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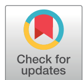

Establishing a biochemical understanding of the initiation of chromosome replication in bacteria

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In the mid-1950s, Arthur Kornberg elucidated the enzymatic synthesis of DNA by DNA polymerase, for which he was recognized with the 1959 Nobel Prize in Physiology or Medicine. He then identified many of the proteins that cooperate with DNA polymerase to replicate duplex DNA of small bacteriophages. However, one major unanswered problem was understanding the mechanism and control of the initiation of chromosome replication in bacteria. In a seminal paper in 1981, Fuller, Kaguni, and Kornberg reported the development of a cell-free enzyme system that could replicate DNA that was dependent on the bacterial origin of DNA replication, *oriC*. This advance opened the door to a flurry of discoveries and important papers that elucidated the process and control of initiation of chromosome replication in bacteria.

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Understanding how DNA is inherited from one cell generation to the next had long been of major interest of Arthur Kornberg, particularly since the 1953 understanding of the double-helical structure of DNA with its implications for DNA replication (1, 2) and his mid-1950s discovery of the first DNA polymerase (3). Among Kornberg's most interesting questions was understanding how the entire duplication of a chromosome begins and the events in the cell that control this important process (4). Achieving these goals required a robust experimental system that allowed the identification of the proteins involved so that their mechanism and regulation could be studied. But this was a tall order when Kornberg first started to address this problem in the late 1960s and early 1970s. After a long struggle, a breakthrough paper appeared in 1981 that reported the enzymatic initiation of DNA replication from a bacterial origin of DNA replication in an isolated cell extract (5). In this paper, Robert (now Roberta) Fuller, Jon Kaguni, and Arthur Kornberg opened up a path toward understanding how bacterial initiation of DNA replication occurs and how it is regulated.

Although it was known that the replication of the bacterial chromosome started at a unique site and the origin of DNA replication had been genetically mapped using F′ deletion mapping, the physical nature of this replication origin was not apparent before the advent of recombinant DNA technology in the mid-1970s. Therefore, Kornberg and many others turned to simpler systems of replication, including the replication of single-stranded bacteriophage DNA. For the most part, these phages rely on host cell enzymes to duplicate their genomes. These bacterial proteins, including DNA polymerase III and other cooperating proteins at the replication fork that copied the single-strand DNA to duplex DNA, were mostly identified by studying the replication of small bacteriophage genomes, including ΦX174, M13, and G4 phage (6–10). Although the single-stranded DNA phages have varied mechanisms for starting replication, they all utilize the bacterial host enzymes that are required for duplication of the bacterial chromosome (11). Key to the identification and purification of many of these host cell proteins was the isolation of mutants of *Escherichia coli* (*E. coli*) that were defective in the replication of phage or chromosomal DNA, including mutants such as *dnaA*, *dnaB*, *dnaC*, *dnaG*, etc. Using the single-stranded viral DNA (ssDNA) templates or replicative form (double-stranded DNA; dsDNA) templates, replication of these small phage genomes in crude cell extracts or in fractions derived from these extracts allowed the biochemical purification of all the essential proteins that function at the DNA replication fork to coordinately synthesize both leading and lagging strands of DNA. Indeed, with the discovery of DNA ligase, it was possible to replicate ΦX174 DNA to produce a synthetic version that had all the infectious properties of the authentic DNA that was isolated from phage particles (12, 13). This creation of an infectious DNA, or as it was reported "creation of life in a test tube," caused a sensation in the press (14).

Despite these major successes, Kornberg noted "in 1971, we had been frustrated in our attempts to put the shattered Humpy Dumpty of bacterial replication back together again" (7). The key missing piece in the puzzle was the process of initiation of bacterial chromosome replication. It should be noted that in the early 1970s, restriction enzyme-mediated cloning of recombinant DNA had not been developed, and using the large bacterial chromosome as a template for biochemical studies was far too complicated and messy. However, the advent of recombinant DNA technology, which allowed cloning of the bacterial origin of DNA replication (*oriC*) and overexpression of proteins in bacterial cells from cloned genes, meant that progress could proceed. It also took determination and steadfastness to continue working on a problem that had, at that point, only produced "10 man-years of utter frustration" (15). Kornberg's persistence paid off in a major way with the development of a protein fraction (called Fraction

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II) derived from a crude cell extract that could support the initiation of DNA replication from the *E. coli* origin of DNA replication, *oriC*. Key to this discovery was the availability of small plasmids that harbored the *oriC* sequence. However, equally important was the remarkable biochemical insight and techniques employed for the preparation of the active enzyme fraction.

The Template

Replication of the circular bacterial chromosome was known to be bidirectional from a unique site (16–20). Physical mapping using fragments from the *E. coli* genome cloned into a plasmid that lacked an origin of DNA replication and antibiotic selection of the plasmid enabled the isolation of a genetic element from the *E. coli* chromosome that conferred on the plasmid the ability to replicate autonomously. As a result, in 1977, the *E. coli oriC* was cloned, and using the recently developed DNA sequencing methods, the DNA sequence of *oriC* was determined (20–22). Later comparison of the minimal origin-containing DNA from several enteric bacterial chromosomes revealed conserved DNA sequence elements within *oriC* (23, 24). Thus, the template for biochemical investigation of the initiation of bacterial chromosome replication was available. To initiate the hunt for biochemical conditions that could support *oriC*-dependent DNA replication, Kornberg obtained the *oriC*-containing plasmids from Seiichi Yasuda. However, this was only one part of the puzzle. Next was a major challenge, how to prepare an enzyme system that could initiate DNA replication from *oriC*?

The Active Fraction

Large amounts of *E. coli*, 300 L at a time, were grown and concentrated, treated with two different lysozyme enzymes, and after a freeze–thaw cycle, the crude cell extract was clarified by ultracentrifugation. This crude extract was not able to support *oriC*-dependent DNA replication, but Fuller, Kaguni, and Kornberg (Fig. 1) then produced from this crude extract a fraction that was active under certain biochemical conditions. To achieve this, some amazing biochemistry was

Jon Kaguni, Arthur, and Bob Fuller in 1981.

Fig. 1. Jon Kaguni, Arthur Kornberg, and Robert (now Roberta) Fuller in 1981. Image credit: Reprinted with permission from Nature.

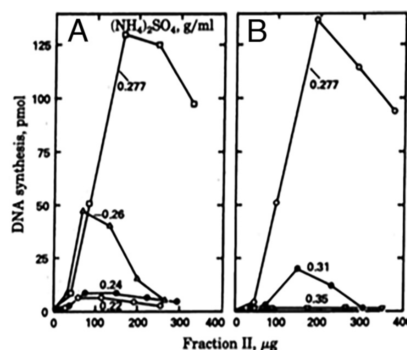

Fig. 2. Use of ammonium sulfate to fractionate the crude *E. coli* extract. From Fuller et al. (5). The original legend is reproduced below the figure. Image credit: Reprinted with permission from ref. 5.

performed. The crude and inactive cell extract was subjected to ammonium sulfate fractionation by slowly adding the salt and precipitating a subset of protein that was then resuspended in a highly concentrated protein fraction. Kornberg had used the ammonium sulfate precipitation "trick" as he called it (12) 30 y earlier for the discovery of the yeast enzyme that converts NAD to NADP (25). Remarkably, the concentration of ammonium sulfate that worked was found to reside in an extremely narrow range (>0.26 to <0.29 g/mL), derived by titrating different amounts of the salt and testing for activity (Fig. 2). Presumably, the precipitation of a subset of protein from the crude extract not only concentrated the essential protein(s) but also removed inhibitors of DNA replication that remained soluble in 0.277 g/mL of $(NH_4)_2SO_4$. It is striking that small changes in the amount of added salt, either too little or too much would yield fractions that were inactive. Moreover, even the most active fraction had an optimal protein concentration in the replication reaction.

Equally important was the observation by Fuller et al. that the concentrated protein extract, called Fraction II, was not active unless a molecular crowding agent such as polyethylene glycol (PEG), polyvinyl alcohol (PVA), or methylcellulose was added. As the authors noted, these polymers may have increased the effective concentration of the active enzymes by an excluded volume effect. The reaction also contained added ATP and an ATP-regenerating system consisting of creatine phosphate and creatine kinase. The DNA replication reaction was dependent on the presence of an *oriC*-containing plasmid, which was inhibited by the RNA polymerase inhibitor Rifampicin, which also inhibited DNA replication in *E. coli* cells, and inhibitors of gyrase, the topoisomerase that supercoils the circular plasmid DNA. Thus, by a number of criteria, origin-dependent DNA replication was achieved by the combination of several biochemical methods, with each alone being insufficient for success. As Kornberg later noted, "after 10-man years of fruitless effort to obtain a cell-free initiation system, Bob Fuller and Jon Kaguni were the ones who finally succeeded" (12).

It is amazing that this worked. Multiple experimental biochemical "tricks" had to work, including the precise amount of ammonium sulfate that was used to produce fraction II, along with the requirement for a molecular crowding reagent. Coupled with the availability of cloned *oriC,* everything came together to open the biochemical door that resulted in many important follow-on discoveries.

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Validating the Initiation Reaction

Additional confidence that the initiation reaction was authentic also came from experiments in which either DnaB protein [later shown to be the replicative helicase, (26)] and SSB, the single-stranded DNA binding protein, were removed from the active Fraction II using either anti-DnaB or anti-SSB antibodies, respectively. These depleted extracts were inactive for DNA replication, but importantly, the addition of purified DnaB or SSB protein, respectively, reconstituted robust DNA replication in the test tube. Since both DnaB and SSB are essential for DNA replication in *E. coli* cells, these experiments further validated the cell-free initiation reaction. Interestingly, the removal of Pol I, the DNA polymerase enzyme Kornberg first discovered and used to demonstrate enzymatic synthesis of DNA, was not required, but this was now an expected observation since the replicative polymerase was known to be DNA polymerase III, the polymerase that also duplicates the single-stranded phage DNAs.

The initiation reaction was dependent on a functional *oriC* origin sequence since the replicative form of ΦX174 DNA or other circular plasmids did not work. Furthermore, physical mapping of the start site of DNA replication in the plasmid showed that replication started near *oriC*. This was achieved by the use of increasing concentrations of the chain terminator 2′, 3′-dideoxythymidine 5′-triphosphate (dTTP) in the reaction and continuous labeling of the replicated DNA by inclusion of [α-^{32}P]dTTP. In the presence of high concentrations of ddTTP, the restriction enzyme fragments near *oriC* were preferentially labeled. Later, in a separate study, the authors showed using electron microscopy that replication from *oriC* in vitro did indeed start at the genetically defined origin and was bidirectional from that sequence (Fig. 3) (27).

In an important experiment that would later lead to key discoveries about the initiation mechanism, Fraction II extracts prepared from the *E. Coli dnaA* mutant were shown to be inactive for *oriC*-dependent DNA replication but could support conversion of ΦX174 ssDNA to the duplex DNA form. Thus, these extracts were capable of supporting DNA replication, just not *oriC*-dependent initiation. When, however, a Fraction II extract prepared from a strain of *E. coli* harboring a plasmid overexpressing the wild-type DnaA protein was added to the reaction, *oriC*-dependent DNA replication was stimulated over 30-fold.

A Flurry of Results

Once the breakthrough of developing a cell-free system for origin-dependent DNA replication had occurred, many paths for experimentation became possible, including the discovery of novel proteins, uncovering the mechanism of the initiation of DNA replication, and understanding the control of the initiation process. An immediate and major down-stream discovery was the purification of the DnaA protein by Fuller and Kornberg using a biochemical assay in which Fraction II from a *dnaA* mutant strain was complemented with protein fractions from a strain of *E. coli* that overexpressed wild-type DnaA protein. The purified 52,000-dalton protein was shown to bind to supercoiled *oriC-containing* DNA (28). In a follow-up study, Fuller, Funnell, and Kornberg showed that DnaA protein bound to a 9-mer sequence located in at least four sites

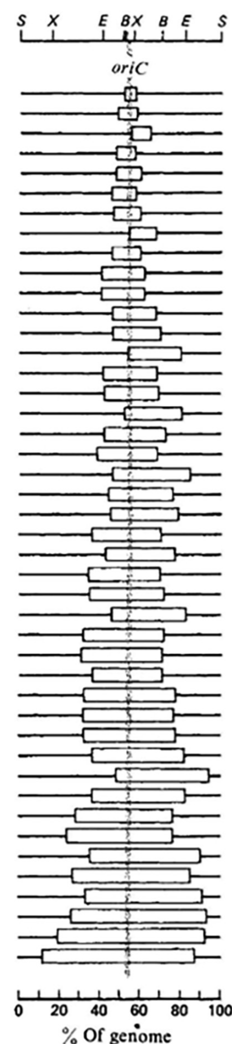

Fig. 3. Summary diagram of electron micrograph observations showing bidirectional DNA replication from *oriC*. From Kaguni et al. (27). The original legend is reproduced below the figure. Image credit: Reprinted with permission from ref. 27.

within *oriC* using deoxyribonuclease footprinting (29). Subsequent studies by many groups, but particularly Kornberg's laboratory, elucidated the mechanism by which DnaA opened the *oriC* DNA and recruited the DnaB–DnaC hexameric proteins to begin more extensive unwinding of DNA during DNA replication (30–33). These studies showed that the DnaA protein bound to about a dozen sites at *oriC* with varying affinities in supercoiled *oriC* DNA, and, in combination with the IHF protein that produced a sizable bend in the origin DNA, unwound a region of the origin called the DNA Unwinding Element (DUE). However, DnaA turned out to have far more complicated functions than just binding to and unwinding the origin. First, the interaction with the origin is controlled by a DnaA•ATP to DnaA•ADP cycle that is controlled by many DnaA binding sites in the *E. coli* genome and by the process of DNA replication initiation itself, implying feedback regulation. Moreover, DnaA is a transcriptional regulator that controls its own levels in the cell (33). These studies on DnaA protein, including the effect of acidic, membrane-associated phospholipids on recycling DnaA•ADP to the apo-DnaA protein that can bind ATP have uncovered many aspects of how the initiation of bacterial chromosome

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replication is regulated and coordinated with cell growth and division (33).

Reconstitution of Complete oriC-Dependent DNA Replication

The breakthrough of establishing *oriC*-dependent initiation of DNA replication in Fraction II, as well as the prior studies on the DNA replication fork proteins identified via biochemical studies of phage DNA replication systems set a path toward reconstitution of complete DNA replication from the *E. coli* origin (34). In addition to DnaA and IHF proteins binding to the origin, the recruitment of DnaB helicase by DnaC promotes more extensive DNA unwinding that allows the ssDNA to bind SSB (32). Once the SSB is loaded, recruitment of primase (DnaG) and the DNA polymerase III holoenzyme can occur, much like the conversion of phage ssDNA to replicative form DNA. Bidirectional replication from *oriC* occurs via coordination between the primase, RNA polymerase, and helicase, with gyrase relieving the torsional strain produced by extensive DNA unwinding (35, 36). In a series of papers, Kornberg and colleagues described the reconstitution of DNA replication from *oriC* using entirely purified enzymes (36–38). In addition to the proteins such as DnaA, DnaB, DnaC, and SSB that promoted origin recognition and unwinding, priming proteins RNA polymerase and primase allow DNA replication by the DNA polymerase III holoenzyme that consists of polymerase along with its DNA polymerase clamp that is loaded by clamp loading proteins (39, 40). One surprise was that the n, n′, and n″ (PriA, B, C) and rep helicase that were identified using the phage systems were not required for *oriC*-dependent DNA replication.

Due to the ability of the biochemist to manipulate reaction conditions, Kornberg and collaborators were able to establish initiation of DNA synthesis by primase alone, which makes a short RNA strand to prime DNA replication, or with RNA polymerase priming DNA replication, or a combination of both priming mechanisms. The reconstituted system also depended on specificity proteins such as protein HU, topoisomerase I, and RNase H to prevent *oriC*-independent priming of DNA replication (36). Finally, enzymes such as DNA polymerase I and DNA ligase allowed the maturation and ligation of Okazaki fragments and the synthesis of complete circular, duplex DNAs (34) and their decatenation by topoisomerase IV to produce monomer, circular DNA products (41). Indeed, conditions exist where these purified proteins can catalyze multiple rounds of DNA replication in the test tube (42). These reconstituted reactions no longer required the addition of a hydrophilic polymer such as PEG or PVA because it was possible to increase the concentration of purified components, particularly the gyrase A protein (36).

Sharing Reagents

One of the remarkable aspects of the environment that Kornberg established in the Biochemistry Department at Stanford University was the cohort of talented biochemists who shared reagents and ideas. As noted above, the donation of the cloned *oriC*-containing plasmid by Seiichi Yasuda, who was a previous postdoctoral researcher in Kornberg's laboratory and along with Yukinori Hirota had cloned *oriC*, was critical to the success of the development of the in vitro replication system. Other critical reagents, including the restriction endonucleases BamH I and EcoR I used to map the start site of replication were obtained from John Carlson and Carl Mann who worked in the Biochemistry Department, and other purified reagents, such as T4 lysozyme and antibody against RecA protein, were also gifts from colleagues. Although by 1981 companies were established to produce and sell enzymes, such as restriction endonucleases, it was then still common for these critical reagents to be shared among researchers, and Kornberg's department was a hub of collaboration and sharing, driven by Kornberg's 10 commandments (43).

Comparison to Eukaryotic DNA Replication

It is instructive to consider whether an approach similar to the one taken by Fuller, Kaguni, and Kornberg could have worked for understanding the initiation of DNA replication in eukaryotic cells. Indeed in 1983, cell extracts were prepared from *Xenopus* eggs and were valuable for studies on the cell division cycle (44). However, efficient initiation of added sperm DNA required decondensation of the DNA, formation of chromatin, and formation of a nucleus (45). Much later, in 1998, a completely soluble cell-free system was developed from *Xenopus* egg extracts in which a cytoplasmic cell extract and a nuclear extract had to be added sequentially to establish initiation of DNA replication on exogenous sperm DNA (46). While this system continues to be of great value to contemporary DNA replication research [(47); e.g., see a recent discovery (48)], it did not result in the reconstitution of eukaryotic DNA replication with purified proteins or understanding of the control of initiation of eukaryotic DNA replication. Moreover, the nature of origins of DNA replication in this *Xenopus* cell-free system is not clear.

Like studies of DNA replication in bacteria that utilized phage DNAs, studies of eukaryotic DNA replication and replication-dependent chromatin assembly emerged from the use of small DNA viruses such as Simian Virus 40 (SV40). A soluble, nucleus-free extract from simian or human cells was developed by Joachim Li and Tom Kelly that could initiate DNA replication from the SV40 origin of DNA replication in the presence of SV40 T antigen (49). This extract system, with the addition of a nuclear extract, is capable of DNA replication-coupled chromatin assembly (50). Using this system, a number of essential DNA replication proteins, such as a DNA polymerase δ, Replication Protein A, the eukaryotic single strand DNA binding protein, the polymerase clamp PCNA, the clamp loader RFC, as well as FEN1, an Okazaki fragment maturation factor, were discovered (51, 52).

To understand eukaryotic chromosome replication, the budding yeast *Saccharomyces cerevisiae* turned out to be the system of choice. DNA sequences were isolated from *S. cerevisiae* that could confer on small plasmids the ability to replicate in cells like the endogenous chromosomes (53) and these Autonomously Replication Sequences (*ARSs*) were shown to define the origins of DNA replication (54). The 1981 Fuller et al. paper spurred interest in taking a similar approach to understand DNA replication in yeast and, indeed, a paper appeared that reported the initiation of *S. cerevisiae ARS* DNA replication in vitro, using an extract obtained from yeast cells by precipitation of protein

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using 0.277 g/mL ammonium sulfate, the same concentration that worked for *E. coli* (55). In retrospect, these extracts were unlikely to have promoted authentic initiation of DNA replication from yeast origins because they contained active Cyclin-Dependent Protein Kinases that are now known to inhibit the initiation of DNA replication from yeast origins in vitro.

The route toward reconstitution of complete eukaryotic cell DNA replication in vitro with purified enzymes took a very different route than the studies that resulted in the reconstitution of *oriC*-dependent DNA replication. The key to early success was the detailed characterization of the DNA sequences in a *S. cerevisiae* origin of DNA replication (*ARS1*), and the identification of the origin recognition complex (ORC) that bound to the origin DNA (56, 57). In addition, it was shown that a complex later called the pre-Replicative Complex (pre-RC) was regulated during the cell division cycle and assembled in the G1 phase (58). Biochemical and genetic studies in yeast resulted in the identification of ORC interacting proteins [e.g., Cdc6 (59)] that eventually resulted in the reconstitution of pre-RC assembly with purified proteins [ORC, Cdc6, Cdt1, Mcm2-7 (60, 61)]. Then, the initiation and entire replication of DNA from yeast origins was reconstituted with purified proteins, including many proteins found using the SV40 system (62–64). Paradoxically, Cyclin-Dependent Protein Kinases are required to activate origin firing, but they also inhibit pre-RC assembly, which is required for the initiation of DNA replication. Thus, the entire process and control of the initiation of DNA replication in eukaryotic cells turned out to be very different from that in *E. coli*. The *S. cerevisiae* system is well understood, primarily because origins require specific DNA sequences, but the nature of origins of DNA replication in most eukaryotes are not DNA sequence specific and how ORC determines the location of these start sites for the initiation of DNA replication is under active investigation (65–68).

Understanding the biochemistry and enzymology of the initiation of DNA replication in bacteria capped off a truly remarkable career for Arthur Kornberg. In reviewing the literature and the history, it is clear that Kornberg was not only an extraordinarily talented biochemist but also a mentor to a remarkable group of scientists, many of whom went on to make their own major contributions to science (see https://arthurkornberg.stanford.edu/labAK.html). In addition, he created a research environment that was conducive to major breakthroughs, including the 1981 paper discussed herein.

Data, Materials, and Software Availability. All study data are included in the main text.

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1. J. D. Watson, F. H. C. Crick, Genetical implications of the structure of deoxyribonucleic acid. *Nature* **171**, 964–967 (1953).
2. J. D. Watson, F. H. C. Crick, Molecular structure of nucleic acids; a structure for deoxyribose nucleic acid. *Nature* **171**, 737–738 (1953).
3. A. Kornberg, I. R. Lehman, M. J. Bessman, E. S. Simms, Enzymic synthesis of deoxyribonucleic acid. *Biochim. Biophys. Acta* **21**, 197–198 (1956).
4. A. Kornberg, *For the Love of Enzymes. The Odyssey of a Biochemist* (Harvard University Press, 1991).
5. R. S. Fuller, J. M. Kaguni, A. Kornberg, Enzymatic replication of the origin of the *Escherichia coli* chromosome. *Proc. Natl. Acad. Sci. U.S.A.* **78**, 7370–7374 (1981).
6. K. Arai, N. Arai, J. Shlomai, A. Kornberg, Replication of duplex DNA of phage phi X174 reconstituted with purified enzymes. *Proc. Natl. Acad. Sci. U.S.A.* **77**, 3322–3326 (1980).
7. A. Kornberg, Aspects of DNA replication. *Cold Spring Harb. Symp. Quant. Biol.* **43**, 1–9 (1979).
8. R. Schekman, J. H. Weiner, A. Weiner, A. Kornberg, Ten proteins required for conversion of phiX174 single-stranded DNA to duplex form in vitro. Resolution and reconstitution. *J. Biol. Chem.* **250**, 5859–5865 (1975).
9. R. Schekman, A. Weiner, A. Kornberg, Multienzyme systems of DNA replication. *Science* **186**, 987–993 (1974).
10. S. Eisenberg, J. F. Scott, A. Kornberg, Enzymatic replication of X174 duplex circles: Continuous synthesis. *Cold Spring Harb. Symp. Quant. Biol.* **43**, 295–302 (1979).
11. P. D. Baas, DNA replication of single-stranded *Escherichia coli* DNA phages. *Biochim. Biophys. Acta Gene Struct. Expr.* **825**, 111–139 (1985).
12. A. Kornberg, Never a dull enzyme. *Annu. Rev. Biochem.* **58**, 1–31 (1989).
13. M. Goulian, A. Kornberg, R. L. Sinsheimer, Enzymatic synthesis of DNA, XXIV. Synthesis of infectious phage phi-X174 DNA. *Proc. Natl. Acad. Sci. U.S.A.* **58**, 2321–2328 (1967).
14. K. Grens, Meet the press, *The Scientist* **31**, 68 (2017).
15. A. Kornberg, Ten commandments: Lessons from the enzymology of DNA replication. *J. Bacteriol.* **182**, 3613–3618 (2000).
16. J. Cairns, The bacterial chromosome and its manner of replication as seen by autoradiography. *J. Mol. Biol.* **6**, 208-IN5 (1963).
17. M. Masters, P. Broda, Evidence for the bidirectional replication of the *Escherichia coli* chromosome. *Nat. N. Biol.* **232**, 137–140 (1971).
18. R. E. Bird, J. Louarn, J. Martuscelli, L. Caro, Origin and sequence of chromosome replication in *Escherichia coli*. *J. Mol. Biol.* **70**, 549–566 (1972).
19. D. M. Prescott, P. L. Kuempel, Bidirectional replication of the chromosome in *Escherichia coli*. *Proc. Natl. Acad. Sci. U.S.A.* **69**, 2842–2845 (1972).
20. K. von Meyenburg *et al.*, Origin of replication, oriC, of the *Escherichia coli* K12 chromosome: Genetic mapping and minichromosome replication. *Cold Spring Harb. Symp. Quant. Biol.* **43**, 121–128 (1979).
21. S. Yasuda, Y. Hirota, Cloning and mapping of the replication origin of *Escherichia coli*. *Proc. Natl. Acad. Sci. U.S.A.* **74**, 5458–5462 (1977).
22. Y. Hirota *et al.*, Structural and functional properties of the *Escherichia coli* origin of DNA replication. *Cold Spring Harb. Symp. Quant. Biol.* **43**, 129–38 (1979).
23. J. W. Zyskind, D. W. Smith, The bacterial origin of replication, oriC. *Cell* **46**, 489–490 (1986).
24. S. Chodavarapu, J. M. Kaguni, Chapter one replication initiation in bacteria. *Enzymes* **39**, 1–30 (2016).
25. A. Kornberg, Enzymatic synthesis of triphosphopyridine nucleotide. *J. Biol. Chem.* **182**, 805–813 (1950).
26. J. H. LeBowitz, R. McMacken, The *Escherichia coli* dnaB replication protein is a DNA helicase. *J. Biol. Chem.* **261**, 4738–4748 (1986).
27. J. M. Kaguni, R. S. Fuller, A. Kornberg, Enzymatic replication of *E. coli* chromosomal origin is bidirectional. *Nature* **296**, 623–627 (1982).
28. R. S. Fuller, A. Kornberg, Purified dnaA protein in initiation of replication at the *Escherichia coli* chromosomal origin of replication. *Proc. Natl. Acad. Sci. U.S.A.* **80**, 5817–5821 (1983).
29. R. S. Fuller, B. E. Funnell, A. Kornberg, The dnaA protein complex with the *E. coli* chromosomal replication origin (oriC) and other DNA sites. *Cell* **38**, 889–900 (1984).
30. D. Bramhill, A. Kornberg, A model for initiation at origins of DNA replication. *Cell* **54**, 915–918 (1988).
31. D. Bramhill, A. Kornberg, Duplex opening by dnaA protein at novel sequences in initiation of replication at the origin of the *E. coli* chromosome. *Cell* **52**, 743–755 (1988).
32. W. Messer, The bacterial replication initiator DnaA. DnaA and oriC, the bacterial mode to initiate DNA replication. *FEMS Microbiol. Rev.* **26**, 355–374 (2002).
33. T. Katayama, K. Kasho, H. Kawakami, The DnaA cycle in *Escherichia coli*: Activation, function and inactivation of the initiator protein. *Front. Microbiol.* **8**, 2496 (2017).
34. B. E. Funnell, T. A. Baker, A. Kornberg, Complete enzymatic replication of plasmids containing the origin of the *Escherichia coli* chromosome. *J. Biol. Chem.* **261**, 5616–5624 (1986).
35. H. Hiasa, K. J. Marians, Initiation of bidirectional replication at the chromosomal origin is directed by the interaction between helicase and primase*. *J. Biol. Chem.* **274**, 27244–27248 (1999).
36. J. M. Kaguni, A. Kornberg, Replication initiated at the origin (oriC) of the *E. coli* chromosome reconstituted with purified enzymes. *Cell* **38**, 183–90 (1984).
37. A. van der Ende, T. A. Baker, T. Ogawa, A. Kornberg, Initiation of enzymatic replication at the origin of the *Escherichia coli* chromosome: Primase as the sole priming enzyme. *Proc. Natl. Acad. Sci. U.S.A.* **82**, 3954–3958 (1985).
38. T. Ogawa, T. A. Baker, A. van der Ende, A. Kornberg, Initiation of enzymatic replication at the origin of the *Escherichia coli* chromosome: Contributions of RNA polymerase and primase. *Proc. Natl. Acad. Sci. U.S.A.* **82**, 3562–3566 (1985).
39. P. McInerney, A. Johnson, F. Katz, M. O'Donnell, Characterization of a triple DNA polymerase replisome. *Mol. Cell* **27**, 527–538 (2007).
40. M. O'Donnell, Replisome architecture and dynamics in *Escherichia coli**. *J. Biol. Chem.* **281**, 10653–10656 (2006).

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41. H. Peng, K. J. Marians, Decatenation activity of topoisomerase IV during oriC and pBR322 DNA replication in vitro. *Proc. Natl. Acad. Sci. U.S.A.* **90**, 8571–8575 (1993).
42. M. Su'etsugu, H. Takada, T. Katayama, H. Tsujimoto, Exponential propagation of large circular DNA by reconstitution of a chromosome-replication cycle. *Nucleic Acids Res.* **45**, gkx822 (2017).
43. A. Theologis, R. W. Davis, To give or not to give? That is the question. *Plant Physiol.* **135**, 4–9 (2004).
44. M. J. Lohka, Y. Masui, Formation in vitro of sperm pronuclei and mitotic chromosomes induced by amphibian ooplasmic components. *Science* **220**, 719–721 (1983).
45. J. J. Blow, R. A. Laskey, Initiation of DNA replication in nuclei and purified DNA by a cell-free extract of Xenopus eggs. *Cell* **47**, 577–587 (1986).
46. J. Walter, L. Sun, J. Newport, Regulated chromosomal DNA replication in the absence of a nucleus. *Mol. Cell* **1**, 519–529 (1998).
47. J. J. Blow, R. A. Laskey, Xenopus cell-free extracts and their contribution to the study of DNA replication and other complex biological processes. *Int. J. Dev. Biol.* **60**, 201–207 (2016).
48. Y. Lim *et al.*, In silico protein interaction screening uncovers DONSON's role in replication initiation. *Science* **381**, eadi3448 (2023).
49. J. J. Li, T. J. Kelly, Simian virus 40 DNA replication in vitro. *Proc. Natl. Acad. Sci. U.S.A.* **81**, 6973–6977 (1984).
50. B. Stillman, Chromatin assembly during SV40 DNA replication in vitro. *Cell* **45**, 555–565 (1986).
51. S. Waga, B. Stillman, The DNA replication fork in eukaryotic cells. *Ann. Rev. Biochem.* **67**, 721–751 (1998).
52. T. Kelly, Historical perspective of eukaryotic DNA replication" in *DNA Replication: From Old Principles to New Discoveries*, H. Masai, M. Foiani, Eds. (Springer, 2017), pp. 1-41.
53. D. T. Stinchcomb, K. Struhl, R. W. Davis, Isolation and characterisation of a yeast chromosomal replicator. *Nature* **282**, 39–43 (1979).
54. B. J. Brewer, W. L. Fangman, The localization of replication origins on ARS plasmids in *S. cerevisiae*. *Cell* **51**, 463–471 (1987).
55. S. E. Celniker, J. L. Campbell, Yeast DNA replication in vitro: Initiation and elongation events mimic in vivo processes. *Cell* **31**, 201–213 (1982).
56. Y. Marahrens, B. Stillman, A yeast chromosomal origin of DNA replication defined by multiple functional elements. *Science (New York, NY)* **255**, 817–823 (1992).
57. S. P. Bell, B. Stillman, ATP-dependent recognition of eukaryotic origins of DNA replication by a multiprotein complex. *Nature* **357**, 128–134 (1992).
58. J. F. X. Diffley, J. H. Cocker, Protein-DNA interactions at a yeast replication origin. *Nature* **357**, 169–172 (1992).
59. C. Liang, M. Weinreich, B. Stillman, ORC and Cdc6p interact and determine the frequency of initiation of DNA replication in the genome. *Cell* **81**, 667–676 (1995).
60. D. Remus *et al.*, Concerted loading of Mcm2–7 double hexamers around DNA during DNA replication origin licensing. *Cell* **139**, 719–730 (2009).
61. C. Evrin *et al.*, A double-hexameric MCM2-7 complex is loaded onto origin DNA during licensing of eukaryotic DNA replication. *Proc. Natl. Acad. Sci. U.S.A.* **106**, 20240–20245 (2009).
62. J. T. P. Yeeles, T. D. Deegan, A. Janska, A. Early, J. F. X. Diffley, Regulated eukaryotic DNA replication origin firing with purified proteins. *Nature* **519**, 431–435 (2015).
63. J. T. P. Yeeles, A. Janska, A. Early, J. F. X. Diffley, How the eukaryotic replisome achieves rapid and efficient DNA replication. *Mol. Cell* **65**, 105–116 (2017).
64. C. F. Kurat, J. T. P. Yeeles, H. Patel, A. Early, J. F. X. Diffley, Chromatin controls DNA replication origin selection, lagging-strand synthesis, and replication fork rates. *Mol. Cell* **65**, 117–130 (2017).
65. Y. Hu, B. Stillman, Origins of DNA replication in eukaryotes. *Mol. Cell* **83**, 352–372 (2023).
66. A. Costa, J. F. X. Diffley, The initiation of eukaryotic DNA replication. *Annu. Rev. Biochem.* **91**, 107–131 (2022).
67. U. Bastolla *et al.*, The topography of DNA replication origins in Eukarya: GGN clusters, landmark nucleosomes, CDC6 and G4 structures. bioRxiv [Preprint] (2024). https://doi.org/10.1101/2024.01.04.574144. Accessed 1 March 2024.
68. O. Ganier, P. Prorok, I. Akerman, M. Méchali, Metazoan DNA replication origins. *Curr. Opin. Cell Biol.* **58**, 134–141 (2019).

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