other proper explanations available for the lagging-strand synthesis, the discontinuous replication model itself still lacks evidence for *de novo* synthesis of Okazaki fragments! We defended the Okazaki fragment hypothesis by focusing the possible structural difference at the 5′ ends between the repair molecules and the Okazaki fragments as follows. Firstly, alkaline treatment of the short DNA fragments generated during the DNA repair reaction (*i.e.*, pseudo-Okazaki fragments) produced the 5′-phosphoryl ended DNA — while the 5′-OH ended DNA would be produced from the nascent DNA molecules with RNA primer. Therefore, by the alkaline treatment test, the RNA-linked short DNA fragments produced during DNA replication (*i.e.*, Okazaki fragments) and the fragments synthesized during the DNA excision repair reaction (*i.e.*, pseudo-Okazaki fragments) would be distinguishable. Secondly, the ratio of pseudo-Okazaki fragments to Okazaki fragments in the wild type *E. coli* cells was very low compared to the *sof* strain even though these strains showed similar DNA replication rate.[31)–33)]

The discontinuous replication model was originally proposed to explain the mechanism of the lagging strand synthesis. However, based on the observations that DNA chains synthesized in bacteria deficient of DNA ligase or DNA polymerase I were all short, the possibility of the both-strand discontinuous replication was once considered. However, because both of DNA ligase and DNA polymerase I are involved in the DNA repair process, it was later interpreted that the incorporation of the ^{3}H-labeled thymidylate into exclusively into short DNA fragments in the absence of these enzymes would not necessarily support the double-strand discontinuous replication. Extrapolating from the products in the *in vitro* reaction with purified replication enzymes, majority of investigators now believe that the leading strand is synthesized in the continuous manner only, and that the leading strand-derived radioactive short DNA fragments generated *in vivo* are likely to be produced in the process of DNA repair reaction.

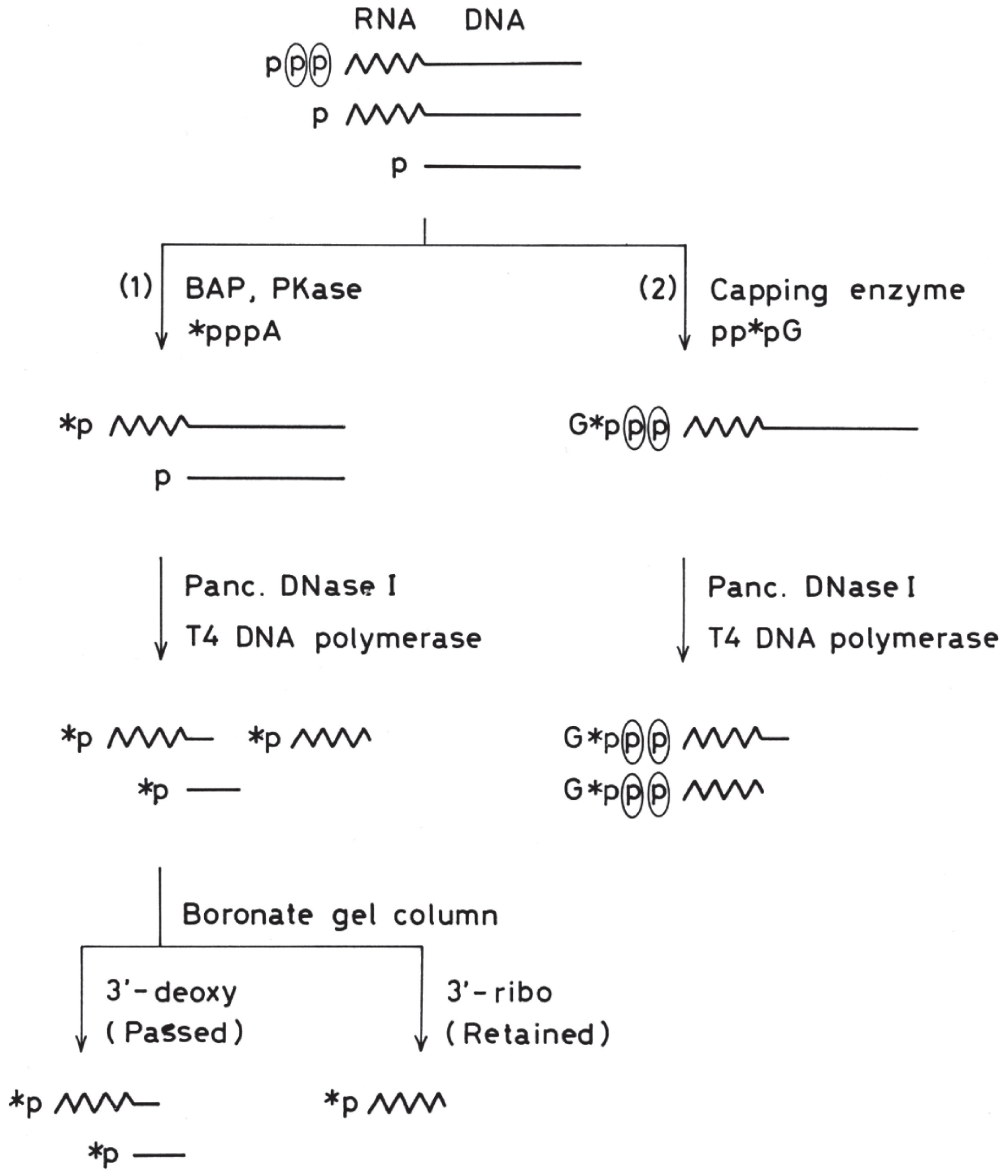


Fig. 10. Detection of intact Primer RNA.

The structure of primer RNA

The discontinuous replication mechanism would not be established unless the nature of the primer was unveiled. Because the amount of the primer RNA was very low, we attempted to accumulate the primer using an inhibitor of the primer-degrading enzyme and purify it to determine its nucleotide sequence.[34),35)] We isolated a highly purified Okazaki fragment preparation and introduced the high specific-activity [^{32}P] label to its 5′ end by two *in vitro* reactions — namely, the intact primer molecules with the initiation terminal (5′-triphosphate) were labeled by forming the [^{32}P]-labeled cap structure using the capping enzyme (RNA guanyltransferase) and [α-^{32}P] GTP,[36)] and the partially degraded primer molecules, which have the 5′-monophosphate end, was treated with phosphatase to remove the 5′ phosphate and then labeled by a kinase reaction using polynucleotide kinase and [γ-^{32}P] ATP.[37),38)] We then degraded the DNA portion of the 5′-[^{32}P]-labeled Okazaki fragments to trim them to the junction between RNA and DNA, and the primary structure of the primer RNA was determined (Figs. 9, 10). Results obtained from

Table 1. Structure of primer RNA and core recognition sequence

System	Structure	Core Recognition Site
Phage T7	$\mathrm{pppApCp}\frac{\mathrm{C}}{\mathrm{A}}(\mathrm{pN})_{1\sim2}$ (N: A, C rich)	$3'$–C–$\overset{\star}{\mathrm{T}}$–G–$5'$
Phage T4	$\mathrm{pppApC(pN)_3}$	$3'$–T–$\left(\frac{\overset{\star}{\mathrm{C}}}{\mathrm{T}}\right)$–G–$5'$ (HMC not used)
E. coli	$\mathrm{ppp}\frac{\mathrm{A}}{\mathrm{G}}(\mathrm{pN})_{9\sim11}$	$3'$–G–$\overset{\star}{\mathrm{T}}$–C–$5'$ (Pu–$\overset{\star}{\mathrm{P}}$y–Py)
Eukaryote	$\mathrm{ppp}\frac{\mathrm{A}}{\mathrm{G}}(\mathrm{pN})_{7\sim9}$	$3'$–Pu–$\overset{\star}{\mathrm{P}}$y–Py–$5'$ (T > C)

several systems are shown in Table 1. In 1978, I was invited to the Cold Spring Harbor Symposium, where I reported the structure of the primer RNA.[39] Several overseas laboratories were working on the primer RNA structure using *in vitro* systems, and their results agreed well with ours. After this symposium, no one expressed doubt about the nature of Okazaki fragments as intermediate molecules synthesized during the process of the discontinuous DNA replication, and the criticism that Okazaki fragments could be intermediate molecules produced in the course of the DNA repair reaction was no longer voiced.

Location of primer synthesis and the signal sequence

The primary structure of the primer, by itself, did not contain enough information to determine the frequency of initiation of Okazaki fragment synthesis, which was estimated from the length of the fragment to be once every 1,000–2,000 nucleotides (Table 1). To understand the mechanism that determines the length of Okazaki fragments, we attempted to map the sites of the primer RNA synthesis on the genomic DNA and identify the signal sequence for initiation of the primer RNA synthesis including the non-transcribed region of the DNA template. It was about the beginning of 1980, when several new and powerful experimental techniques — *e.g.*, nucleotide sequence determination and the recombinant DNA

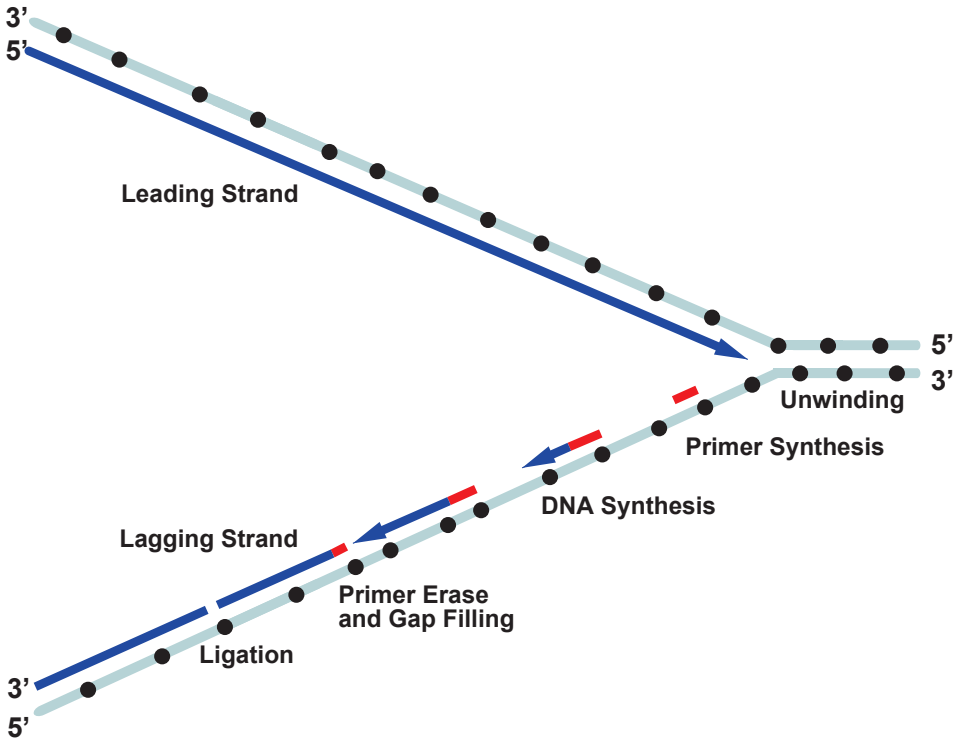


Fig. 11. Steps of the discontinuous DNA replication reaction. The leading strand is synthesized continuously while the lagging strand is synthesized discontinuously. The elongation reaction of the lagging strand consists of five steps: I, Unwinding of the DNA template; II, Primer synthesis; III, DNA (Okazaki fragment) synthesis; IV, Primer degradation and gap filling; and V, Ligation of Okazaki fragments. The dots on the template DNA indicate the signal sequences for primer RNA synthesis.

technologies — became widely available to obtain long-awaited answers of tough problems. Here, I only present a brief summary of the major achievements accomplished in those days. It has been established that the signal sequences consisting of three nucleotides (Table 1) are distributed along the genomic DNA, and Okazaki fragments are synthesized when the primer-synthesizing RNA polymerase (*i.e.*, primase) recognizes these signal sequences at the replication fork. The frequency of the signal sequences appearing in the genomic DNA is not reflected in the length of Okazaki fragments, which is 1,000–2,000 nucleotides. Different signal sequences are chosen at round of replication, and this choice seems dependent on the replication machinery that forms the replication fork. The flexibility of the mechanism of Okazaki fragment initiation is advantageous to replicate the whole genomic DNA without a hole.

The biochemical mechanism of the discontinuous replication

Prompted by the discovery of the discontinuous replication, the biochemical research on DNA replication after the 1970s was led by efforts to reconstitute the reactions at the replication fork *in vitro*. The major achievements of this era include elucidation of the mechanism of the primer synthesis, *in vitro* reconstitution of the processive DNA-synthesizing machinery that mimics the *in vivo* velocity of replication (1,000 nucleotides per second), and reconstitution of the replication fork protein complex that synthesizes both the leading and lagging strands simultaneously. The precise device of the fork reactions and the common mechanism of DNA replication conserved among the prokaryotes and eukaryotes are examples of research themes that have long attracted investigators.[40)–43)]

Concluding remarks

The steps of the discontinuous replication mechanism elucidated by the above research are shown in Fig. 11. Our research, from the discovery of Okazaki fragments to the establishment of the series of steps in the replication reaction, was performed by employing the approach of analyzing the DNA products of the reactions occurring in cells.

Acknowledgement

I thank many collaborators for their devoted effort and School of Science, Nagoya University where main part of this work was performed.

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(Received Feb. 15, 2017; accepted Mar. 22, 2017)

Profile

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