Replication factors A and C (RF-A and RF-C) and the proliferating cell nuclear antigen (PCNA) differentially augment the activities of DNA polymerases α and δ. The mechanism of stimulation by these replication factors was investigated using a limiting concentration of primed, single-stranded template DNA. RF-A stimulated polymerase α activity in a concentration-dependent manner, but also suppressed nonspecific initiation of DNA synthesis by both polymerases α and δ. The primer recognition complex, RF-C-PCNA-ATP, stimulated pol δ activity in cooperation with RF-A, but also functioned to prevent abnormal initiation of DNA synthesis by polymerase α. Reconstitution of DNA replication with purified factors and a plasmid containing the SV40 origin sequences directly demonstrated DNA polymerase α dependent synthesis of leading strands and DNA polymerase δ/PCNA/RF-C dependent synthesis of leading strands. RF-A and the primer recognition complex both affected the relative levels of leading and lagging strands. These results, in addition to results in an accompanying paper (Tsurimoto, T., and Stillman, B. (1991) J. Biol. Chem. 266, 1950–1960), suggest that an exchange of DNA polymerase complexes occurs during initiation of bidirectional DNA replication at the SV40 origin.

The identification and characterization of the cellular proteins required for simian virus 40 (SV40) DNA replication in vitro has opened the way for detailed studies on the mechanism of DNA replication (reviewed in Chailberg and Kelly, 1989; Stillman, 1988; Borowiec et al., 1990). SV40 DNA replication has been reconstituted in vitro with purified SV40 large tumor antigen (TAg) and seven essential cellular proteins (Tsurimoto et al., 1990). Based upon mechanistic studies to date, the process of DNA replication has been divided into several distinct stages. The first stages, origin recognition and unwinding of the origin proximal DNA, require SV40 large tumor antigen (TAg), a cellular topoisomerase, and a cellular protein called replication factor A (RF-A) (Wold et al., 1987; Tsurimoto et al., 1989; Borowiec et al., 1990). RF-A is a multisubunit replication factor comprising protein subunits of relative molecular masses of 70,000 (70 kDa), 34,000 (34 kDa), and 11,000 (11 kDa) (Wobbe et al., 1987; Fairman and Stillman, 1988; Wold and Kelly, 1988), with the 70-kDa subunit functioning as a single strand-specific DNA binding protein (Brill and Stillman, 1989; Wold et al., 1989; Kenny et al., 1990). In the absence of DNA synthesis, TAg, topoisomerase, and RF-A cooperate to extensively unwind plasmid DNAs containing the SV40 origin of replication (Dean et al., 1987; Dodson et al., 1987; Wold et al., 1987; Borowiec and Hurwitz, 1988; Goetz et al., 1988; Wiekowski et al., 1988; Roberts, 1989) but in the presence of the full complement of replication factors, origin unwinding and initiation of DNA replication are coupled (Tsurimoto et al., 1989).

The initiation of actual DNA synthesis at the replication origin first involves the interaction of DNA polymerase α-prime (pol α) with the "unwound complex," a complex of TAg and RF-A stably bound at the origin of DNA replication (Lee et al., 1989; Tsurimoto et al., 1989; Borowiec et al., 1990; Tsurimoto et al., 1990). Initiation of DNA synthesis most likely involves the formation of a primer by the primase activity associated with DNA polymerase α and synthesis of an Okazaki fragment at the origin. Pol α then continues to synthesize Okazaki fragments exclusively on the lagging strand template (Tsurimoto et al., 1990), although under some abnormal conditions, pol α can copy both leading and lagging strand templates (Ishimi et al., 1988).

For complete DNA replication of the leading strand DNA template, three additional replication factors have been identified. Replication factor C (RF-C) is a multisubunit enzyme consisting of polypeptides with relative molecular masses of 140,000, 41,000, and 37,000 (140 kDa, 41 kDa, and 37 kDa, respectively) (Tsurimoto and Stillman, 1989a). RF-C binds in a structurally specific manner to a primer-template junction and also has a DNA-dependent ATPase activity (Tsurimoto and Stillman, 1990, 1991). The RF-C-DNA-dependent ATPase activity is stimulated by another cellular replication factor, the proliferating cell nuclear antigen (PCNA) (Tsurimoto and Stillman, 1990). PCNA was first identified as a SV40 replication factor required for coordinated leading and lagging strand synthesis (Prelch et al., 1987a; Prelch and Stillman, 1988). Under some circumstances, PCNA also stimulates the processivity of DNA polymerase δ (pol δ), implicating this polymerase in the replication of DNA. Indeed, several recent studies have directly demonstrated a role for DNA polymerase δ in SV40 DNA replication (Lee et al., 1989; Weinberg and Kelly, 1989; Tsurimoto et al., 1990; Melendy and Stillman, 1991).

The three replication factors, RF-A, RF-C, and PCNA, affect the activities of pol α and pol δ in fundamentally different ways. RF-A and RF-C both stimulate pol α activity under some circumstances, whereas RF-A, RF-C, and PCNA cooperatively stimulate pol δ activity (Kenny et al., 1989; Tsurimoto and Stillman, 1989b).
The characterization of these proteins suggested functional similarities between the RF-C and PCNA factors and the bacteriophage T4 DNA polymerase accessory proteins encoded by genes 44/62 and gene 45, respectively (Tsurimoto and Stillman, 1990). Omission of either PCNA or RF-C from the replication reactions resulted in the accumulation of short nascent strands that hybridized to the lagging strand template DNA (Prelich and Stillman, 1988; Tsurimoto and Stillman, 1989a). Furthermore, pol α and pol δ synthesize short and long DNA strands, respectively, on singly primed single-stranded phage M13 DNA in the presence of various combinations of RF-A, PCNA, and RF-C (Tsurimoto and Stillman, 1989b). These results strongly suggested that during SV40 DNA replication, pol α and pol δ synthesize the lagging and leading strands, respectively. The involvement of two separate DNA polymerases that appear to cooperatively replicate leading and lagging strands at the replication fork raises the question of how initiation of DNA replication occurs at the origin. For example, instead of a single initiation event for DNA synthesis, there must be at least two initiation events, one for lagging strand synthesis and one for leading strand synthesis. Recent studies have demonstrated sequential roles for pol α and pol δ polymerase complexes in initiation of DNA replication from the SV40 origin (Tsurimoto et al., 1990). In an accompanying paper, we carried out mechanistic studies on the interaction of replication factors and DNA polymerases at a primer-template junction (Tsurimoto and Stillman, 1991). Highly specific primer binding of a primer recognition protein complex (RF-C-PCNA) in cooperation with RF-A and dynamic assembly and disassembly of these components coupled with ATP hydrolysis have been demonstrated. In this paper, we have studied the primer-template junction recognition by DNA polymerase complexes via their interaction with the replication factors. A DNA polymerase switching model for initiation of leading strand DNA synthesis from an Okazaki fragment synthesized at the replication origin is demonstrated. Furthermore, we have reconstituted SV40 DNA replication in vitro with purified replication factors and two DNA polymerases based on this model. The mode of DNA synthesis is controlled by a balance of replication factor activities.

**MATERIALS AND METHODS**

**DNA Polymerase Assays—**A standard reaction mixture for DNA polymerase assays with poly(dA)/oligo(dT) template contained 30 mM HEPES, pH 8.0, 7 mM MgCl₂, 0.5 mM dithiothreitol, 0.1 mg/ml bovine serum albumin, poly(dA) (average length 400), oligo(dT) (average length 12-15) (20;4 μM nucleotide), and 0.05 mM [α-³²P]dTP (approximately 3,000 cpm/nmol). Note that the pH of these reactions is different from those used previously (Tan et al., 1986; Prelich et al., 1987) to demonstrate an effect of PCNA on the processivity of pol δ. The reaction mixture was incubated at 37°C for 15 min, and one-fifth of the sample was spotted on DEAE-paper (DE81, Whatman) to measure the incorporated radioactivity (Tsurimoto et al., 1990). The remainder of the sample was used for product analysis as described below. DNA polymerase assays with a singly primed, single-stranded DNA was carried out with the same reaction mixture except for the addition of 0.05 mM [α-³²P]dATP (specific activity 3,000 cpm/nmol), 0.05 mM dGTP, 0.05 mM dCTP, and 0.05 mM dTTP and 0.6 μg/ml single-stranded pUC118 DNA primed with a 3-fold molar excess of an 17-base sequencing primer (primer 1211 from New England Biolabs) instead of TTP and poly(dA)/oligo(dT).

**DNA Replication in Vitro with a Plasmid Containing the SV40 Replication Origin—**SV40 DNA replication in vitro was assayed under standard conditions as described previously (Tsurimoto et al., 1989). Components added were 80 μg/ml SV40 Tag, 4 μg/ml calf thymus topoisomerase I, 1.8 μg/ml calf thymus topoisomerase II, 6.25 μg/ml pSV011 DNA, and 57 units/ml calf thymus pol λ (5.5 × 10⁸ units/ mg) and various amounts of RF-A, PCNA, RF-C, and pol α-prime as indicated. The reaction mixture was incubated at 37°C for 1 h, and the incorporated dAMP was measured by absorption of a sample of the reaction to DE81 filters.

**Product Analysis—**The remaining portion of each reaction mixture from either DNA polymerase assays or SV40 DNA replication assays was mixed with an equal volume of a stop mixture (0.2 mg/ml protease K, 2% sodium dodecyl sulfate, and 20 mM EDTA) and incubated at 37°C for 30 min. DNA in a sample was extracted with phenol/chloroform (1:1), precipitated with ethanol, and subjected to electrophoresis in an alkaline agarose gel (1% or 2%) in 30 mM NaOH, 1 mM EDTA as described previously (Maniatis et al., 1982; Tsurimoto and Stillman, 1989b). After electrophoresis, the gel was fixed, dried, and autoradiographed.

**Replication Factors—**Highly purified replication factors, RF-A, PCNA, RF-C, pol α-prime, pol δ, topoisomerases I and II, and Tag were obtained by published procedures (Tsurimoto et al., 1989; Tsurimoto and Stillman, 1991).

**RESULTS**

**RF-A Affects DNA Synthesis by Pol α and δ—**Nuclease footprinting experiments demonstrated that the binding of pol α and pol δ-PCNA to a primer-template junction is affected by the amounts of RF-A present in the reaction; most notably, RF-A inhibits binding by pol δ-PCNA and decreases binding by pol α (Tsurimoto and Stillman, 1991). Under some conditions, however, RF-A also functions as a stimulatory factor for both DNA polymerases on a primed template DNA (Tsurimoto and Stillman, 1989b). These apparently contradictory results suggested that the stimulatory and inhibitory effects of RF-A may be dependent on its concentration. Indeed, an excess of primer-template DNA was used for previously reported DNA polymerase assays, but limiting amounts of the primer-template DNA were used in the footprinting assays, indicating that the ratio of RF-A to DNA was different in the two experiments. To test the effect of saturated amounts of RF-A in DNA polymerase reactions, the concentration of a primer-template DNA was decreased by 20-fold relative to the amount used in previous experiments (Tsurimoto and Stillman, 1989b). As shown in Fig. 1A, RF-A stimulated pol α activity, but at higher concentrations, incorporation was reduced.

Analysis of the replication products by alkaline agarose gel electrophoresis revealed that pol α was poorly processive without RF-A (less than 20 nucleotides; Fig. 2, lane 1) but the length of the product was increased to near full length (approximately 3,000-4,000 nucleotides, Fig. 2, lanes 2 and 3) with RF-A present at 12.5-25 μg/ml. Interestingly, the length of the product was reduced to 100 to 200 nucleotides when higher amounts of RF-A were used (Fig. 2, lane 4). Since the processivity of pol α in the presence of RF-A was measured to be 100 to 200 nucleotides in previous experiments with higher concentrations of primed template DNA, the longer products with an intermediate concentration of RF-A most likely were a result of re-initiation of DNA synthesis by pol α at the 3'-end of a newly synthesized strand. Thus, inhibition of pol α activity by high amounts of RF-A was probably caused by suppression of reinitiation by pol α.

The effect of RF-A on pol δ activity was also determined. Pol δ activity was reduced by the addition of RF-A, even in the presence of either PCNA or RF-C (Fig. 1B). The inhibition was substantially more than the reduction of pol α activity. This was consistent with the results obtained by footprinting experiments, which demonstrated that primer-template recognition by the pol δ-PCNA complex was completely inhibited by RF-A, whereas the pol α complex was only partially reduced. Therefore, RF-A at high concentrations has a suppressing function for initiation of DNA synthesis at the 3'-end of a primer by both pol δ and pol α, although it functions as a processivity factor for pol α.

**DNA Synthesis by Pol δ and the Prime Recognition Com-
FIG. 1. Effect of replication factors and ATP on DNA synthesis by pol α and pol δ. A, titration of the amount of RF-A in DNA synthesis reactions containing pol α and poly(dA)/oligo(dT) as template-primer DNA. Reaction mixtures contained 26 units/ml pol δ and various amounts of RF-A. B and C, titration of the amount of RF-A in DNA synthesis reactions containing pol δ and poly(dA)/oligo(dT) as template-primer DNA. Reaction mixtures contained 26 units/ml pol δ, 6.7 μg/ml PCNA, 1 μg/ml RF-C, and various amounts of RF-A as indicated. 1 mM ATP was added to reactions in B and +ATP in C. DNA synthesis was expressed as picomoles of TMP incorporated in a 25-μl reaction mixture following incubation for 15 min.

FIG. 2. Products of DNA synthesis with pol α. Products obtained from reactions containing pol α, as described in Fig. 1 and Table I, were subjected to an alkaline agarose gel electrophoresis. The single-stranded DNA marker was from HpaII-digested, denatured pBR322 DNA run in parallel, and the length in nucleotides is indicated.

Complex Coupled with ATP Hydrolysis—Previous experiments demonstrated that pol δ activity on a primed, single-stranded DNA template required RF-C, PCNA, RF-A, and hydrolysis of ATP (Tsurimoto and Stillman, 1989b). We therefore determined the effect of ATP on DNA synthesis by pol δ in the presence of these factors. Fig. 1C shows a titration of increasing amounts of RF-A and its effect on pol δ activity in the presence of RF-C and PCNA and limiting amounts of primer-template DNA. In the absence of ATP, pol δ activity was inhibited by increasing amounts of RF-A. If ATP was present, however, pol δ activity was greatly stimulated. This suggests RF-C and PCNA did not actively function as a primer recognition complex without ATP. Furthermore, when pol δ was correctly loaded onto the primed single-stranded DNA, RF-A functioned as a stimulatory factor for DNA synthesis.

The results of footprinting experiments (Tsurimoto and Stillman, 1991) demonstrate that pol δ-PCNA protected the same region of the primer-template junction as the active primer recognition complex which contains RF-C-PCNA and ATP. This raises the question of the fate of RF-C once pol δ interacts with the primer. One scenario is that only pol δ-PCNA translocates along the DNA alone, or alternatively, RF-C might remain an active part of the polymerase complex. To begin to address this question, we tested if the ATPase activity of RF-C functions during the elongation stage of DNA synthesis from a primer-template junction with pol δ by determining the effect of ATPγS on DNA synthesis. When DNA synthesis by pol δ in the presence of RF-A, RF-C, and PCNA on a primed template DNA was started and then ATPγS was added after 2 min, DNA synthesis stopped completely, immediately after the addition (Fig. 3A). The addition of an excess of ATP, however, did not affect continued DNA synthesis. When these products were analyzed by alkali agarose gel electrophoresis, the extension of the nascent DNA strand also ceased immediately (Fig. 3A). This result demonstrated that ATP hydrolysis is required to translocate the pol δ complex on a template. ATPγS did not have an effect.

Fig. 3. DNA replication time course. A, effect of blocking ATP hydrolysis during DNA synthesis by pol δ on a singly primed, single-stranded DNA in the presence of RF-A, PCNA, and RF-C. A reaction mixture containing 0.6 μg/ml single-stranded pUC118 DNA primed with a sequencing primer (New England Biolabs), 260 units/ml pol δ, 70 μg/ml RF-A, 70 μg/ml PCNA, 3 μg/ml RF-C, and 0.1 mM ATP was preincubated at 0 °C for 5 min and then incubated at 37 °C. At time 2 min, ATP or ATPγS was added to the mixture to a final concentration of 1 mM (as indicated), and the incubations were continued at 37 °C. At each indicated time, an aliquot of the mixture was withdrawn, and a sample was spotted onto DEAE-paper to measure incorporation of label, and another sample was analyzed by gel electrophoresis (see Fig. 4). DNA synthesis was expressed as picomoles of DMP incorporated in a 25-μl reaction mixture. B, effect of formation of the active primer recognition complex on pol α activity using a singly primed, single-stranded template DNA in the presence of RF-A and ATP. A reaction mixture containing 15 μg/ml pol α, 70 μg/ml RF-A, 3 μg/ml RF-C, and 1 mM ATP was preincubated at 0 °C for 5 min and incubated at 37 °C. At time 2 min, PCNA was added to a final concentration of 70 μg/ml (+), or the same volume of buffer A (Tsurimoto et al., 1989a) was added to the mixture (−). Both reactions continued to be incubated at 37 °C, and DNA synthesis and product analysis at each time point were performed as described in A.
of denatured adenovirus DNA that was digested with HindIII.

6. alkaline agarose gel (1%) electrophoresis. The positions are indicated by the length stranded DNA template.

confirmation of this will require immunological reagents to on the low amount of DNA synthesis by pol δ and PCNA on the primed template DNA (data not shown), suggesting that the effect of ATPγS was RF-C-dependent. This suggests that RF-C translocates with the pol δ during DNA synthesis, but confirmation of this will require immunological reagents to detect RF-C, which are currently not available. In the analogous polymerase complex from bacteriophage T4, ATP hydrolysis by gene 44/62 protein complex is required for the assembly of the active DNA polymerase holoenzyme at a 3'-end and is also required during the elongation stage (Huang et al., 1981; Mace and Alberts, 1984). It has been proposed that hydrolysis of ATP is involved in a timing mechanism that accounts for the recycling of DNA polymerase required for lagging strand DNA synthesis, since ATPγS did not block translocation of the DNA polymerase directly (Selick et al., 1987). Alternatively, it is possible that ATP hydrolysis by the gene 44/62 protein is required for translocation on both the leading and lagging strands, since ATP hydrolysis by RF-C is required for leading strand synthesis by pol δ.

ATP-dependent Blocking of Pol α DNA Synthesis by the Primer Recognition Complex—As shown in Table I, DNA synthesis by pol α in the presence of a high amount of RF-A was not significantly affected by PCNA or ATP, except for slight inhibition by ATP. This inhibition with ATP seems to be intrinsic to pol α, since the same result was obtained without RF-A (data not shown). The length of the major product synthesized under these conditions was 50–200 bases, which is the same as the products obtained by pol α and RF-C alone (Fig. 2, lanes 8–9). Addition of RF-C slightly stimulated the incorporation, but significantly, full length product was produced (Fig. 2, lane 9). This demonstrates that RF-C stimulates not only pol δ but also pol α, as we have published previously (Tsurimoto and Stillman, 1989b). It is not yet clear from these experiments whether RF-C increased the processivity of pol α directly or increased the efficiency of reinitiation of pol α on a previously synthesized nascent DNA strand.

In contrast to these results, the effect of the active primer recognition complex (RF-C·PCNA·ATP) on pol α was completely different and opposite to the effect found on pol δ. If the priming complex was in the inactive form lacking ATP, pol α exhibited the same mode of DNA synthesis as with RF-C alone (Table I). If, however, the complex was activated with ATP, pol α activity was strongly inhibited (Table I) and no products were detected on an alkaline agarose gel (Fig. 2, lane 12).

The same result was obtained by following a time course of DNA synthesis by pol α on a singly primed template DNA (Fig. 3). Pol α synthesized DNA constantly for at least 14 min in the presence of RF-C and ATP, and relatively long DNA strands were produced (Figs. 3B and 4B). If PCNA was added to the reaction after DNA synthesis for 2 min, this allowed RF-C and ATP to form the primer recognition complex and DNA synthesis stopped immediately (Figs. 3B and 4B). This confirmed the result that the primer recognition complex blocked DNA synthesis by pol α, but also suggested that the elongation of DNA synthesis by pol α might be regulated by PCNA or by the interaction of ATP with RF-C. However, since the intrinsic processivity of pol α is low, this experiment does not determine whether the active primer recognition complex blocks pol α translocation or whether it simply blocks reinitiation on preformed nascent DNA strands.

Reconstitution of SV40 Replication with Two DNA Polymerases—As shown above, initiation of DNA synthesis at the 3'-end of a primer by pol α or δ is controlled by two different mechanisms: the amount of RF-A and the formation of an active primer recognition complex. We therefore investigated the effect of these two replication components on leading and lagging strand synthesis during SV40 DNA replication. The results above and those recently obtained (Tsurimoto et al., 1990) suggest that initiation of DNA replication by pol α results in the synthesis of the first Okazaki fragment at the replication origin and then the primer recognition complex appears to be involved in a switching mechanism to remove pol α and load pol δ onto the 3'-end of this Okazaki fragment to initiate leading strand DNA synthesis. This could explain why leading strand synthesis was compromised by the absence of PCNA and RF-C: however, it does not explain why pol α did not continue to self-prime and copy the leading strand template DNA, as has been observed by Ishimi et al. (1988).

To address this point, and taking note of the effect of RF-A on pol α activity, we determined whether RF-A would suppress nonspecific leading strand synthesis by pol α. Increasing amounts of RF-A were added to reconstituted replication reactions containing various combinations of essential replication factors. In the first series of experiments, all reactions contained a plasmid DNA harboring the SV40 replication origin, TAg, topoisomerases I and II, and pol α (Fig. 5A). In all cases, DNA replication absolutely required RF-A, and the optimum amount of RF-A for incorporation was less than 12.5 μg/ml (Fig. 5A). In the absence of any additional replication components and with 12.5 μg/ml RF-A concentrations, a bimodal distribution of nascent DNA strands was
detected by alkaline agarose gel electrophoresis (Fig. 6A, lane 1). These short and long DNAs correspond to the lagging and leading strand nascent DNAs, respectively, as has been reported by Ishimi et al. (1988). Increasing the amount of RF-A in the reaction decreased the incorporation slightly (Fig. 5A), but more importantly, this led to a dramatic elimination of the long nascent strands (Fig. 6A, lanes 2–4). Thus, RF-A modulates that activity of pol α by blocking nonspecific initiation on a leading strand template from the 3'-end of Okazaki fragments. The addition of the primer recognition complex (PCNA-RF-C) also blocked the long leading strands, even in the presence of low amounts of RF-A (Fig. 6A, lanes 5–9). This suggests that both RF-A and RF-C-PCNA limit pol α to the lagging strand template. The addition of pol δ, RF-C, and PCNA to the reactions restored the synthesis of the long leading strands, and their synthesis became insensitive to high concentrations of RF-A (Fig. 6A, lanes 9–12).

The amount of DNA replication obtained with these eight purified proteins was comparable to the levels obtained with previously reconstituted SV40 replication reactions containing six purified proteins and one crude fraction (Tsurimoto et al., 1989). Furthermore, this reconstituted replication system appeared, by several criteria, to reflect the mechanism of SV40 DNA replication observed with a crude lysate, except for the lack of production of mature lagged strand replicated DNA (Tsurimoto et al., 1990). Most importantly, this purified replication system completely reproduced the requirement for RF-C and PCNA and directly demonstrated that the leading and lagging strands are synthesized by pol δ and pol α, respectively.

The Effect of Replication Factors on the Mode of SV40 DNA Replication—The reconstituted replication system was sensitive to the ratio of various components in addition to the amount of RF-A. As shown in Fig. 6B (lanes 1–4), short lagging strands were synthesized by pol α in the presence of high amounts of RF-A and in the absence of RF-C and PCNA (Figs. 5B and 6B, lanes 1–4). These fragments increased in length with increasing amounts of pol α, as reported previously on a singly primed template DNA (Tsurimoto and Stillman, 1989b). This suggests that high amounts of pol α initiated DNA synthesis multiple times on the lagging strand template DNA. In the presence of the active primer recognition complex (RF-C-PCNA-ATP), pol δ was activated, but the amount of DNA replication was also dependent upon the amount of pol α (Fig. 5B). Interestingly, with low amounts of pol α, the short lagging strand DNA disappeared, but the longer leading strand DNA was synthesized (Fig. 6B, lanes 5–8). At the lowest concentration of pol α, a significant amount of product equivalent to full length plasmid was produced (Fig. 6B, lane 5). In the absence of enzymes that mature Okazaki fragments into a continuous strand, this unit length DNA must be produced by abnormal leading strand synthesis by pol δ all the way around the template DNA, except for the initiation reaction by pol α at the replication origin. Efficient and coordinated leading and lagging strand DNA synthesis with purified proteins was seen in the presence of relatively high amounts of pol α (Fig. 6B, lane 8). With higher amounts of pol δ, more efficient DNA synthesis was obtained, but the product was changed from the bimodal distribution to almost all of the product migrating in the position of the unusual unit length leading strand (data not shown). Therefore, coordinated leading and lagging DNA synthesis in vitro requires balanced leading and lagging strand DNA polymerase activities.

The amount of the primer recognition complex also affects the type of DNA synthesis. When the complex was absent, DNA replication with either pol α alone or pol α plus pol δ yielded similar products consisting of short, lagging DNA strands (Fig. 6C, lanes 1 and 6). However, addition of increasing amounts of the primer recognition complex suppressed DNA synthesis dependent upon pol α alone and shortened the product to 200–300 nucleotides in length (Figs. 5C and 6C, lanes 7–10). In contrast, DNA synthesis in the presence of both pol α and δ was stimulated by the addition of optimal amounts of RF-C and PCNA, yielding a bimodal distribution of DNA strands (Figs. 5C and 6, lane 2). Higher amounts of the complex increased the population of the long products, equal to or longer than unit length DNA strands (Fig. 6, lane 5). This result again confirmed that the primer recognition complex affected DNA polymerase switching for initiation of leading strand DNA synthesis at the replication origin. Furthermore, the concentration of the primer recognition complex is an important factor for balancing the activity of the two polymerases, resulting in coordinated leading and lagging strands synthesis.

**Fig. 5.** Titration of replication factors in SV40 DNA replication reactions reconstituted with purified components. Replication reactions were performed in a 25-μl reaction mixture at 37°C for 60 min, and DNA synthesis was measured and is indicated as picomoles of dAMP incorporated. A, titration of RF-A. Components used are indicated next to each line. Reactions contained 24 μg/ml pol α, 2 μg/ml RF-C, 6.7 μg/ml PCNA, 87 units/ml pol δ, and the amounts of RF-A indicated in the graph. B, titration of pol α-prime complex. Components used are indicated next to each line. Reactions contained 50 μg/ml RF-A, 24 μg/ml RF-C, 5 μg/ml PCNA, and 87 units/ml pol δ and the amounts of pol α-prime complex indicated in the graph. C, titration of the primer recognition complex (RF-C-PCNA). Amount 1.0 represents 6 μg/ml RF-C and 20 μg/ml PCNA, and both were changed to maintain this ratio. Other components used are 18 μg/ml pol α, 50 μg/ml RF-A, and 87 units/ml pol δ as indicated in the graph.
FIG. 6. Product analysis of the reconstituted SV40 DNA replication reaction. The reaction products are derived from the experiments described in Fig. 5. Components used are indicated above each lane. Increasing amounts of RF-A in column A were 12.5, 25, 37.5, and 50 μg/ml. Increasing amounts of pol α-primase in column B were 6, 12, 18, and 24 μg/ml. Increasing amounts of RF-C, PCNA in column C were 0, 0.125, 0.25, 0.5, and 1.0 (1.0 = 6 μg/ml RF-C and 20 μg/ml PCNA). Marker positions are indicated by the length of single-stranded DNA obtained from denatured adenovirus DNA that had previously been digested with HindIII. ssl is the position of a single-stranded linear template DNA (2.9 kilobases).

As published previously, RF-A is a processivity factor for pol α (Tsurimoto et al., 1989b), but as described herein, it also blocks initiation of DNA synthesis by pol α on the end of a nascent strand that was just synthesized. This latter function accounts for the suppression of nonspecific DNA synthesis from the 3'-end of Okazaki fragments by this polymerase. As shown in Fig. 6A, this suppressing function of RF-A was also revealed during SV40 DNA replication in vitro, since high amounts of RF-A restricted DNA synthesis by pol α to the lagging strand template.

The results demonstrating an effect of RF-A on primed DNA synthesis by pol α also presented a paradox. RF-A did not completely block DNA synthesis by pol α when primed by the unique oligonucleotide primer (the initial primer), but reinitiation at the 3' end of the newly synthesized strand was blocked by RF-A. This must mean that the two potential primers were not equally available to pol α. One interesting explanation for this paradox is that a pol α-RF-A complex might stably bind to the 3' end of a newly synthesized strand and block reinitiation. This complex might then be released by the combined action of RF-C and PCNA, noting that RF-C may indeed be a part of the lagging strand complex (Tsurimoto and Stillman, 1989a). Therefore, PCNA may play a role in lagging strand DNA synthesis by cycling the lagging strand polymerase complex from the 3'-end of a newly synthesized Okazaki fragment to a new location on the lagging strand template for self-priming by primase. Such a mechanism is consistent with the results in Figs. 3B and 4B. This model has been suggested for phage T4 and E. coli lagging strand DNA replication. This model also implies that the initial oligonucleotide primer cannot tightly bind pol α, as we have observed (Tsurimoto and Stillman, 1991). Furthermore, during DNA replication, pol α would not normally need to encounter such preformed primers, unlike the pol δ complex.

RF-A also blocks initiation of DNA synthesis by pol δ on a preformed primer. But a specific mechanism exists to load pol δ onto the preformed primer that requires RF-C, PCNA, and ATP. Once the primer recognition complex is formed, pol δ binds to it, and RF-A then stimulates DNA synthesis by the leading strand polymerase complex. We suggest that this is the mechanism for initiation of leading strand DNA replication at the replication origin.

A Model for DNA Polymerase Switching during Initiation of Bidirectional DNA Replication—We have recently demonstrated that the initiation of DNA replication in vitro from
the SV40 origin requires the sequential initiation by pol α, followed by pol δ. The pol α complex then moves from the origin to copy the lagging strand template, whereas the pol δ complex copies the leading strand template. Taking these results and the results from this and the accompanying paper (Tsurimoto and Stillman, 1991) into account, we propose a polymerase switching model for the initiation of leading strand synthesis. Only a helicase (Tag) moving with the lagging strand polymerase complex is shown, although it is likely that a 5′ → 3′ helicase functions in vivo. Waved lines indicate primer RNA. Thick and thin solid lines are template and nascent DNAs, respectively.

After the synthesis of the first Okazaki fragment, PCNA and ATP interact with the lagging strand polymerase complex and releases the pol α complex, forming the active primer recognition complex. Saturated amounts of RF-A also contribute to the arrest of DNA synthesis by blocking pol α and its release from the template. Once the primer recognition complex is formed, pol δ efficiently recognizes the 3′-end of the first Okazaki fragment by direct protein-protein interactions resulting in an exchange of DNA polymerase from the lagging strand complex to the leading strand complex. The leading strand polymerase complex then translocates in a ATP-dependent manner along the template to continuously synthesize DNA. The transiently released pol α complex could bind to the lagging strand template and move away from the origin to synthesize Okazaki fragments, perhaps by cycling on the template in a PCNA-dependent manner (see Tsurimoto and Stillman, 1991). Such coordinated replication of both strands at a replication fork has been proposed by Alberts et al. (1982) and Kornberg (1982).

This model is consistent with the data described in this report and in an accompanying paper (Tsurimoto and Stillman, 1991). It also draws upon the extensive biochemical analysis of the leading and lagging polymerase complexes from E. coli and phage T4, where the cycling of the lagging strand polymerase complex has been investigated (reviewed by McHenry, 1988). The novel feature of this model is the switching of DNA polymerase complexes that occurs at the replication origin to establish continuous leading strand synthesis. This model, however, could certainly apply to the initiation of bidirectional DNA replication that occurs at the
E. coli oriC and phage λ replication origins, even though E. coli polymerase III functions to synthesize both strands at the replication fork.

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