Proc. Natl. Acad. Sci. USA
Vol. 82, pp. 3954–3958, June 1985
Biochemistry

Initiation of enzymatic replication at the origin of the *Escherichia coli* chromosome: Primase as the sole priming enzyme

(DNA/*oriC*/plasmids)

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Contributed by Arthur Kornberg, January 28, 1985

ABSTRACT **The enzymatic replication of plasmids containing the unique (245 base pair) origin of the *Escherichia coli* chromosome (*oriC*) can be initiated with any of three enzyme priming systems: primase alone, RNA polymerase alone, or both combined (Ogawa, T., Baker, T. A., van der Ende, A. & Kornberg, A. (1985) *Proc. Natl. Acad. Sci. USA* 82, 3562–3566). At certain levels of auxiliary proteins (topoisomerase I, protein HU, and RNase H), the solo primase system is efficient and responsible for priming synthesis of all DNA strands. Replication of *oriC* plasmids is here separated into four stages: (*i*) formation of an isolable, prepriming complex requiring *oriC*, dnaA protein, dnaB protein, dnaC protein, gyrase, single-strand binding protein, and ATP; (*ii*) formation of a primed template by primase; (*iii*) rapid, semiconservative replication by DNA polymerase III holoenzyme; and (*iv*) conversion of nearly completed daughter molecules to larger DNA forms. Optimal initiation of the leading strand of DNA synthesis, over a range of levels of auxiliary proteins, appears to depend on transcriptional activation of the *oriC* region by RNA polymerase prior to priming by primase.**

Initiation of replication at the *Escherichia coli* origin of replication (*oriC*) is a key regulatory event in the bacterial growth cycle (1, 2). Biochemical studies of the mechanism of initiation became possible with the construction of *oriC*-containing plasmids (3, 4) and the development of a soluble enzyme system that initiates replication of these plasmids at *oriC* (5, 6).

oriC-specific DNA replication was achieved by using three categories of purified proteins (7): (*i*) initiation proteins [e.g., RNA polymerase (RNA pol), dnaA protein, and gyrase] that enable priming of the leading strand of a replication fork at the *oriC* sequence; (*ii*) replication (elongation) proteins (e.g., priming proteins and DNA polymerase III holoenzyme) that advance the fork; and (*iii*) auxiliary (specificity) proteins [e.g., topoisomerase I (Topo I) (8) and RNase H (9)] that suppress potential origins of replication throughout the DNA duplex while permitting initiation at the *oriC* sequence complexed by the dnaA protein (10). Further studies of this reconstituted system have shown that any of three priming systems can be used (11): primase alone, RNA pol alone, and both combined.

Here we show that the solo primase system can be highly efficient in replication and that the overall reaction is divisible into four stages: (*i*) prepriming, (*ii*) priming, (*iii*) elongation to unit-length products, and (*iv*) conversion to large DNA structures. RNA pol can also contribute significantly to the early priming events, presumably by transcriptional activation of the *oriC* region.

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MATERIALS AND METHODS

DNAs and Reagents. pCM959 (4) was a gift from M. Meijer (University of Amsterdam, The Netherlands); pTOA7 (T. Ogawa) was constructed by inserting the *Hae* II–*Acc* I *oriC*-containing fragment from M13*oriC*26 (7) via *Eco*RI linkers into *Eco*RI-cleaved pMAPCdSG10, a deletion derivative of pBR327 (W. A. Segraves, personal communication); pSY317, M13*oriC*26, M13*oriC*2LB5, and M13ΔE101 are described in Table 1 and elsewhere (3, 7). Tricine, creatine phosphate, ribo- and deoxyribonucleoside triphosphates (rNTPs and dNTPs) were from Sigma; α-^{32}P-labeled dTTP, rATP, rUTP, rGTP, and rCTP (>400 Ci/mmol; 1 Ci = 37 GBq) were from Amersham. Purified replication proteins were as described (7).

Assay for DNA Replication (Solo Primase System). The standard reaction (25 μl) contained 30 mM Tricine/KOH (pH 7.6); 2 mM ATP; GTP, CTP, and UTP, each at 500 μM; 400 μg of bovine serum albumin per ml; 2 mM creatine phosphate; dATP, dGTP, dCTP, and dTTP, each at 100 μM with [α-^{32}P]dTTP at 50–150 cpm/pmol of total deoxynucleotides; 8 mM magnesium acetate; 5 mM dithiothreitol; creatine kinase, 500 ng; single-stranded DNA binding protein (SSB), 320 ng; dnaB, 60 ng; dnaC, 120 ng; primase, 21 ng; DNA polymerase III holoenzyme, 100 ng; gyrase A subunit, 480 ng; gyrase B subunit, 750 ng; dnaA protein, 100 ng; Topo I, 12.5 ng; protein HU, 20 ng; RNase H, 1.4 ng; and supercoiled DNA, 200 ng (600 pmol as nucleotide). Reactions were assembled at 0°C; temperature and time are specified for each experiment. Nucleotide incorporation was measured as described (7).

Assay for RNA Synthesis. The standard reaction (25 μl) conditions were essentially as for DNA replication except that rGTP, rCTP, and rUTP were 25 μM and α-^{32}P-labeled at 1000–3000 cpm/pmol of total ribonucleotide; dNTPs, RNase H, and Topo I were omitted. Reactions were assembled at 0°C and initiated by incubation at 30°C for 30 min. Ribonucleotide incorporation was measured as described (12).

Isolation of Prepriming Complex. A standard DNA replication reaction lacking primase, DNA polymerase III holoenzyme, Topo I, RNase H, rGTP, rCTP, and rUTP was incubated for 10 min at 35°C, then chilled to 0°C, and filtered at 4°C over a 5-ml Bio-Gel A-5m column in 30 mM Tricine/KOH (pH 7.6), 400 μg of bovine serum albumin per ml, 0.5 mM ATP, 5 mM dithiothreitol, 8 mM magnesium acetate, and 10% glycerol. Void-volume fractions were assayed for

Abbreviations: SSB, single-stranded DNA binding protein; rNTP and dNTP, ribo- and deoxyribonucleoside triphosphate; Topo I, topoisomerase I; RNA pol, RNA polymerase.

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DNA replication by adding primase, DNA polymerase III holoenzyme, SSB, and dNTPs and incubating for 1 min at 30°C.

RESULTS

Contributions of RNA pol and Primase Are Influenced by Auxiliary Proteins and Plasmid Size. The previous dependence of DNA synthesis on RNA pol (7) was observed under reaction conditions that contained a level of protein HU (in a weight ratio to DNA near 0.5–1) that virtually completely inhibits primase action in initiation in the absence, but not in the presence, of RNA pol (11). At about 1/10th this level of protein HU, primase action at *oriC* is stimulated and generally shows no dependence on RNA pol (11). At such optimal levels of protein HU, a dependence on RNA pol was still demonstrable on the large templates (Table 1). The extent of incorporation was greater when the smaller plasmids were used as templates, perhaps in part due to the relatively higher *oriC* concentration in the fixed amount of DNA (as nucleotide residues) used in these assays. A comparison of these various *oriC* templates (Table 1, legend) suggests that their differing activities are more likely due to differences in size and topology than to variation in the sequence surrounding the 245-base-pair minimal *oriC* sequence (13).

Upon omission of the auxiliary proteins, Topo I and RNase H, no dependence on RNA pol was evident, even on the larger plasmids (Table 2). Notably, a significant reaction was observed on the plasmid lacking *oriC* (M13ΔE101) only when RNA pol was present. The specificity for *oriC* and dnaA protein, when initiation was by primase alone, was evident with or without the auxiliary proteins.

Topo I lengthened the lag time of the reaction (Fig. 1) and may be responsible for the requirement for RNA pol on the larger templates (Tables 1 and 2). The effect of Topo I is likely due to its relaxing of negative supercoils, inasmuch as relaxed templates were less active in replication because of an extended lag time in the reaction (data not shown). This action by Topo I might have a relatively greater effect on the larger templates.

An Initiation Stage of RNA Synthesis by Primase. The enormous amount of nonspecific transcription by RNA pol (10 times that of DNA synthesis) has thwarted efforts to identify the *oriC*-specific transcription responsible for initiation of DNA synthesis (7). However, in the solo primase (RNA pol-independent) system, RNA synthesis dependent on *oriC* and dnaA protein was demonstrated. In the absence of Topo I, RNase H, dNTPs, and RNA pol, RNA synthesis proceeded linearly with time to a level of about 2 pmol on the various *oriC* templates, an average incorporation of 20 nucleotides per input of a 4-kilobase-pair plasmid. The plasmid lacking *oriC* (M13ΔE101) showed no RNA synthesis above the background level (0.2 pmol). This *oriC*-specific synthesis (in the absence of auxiliary proteins) depended on dnaA protein, dnaB protein, dnaC protein, primase, gyrase, and, to some extent, protein HU and SSB (Table 3); the optimal SSB concentration was about 11 tetramers per template, an amount sufficient to cover about 400 nucleotides of single-stranded DNA.

The amount of RNA synthesis observed was increased by using increased amounts of primase. At a level of 74 primase molecules per template (pCM959), the amount of RNA synthesized was 10-fold that obtained with 5 primase molecules per template, the optimal primase level for DNA replication. The amounts of the other proteins required for the extensive RNA synthesis were the same as for the low level, but in either case only a small fraction of the RNA was found linked to DNA (data not shown). Thus, most of the RNA produced was not used for priming, presumably because it is dissociated or displaced from the template.

Stage of RNA Synthesis Is Followed by a Burst of DNA Synthesis. Upon addition of dNTPs, a burst of DNA synthesis

Table 1. Influence of size on the template activity of various supercoiled plasmids

Template*	Length, kb	DNA synthesis, pmol	
		With RNA pol	Without RNA pol
pSY317 dimer	24.0	110	21
M13*oriC*26	12.3	228	46
M13*oriC*2LB5	6.7	544	688
pCM959	4.0	656†	512‡
pTOA7	2.2	403	758
M13ΔE101	6.4	7	5

Assays were incubated for 30 min at 30°C. kb, Kilobase pairs.
*All except for M13ΔE101 (vector for M13*oriC*2LB5) contain the minimal *oriC* sequence; pSY317 contains Km^r sequences from pR6-5; M13*oriC*26 and M13*oriC*2LB5 contain M13 sequences; pTOA7 contains pBR322 sequences; pCM959 contains only flanking *E. coli* DNA; the M13 chimeric phage-plasmids are the replicative form I DNAs.
†Value without dnaA protein was 4 pmol.
‡Value without dnaA protein was 6 pmol.

Table 2. RNA pol-independent template activity of various supercoiled plasmids in the absence of auxiliary factors, RNase H and Topo I

Template*	DNA synthesis, pmol	
	With RNA pol	Without RNA pol
pSY317 dimer	151	171
M13*oriC*26	204	211
M13*oriC*2LB5	186	265
pCM959	202	182†
pTOA7	162	172
M13ΔE101	31	5

Incubation was for 30 min at 30°C.
*See legend to Table 1. A strict comparison between the DNA synthesis values in Tables 1 and 2 cannot be made because the experiments, performed at different times, are influenced by variations in the activities of each of the numerous reagents.
†Value without dnaA protein was 9 pmol.

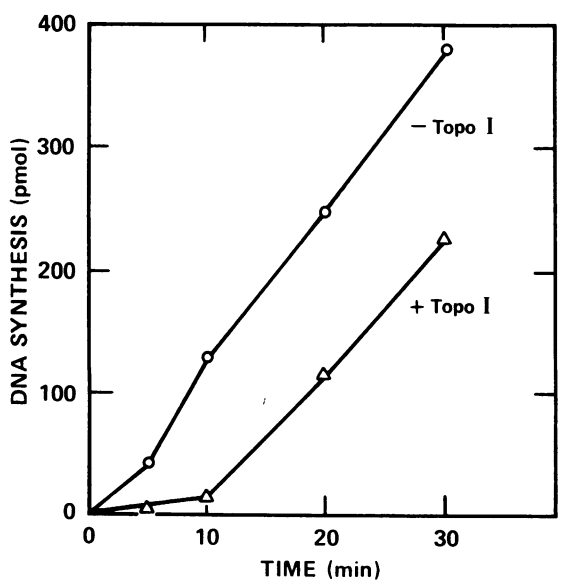

FIG. 1. Influence of Topo I on the time course of RNA pol-independent replication of pCM959 DNA. DNA replication was measured on 25-μl samples from 250-μl reaction mixtures at 30°C.

Table 3. Requirements for RNA pol-independent RNA synthesis on pCM959

Component omitted	RNA synthesis, pmol
None	1.8
Primase	0.3
dnaA	0.4
dnaB	0.4
dnaC	0.5
Gyrase A*	0.6
Gyrase B	0.2
SSB	1.0
HU protein	0.9

*With nalidixate (1 mM) also present, 0.3 pmol was synthesized.

followed RNA synthesis (Fig. 2). This burst was complete within 1 min at 30°C and was followed by a slow rate of synthesis for about 5 min. The extent of this initial DNA synthesis represented replication of 20% or fewer of the template molecules. Upon further incubation, the rate of DNA synthesis again increased, resulting in a large incorporation of dNTPs by 30 min. The products that appeared during the burst of synthesis were unit-length circular duplexes, whereas those produced after prolonged incubation included more complex and greater than unit-length molecules (data not shown).

The amount of DNA synthesized in the initial burst depended on the time and temperature of the prior incubation and may be a measure of the number of plasmid molecules initiated (Fig. 3). Prior incubation below 24°C had no effect but became optimal by 36°C with a transition temperature near 30°C. Such transitions are also observed in the formation of a RNA pol–promoter open complex (14) and are suggestive of an involvement of DNA melting. Addition of RNA pol and rNTPs to the reaction lowered the temperature at which the prior incubation was productive (Fig. 4). Inasmuch as RNA synthesis by primase is not involved in the reaction requiring a 35°C incubation (see below), the need for rNTPs for this RNA pol effect implies that transcription by RNA pol facilitates the reaction. However, this inference is not secure because RNA pol, in the absence of rNTPs, may inhibit the subsequent action of primase (11).

A Prepriming Stage in the Reaction. Since initiations cannot occur at 24°C, the reaction was staged by omitting various components during an incubation at 35°C and then adding them back for a reaction at 24°C (Fig. 5). The prepriming

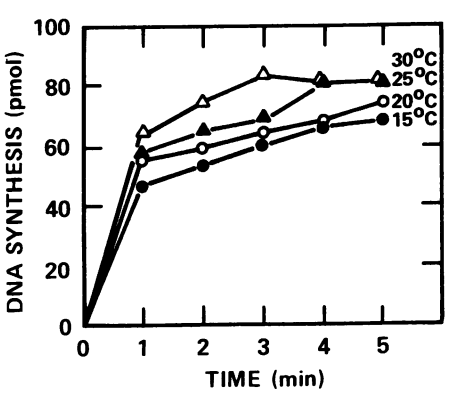

FIG. 2. Burst of DNA synthesis following a prior incubation in an RNA pol-independent replication of pCM959. The mixture lacking Topo I and RNase H was incubated initially without dNTPs for 10 min at 35°C; then dNTPs were added, followed by incubation at the temperatures and times indicated.

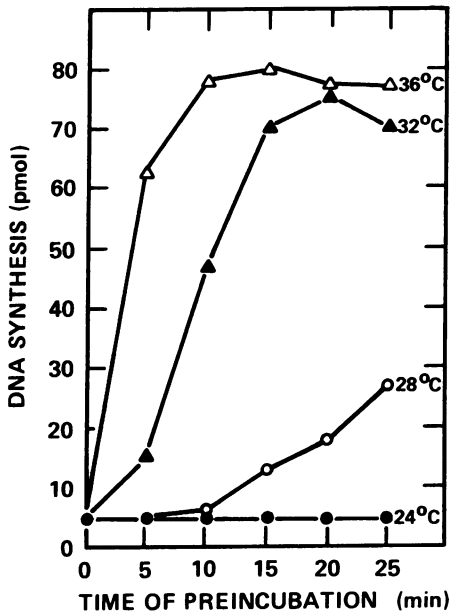

FIG. 3. Influence of temperature and time of a prior incubation on a burst of DNA synthesis. Reactions (200 μl) with pCM959 as template without Topo I, RNase H, and dNTPs were first incubated at the indicated temperatures; 25-μl samples were taken and after addition of dNTPs were incubated for 1 min at 30°C.

stage, clearly separated from priming by primase, required dnaA protein, dnaB protein, dnaC protein, and gyrase; the dependence on SSB was not absolute. There was no effect of including primase or DNA polymerase III holoenzyme during the prepriming stage nor was a requirement for protein HU uniformly demonstrable.

An Isolable Prepriming Complex. Upon filtration on Bio-Gel A-5m, the product of the prepriming stage, found in the void volume, was separated from uncomplexed proteins and

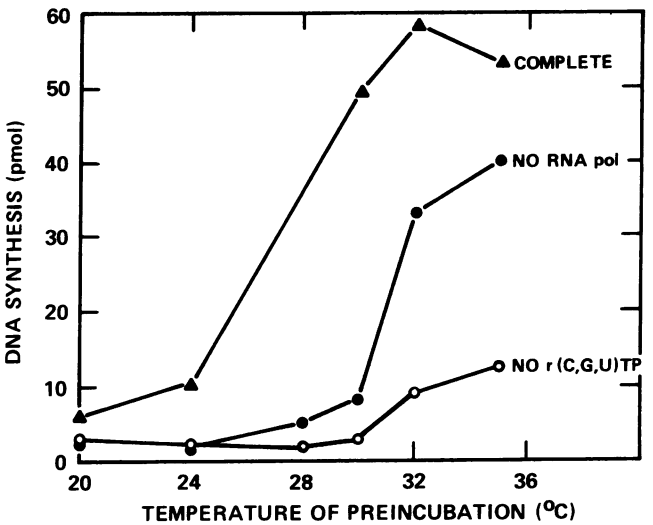

FIG. 4. Effect of RNA pol on the transition temperature of the preincubation stage. Supercoiled pCM959 DNA (600 pmol as nucleotides) was incubated 10 min at 30°C in a 15-μl mixture containing 11 mM magnesium acetate, 50 mM Tricine/KOH (pH 7.8), 3 mM ATP, 450 μg of bovine serum albumin per ml, 2 mM creatine phosphate, and 500 ng of creatine kinase; RNA pol (325 ng) and rGTP, rCTP, and rUTP (500 μM each) were added as indicated. Replication proteins and omitted components were added at 0°C and the mixtures were incubated without dNTPs for 7.5 min at the indicated temperatures. DNA synthesis for 1 min at 30°C followed the addition of dNTPs.

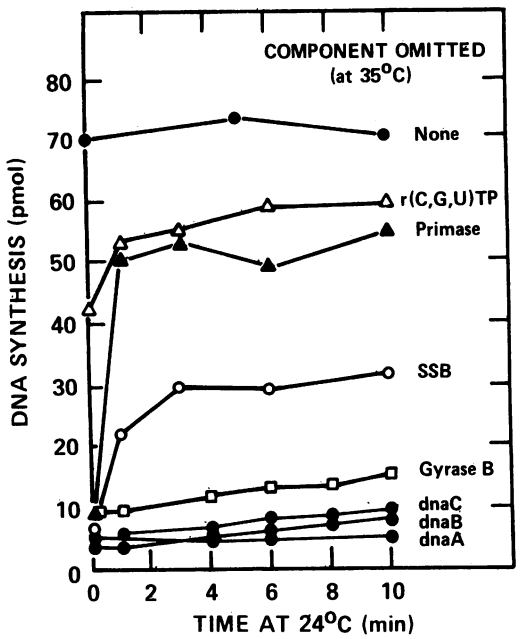

FIG. 5. Requirements in the prepriming stage. DNA synthesis reactions (200 μl) with pCM959 as template but lacking Topo I, RNase H, dNTPs, and the component indicated were incubated at 35°C for 10 min; then after 2 min at 24°C, 25-μl samples (zero time) were removed. The omitted component was added to the reaction mixtures and the incubation at 24°C was continued; 25-μl samples were removed at the indicated times. The samples were incubated for 1 min at 30°C upon addition of dNTPs.

was able to support DNA replication upon addition of primase, DNA polymerase III holoenzyme, SSB, ATP, and dNTPs; the yield measured as DNA synthesis was 80% that of an unfractionated reaction. When the prepriming reaction was incubated at 24°C instead of 35°C, the void volume contained no prepriming complex nor could it be generated by a subsequent incubation at 35°C for 10 min. An attempt with this procedure to isolate a prepriming complex containing functional primase did not succeed.

The reaction was thus divisible into four stages (Fig. 6): (*i*) prepriming, which is rate limiting and requires a temperature higher than 28°C; (*ii*) priming, which is complete within 1 min and occurs at 24°C or lower temperatures (e.g., 16°C); (*iii*) elongation by DNA replication to unit-length products, which is complete within 1 min at 24°C or lower temperatures; and (*iv*) amplification of DNA synthesis to produce more complex and larger than unit-length structures by undefined mechanisms.

DISCUSSION

Mechanisms for initiating replication at the unique origin of the *E. coli* chromosome (*oriC*) have been studied with *oriC* plasmids in purified protein systems (7). Depending on the levels of certain auxiliary proteins, such as protein HU (15) and Topo I, three alternative systems can operate to prime the start of DNA chains (11): primase alone, RNA pol alone, or primase plus RNA pol. At low levels of protein HU and in the absence of Topo I, the solo primase system is highly efficient. In avoiding the massive, nonspecific transcription by RNA pol, the solo primase system is attractive for analysis of the initiation events.

The overall replication reaction has been separated in these studies into four stages (Fig. 6); (*i*) prepriming, (*ii*) RNA priming, (*iii*) DNA replication to form unit-length products, and (*iv*) amplification of DNA synthesis to produce more complex and larger than unit-length molecules. The prepriming stage was identified by exploiting its failure to take place at 24°C (Fig. 3). A complex could be formed at 35°C that required the participation of dnaA protein, gyrase, dnaB protein, dnaC protein, SSB, and ATP (Fig. 5). The isolated complex (the manner of assembly, composition, and structure of which are still largely unknown) functioned by supporting the formation of a primed DNA by primase. Presumably, the binding of dnaA protein to sequences in *oriC* (10), the actions of gyrase, and the interactions with dnaB protein and dnaC protein generate the stable prepriming complex. ATP and incubation at 35°C were also needed. Among the indications that opening of the DNA helix is important for formation of the complex, in addition to the temperature dependence, is the effect of RNA pol, likely by transcription, in lowering the transition temperature for formation of the prepriming complex by several degrees (Fig. 4). The dependence on SSB in the prepriming stage also points to the need for an open DNA structure. The role of gyrase, given a supercoiled template to start with, may be to maintain a stable structure by negative supercoiling to balance the unwinding by a huge, multiprotein complex at *oriC*. Topologic changes, implied in a structure that migrates more rapidly in gel electrophoresis, accompany formation of the prepriming complex (unpublished results).

The prepriming complex differs from that described for initiating the synthesis of the complementary strand on an SSB-coated, phage φX174 circular template. In the φX174 system, protein n′ recognizes a particular sequence in the viral strand and starts the assembly of a mobile primosome in which proteins n′, n, n″, and i participate and dnaB protein, dnaC protein, and primase are included (16). There has been no indication of an effect of adding proteins n, n′, n″, and i to the *oriC* systems nor has the presence of dnaJ protein and dnaK protein had any influence (unpublished results). By contrast with the complexity of the priming systems for *oriC* and φX174, primase alone suffices to prime the unique start of the complementary strand on a phage G4 template (17). The partial homology of sequences within *oriC* with the primase recognition site on G4 (17) is of uncertain significance.

In the solo primase system, both DNA strands are primed by primase action. Although RNA pol can operate alone in initiating DNA replication when primase is absent, the reaction is severely limited (11). The most efficient of the reconstituted systems, over the widest range of auxiliary

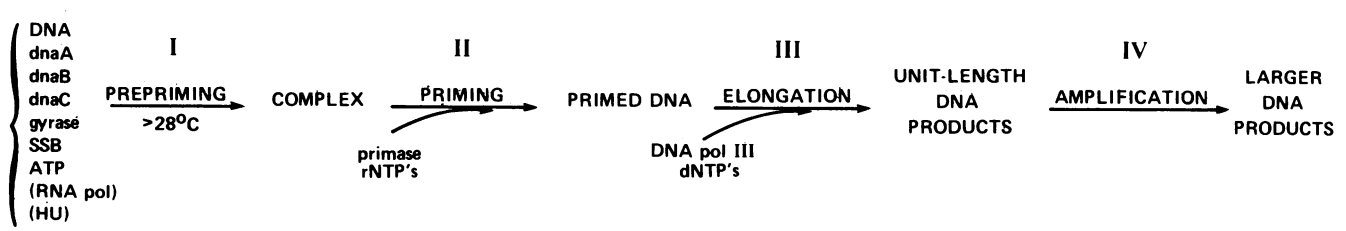

FIG. 6. Stages in DNA replication. See text for details.

factors, combines the actions of both primase and RNA pol. In their synergistic actions, transcription by RNA pol appears to activate the template for priming by primase.

Given alternative systems for priming duplex DNA replication, it is difficult to evaluate under which circumstances each of these operate physiologically. In addition to evidence for RNA pol participation in replication of the *E. coli* chromosome (18–20) and ColE1 plasmids (21), an RNA pol-independent (presumably primase-dependent) system has been described for plasmid R1 replication (22). Most probably, the sequence and topology of the template, as well as numerous protein factors, known and unknown, which interact with the template, determine the choice of initiation mechanisms among those available.

We thank LeRoy L. Bertsch for expert assistance in preparing this manuscript. This work was supported by grants from the National Institutes of Health and the National Science Foundation. A.v.d.E. was supported by the Netherlands Organization for the Advancement of Pure Research (Zuiver Wetenschappelijk Onderzoek) with a North Atlantic Treaty Organization Science Fellowship.

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