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Multiple replication factors augment DNA synthesis by the two eukaryotic DNA polymerases, α and δ

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DNA synthesis by two eukaryotic DNA polymerases, α and δ, was studied using a single-strand M13 DNA template primed at a unique site. In the presence of low amounts of either DNA polymerase α or δ, DNA synthesis was limited and short DNA strands of ~100 bases were produced. Addition of replication factors RF-A, PCNA and RF-C, which were previously shown to be required for SV40 DNA replication *in vitro*, differentially stimulated the activity of both DNA polymerases. RF-A and RF-C independently stimulated DNA polymerase α activity 4- to 6-fold, yielding relatively short DNA strands (<1 kb) and PCNA had no effect. In contrast, polymerase δ activity was stimulated co-operatively by PCNA, RF-A and RF-C ~25- to 30-fold, yielding relatively long DNA strands (up to 4 kb). Neither RF-C nor RF-A appear to correspond to known polymerase stimulatory factors. RF-A was previously shown to be required for initiation of DNA replication at the SV40 origin. Results presented here suggest that it also functions during elongation. The differential effects of these three replication factors on DNA polymerases α and δ is consistent with the model that the polymerases function at the replication fork on the lagging and leading strand templates respectively. We further suggest that co-ordinated synthesis of these strands requires dynamic protein-protein interactions between these replication factors and the two DNA polymerases.

Key words: PCNA/replisome/single-strand DNA binding protein/SV40 DNA replication

Introduction

Plasmid DNAs containing the simian virus 40 (SV40) origin of DNA replication can replicate in a cell-free system containing a human cell extract and a single SV40 encoded protein, the large tumor antigen (TAg) (Li and Kelly, 1984, 1985; Stillman and Gluzman, 1985; Wobbe *et al.*, 1985). Because most of the replication functions rely upon host cell encoded proteins, this has been an ideal system to identify these proteins and to elucidate their functions and how they may be regulated. By systematic fractionation of the human cell extract, a number of essential replication factors and fractions have been identified and some of their functions have been determined (Murakami *et al.*, 1986; Prelich *et al.*, 1987a; Wobbe *et al.*, 1987; Yang *et al.*, 1987; Fairman and Stillman, 1988; Wold and Kelly, 1988; Tsurimoto *et al.*, 1989; Wold *et al.*, 1989; reviewed in Stillman, 1989). One notable discovery was the requirement for the proliferating cell nuclear antigen (PCNA) (Prelich *et al.*, 1987a; Wold *et al.*, 1989) because this protein is a processivity factor for a fourth eukaryotic DNA polymerase, DNA polymerase δ (pol δ; Byrnes *et al.*, 1976; Tan *et al.*, 1986; Prelich *et al.*, 1987b; Bravo *et al.*, 1987). Since DNA polymerase α (pol α) was long thought to be the only replicative DNA polymerase in eukaryotic cells, the idea that two different DNA polymerases were required for eukaryotic DNA replication was unexpected. Recent genetic evidence, however, demonstrating a requirement for two replicative DNA polymerases in the yeast *Saccharomyces cerevisiae*, including the polymerase δ homolog pol III, has lent strong support for this model (Bauer *et al.*, 1988; Boulet *et al.*, 1989; Sitney *et al.*, 1989).

Several observations fit well with the two polymerase model: pol α associates tightly with a primase activity which is required to make a primer for each Okazaki fragment during replication of the lagging strand template, whereas pol δ lacks a DNA primase (reviewed by So and Downey, 1988). Furthermore, in the absence of PCNA, only lagging strand synthesis occurs during SV40 DNA replication *in vitro*, suggesting that pol δ is required for leading strand DNA synthesis in this system (Prelich and Stillman, 1988). According to the model, a multi-protein complex at the replication fork (the replisome) would contain one pol α complex and one pol δ complex functionally linked by accessory proteins. Another replication factor, RF-C, may also be a part of the replisome because it is required during the elongation stage of SV40 DNA replication *in vitro* (Tsurimoto and Stillman, 1989). The biochemical function of RF-C is not yet clear, but like PCNA, this factor is indispensable for synthesis on the leading strand template during elongation, and for co-ordinated synthesis of both strands during DNA replication. Therefore, multiple factors are required to form an active replisome after synthesis of the first nascent DNA strand at the replication origin.

In this report, we demonstrate that three factors, previously shown to be required for DNA replication from the SV40 origin, stimulate the activity of DNA polymerases α and δ. Two of these proteins affect both DNA polymerases, suggesting a mechanism for co-ordinated replication at the fork.

Results

DNA synthesis with pol α and pol δ on primed-ssM13 DNA

During replication, DNA polymerases recognize 3′ ends of primer RNA or DNA and copy templates containing natural DNA sequences. Therefore, we have studied DNA synthesis on primed-ssM13 DNA (single strand M13 DNA primed at a unique site) as a template, rather than synthetic polymer DNA as a template, because it better reflects the mode of synthesis during DNA replication. Furthermore, we employed reaction conditions similar to those used to support

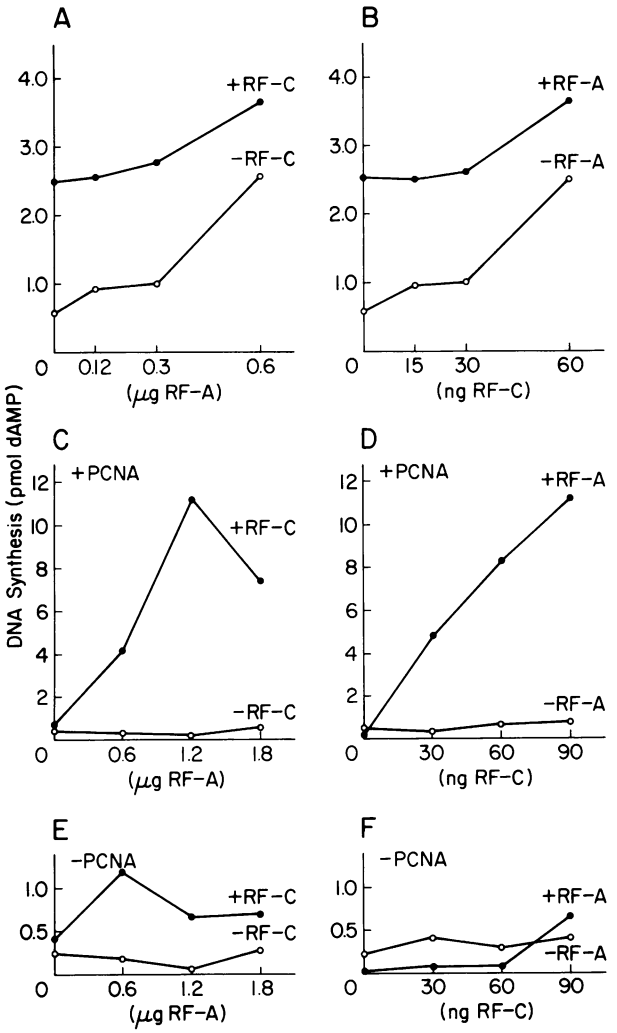

Fig. 1. Titration of RF-A and RF-C in DNA synthesis reactions containing pol α or pol δ. A standard reaction mixture (25 μl) containing 100 ng of primed-ssM13 DNA was incubated with 0.1 U of pol α (**A** and **B**), or 0.27 U of pol δ and 150 ng of PCNA (**C** and **D**), or 0.27 U of pol δ (**E** and **F**). To each reaction, the indicated amounts of RF-A or RF-C were added, and '+RF-C' and '+RF-A' represent further additions of 60 ng (for A) or 90 ng (for C and E) of RF-C and 0.6 μg (for B) or 1.2 μg (for D and F) of RF-A. After incubation for 30 min at 37°C, the acid-insoluble radioactivity was determined and expressed as incorporated nucleotides (pmol of dAMP). NaCl concentrations of the reactions were adjusted to a final concentration of 26 mM in (A) and (B) and 40 mM in (C)−(F).

SV40 DNA replication *in vitro* so that, for any given factor, direct comparisons could be made between their function in the SV40 DNA replication system and their effect on DNA polymerase function. The amounts of pol α or pol δ, when they were used alone in the experiments, supported nucleotide incorporation of <1 pmol dAMP (Figure 1), suggesting that recycling of a DNA polymerase on the same DNA strand was minimal. When either polymerase was used alone on the primed-ssM13 DNA, the major products obtained migrated in an alkaline agarose gel at the position of ~100 bases (Figure 2, lanes 1 and 5). Interestingly, with this template and reaction conditions, PCNA had no effect on pol δ activity (Figure 2, lane 9), although PCNA did stimulate pol δ processivity on a primed synthetic polymer DNA template (Tan *et al.*, 1986; Figure 3). The inability of PCNA to stimulate pol δ on this template was not due

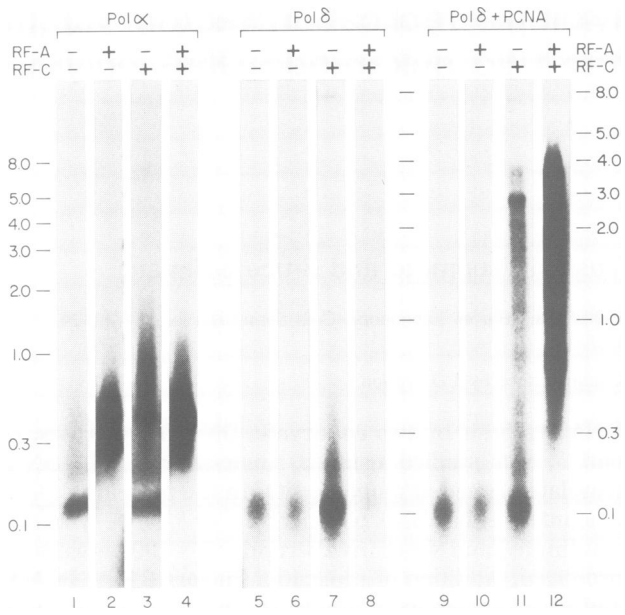

Fig. 2. Product analysis of DNA synthesis on primed-ssM13 DNA by pol α or pol δ in the presence of various combinations of replication factors. The components used were primed-ssM13 DNA (100 ng), pol α (0.1 U), pol δ (0.27 U), RF-A (0.6 μg for **lanes 1−4** and 1.2 μg for **lanes 5−12**), PCNA (250 ng) and RF-C (60 ng for **lanes 1−4** and 90 ng for **lanes 5−12**). Their inclusion in each reaction is indicated above. The NaCl concentration used in these reactions was 26 mM in lanes 1−4 and 40 mM in lanes 5−12. The reaction mixture was incubated at 37°C for 30 min and constant portion of the product DNA (except for lane 12, where one-fifth the amount of the other samples was used) was subjected to electrophoresis in a 1% alkaline agarose gel. Mobilities of denatured DNA fragments were compared to *Hin*dIII-digested adenovirus type 2 DNA and are shown in kilobases on each panel. The results of lanes 1−4 and lanes 5−12 were taken from independent experiments.

to the salt concentration (40 mM NaCl) used in this assay, since this enzyme could not be activated with PCNA even if the salt was lowered to <10 mM (data not shown).

RF-A and RF-C stimulate pol α independently

The three replication factors that had previously been identified as essential for SV40 DNA replication *in vitro* were added to the reaction with pol α. Addition of PCNA to pol α had no effect (data not shown), in agreement with previous results (Tan *et al.*, 1986). In contrast, addition of RF-A or RF-C increased pol α activity at least 4-fold and a combination of both factors increased DNA synthesis at least 6-fold (Figure 1A and B). The stimulation of pol α activity by these replication factors was additive: in the presence of saturating amounts of RF-A (1.2 μg/reaction), RF-C could still stimulate DNA synthesis further (data not shown), suggesting that stimulation by each factor occurred by different mechanisms. These effects were also detected by product analysis (Figure 2, lanes 2−4). It is noteworthy that the size distribution of the DNA strands varied: products obtained in the presence of RF-C were more heterogeneous than those obtained in the presence of RF-A, although both factors stimulated pol α to the same extent.

RF-A, PCNA and RF-C stimulate pol δ co-operatively

Similar experiments were done with pol δ in the presence and absence of PCNA. In the absence of PCNA, RF-A and RF-C had little, if any effect on pol δ activity, even when

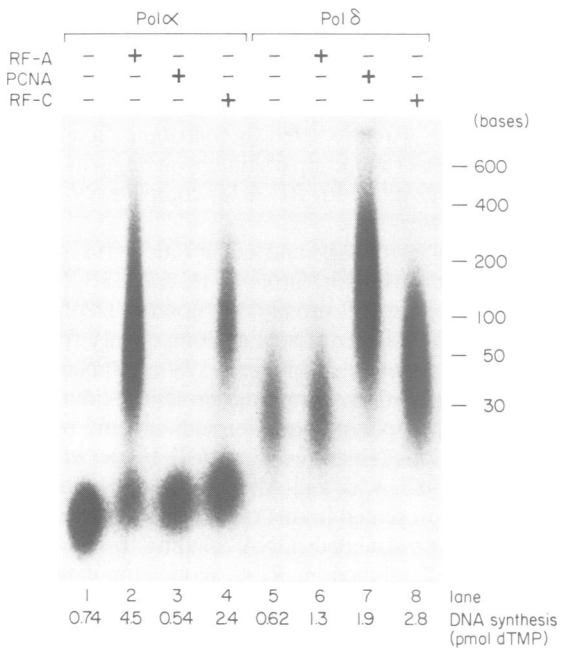

Fig. 3. Product analysis of DNA synthesis on poly(dA)/oligo(dT) by pol α or pol δ in the presence of RF-A, PCNA or RF-C. The components used were poly(dA)/oligo(dT) (20:1; 0.08 mM in nucleotides and 6.7 pmol of 3′ ends), pol α (0.07 U), pol δ (0.025 U), RF-A (1.2 μg), PCNA (250 ng) and RF-C (60 ng) and 26 mM NaCl. The reaction mixture (25 μl) was incubated at 37°C for 15 min and a 5 μl portion was withdrawn to determine the incorporation of dTMP (shown below as pmol dTMP/25 μl reaction), and a 20 μl portion was used for product analysis. The product DNA containing roughly the same amount of radioactivity was separated by electrophoresis in a 2% agarose gel under alkaline conditions. Mobilities of denatured DNA were obtained from *Hpa*II-digested pBR322 DNA and are shown in bases on the left.

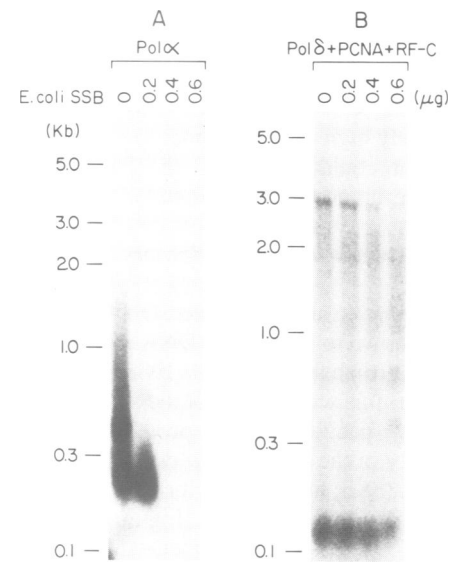

Fig. 4. Substitution of RF-A with *E.coli* SSB for stimulation of DNA polymerases. **(A)** The reaction mixture containing 100 ng primed-ssM13 DNA and 0.2 U of pol α was incubated with the indicated amounts of *E.coli* SSB at 37°C for 30 min. A higher amount of pol α was used in this experiment than the amount used in Figure 2. **(B)** The reaction mixture containing 100 ng primed-ssM13 DNA, 0.27 U of pol δ, 250 ng PCNA and 90 ng RF-C was incubated with the indicated amounts of *E.coli* SSB at 37°C for 30 min. A constant portion of the product DNA was isolated and subjected to 1% agarose gel electrophoresis under alkaline conditions. Mobilities of denatured DNA fragments are shown on the right as for Figure 2.

combined (Figure 1E and F and Figure 2, lanes 5−8). In contrast, when RF-C was added to reactions containing pol δ and PCNA, it increased DNA synthesis slightly (from 0.42 to 0.78 pmol of dAMP) and some of the products were extended and heterogeneous in length (Figure 2, lane 11). When added alone, RF-A seemed to have no effect on pol δ, even in the presence of PCNA, but it if was added to the reaction with RF-C and PCNA, a marked stimulation was observed (Figure 1C and D and Figure 2, lane 12). DNA synthesis increased in proportion to the amount of RF-A or RF-C only when PCNA was present in the reaction, exhibiting a >25-fold stimulation from the basal level. RF-A had an optimum of 1.2 μg/reaction, but saturation could not be achieved with RF-C because it must be stored in a high concentration of NaCl. The length of the product obtained in the presence of all three replication factors ranged from 1 to 4.5 kb, but no full-length product (7.9 kb) was observed. These results suggest that the three replication factors co-operate to stimulate pol δ activity.

Stimulation of pol α and pol δ on poly(dA)/oligo(dT)

To study the mechanisms of stimulation further, similar experiments were done with poly(dA)/oligo(dT) as a template under conditions of template excess, which will measure processivity of the DNA polymerases. It should also be noted that the template:primer ratio with poly(dA)/oligo(dT) was much lower than the ratio with primed-ssM13 DNA as a template. As shown in Figure 3, pol α had very low processivity, synthesizing ~10 bases. Both RF-A and RF-C independently increased the incorporation ~3- to 6-fold,but PCNA had no effect. The length of the majority of the products in the presence of RF-A increased ~100 bases (lane 2), indicating that the major effect of RF-A on pol α was to increase the processivity of the enzyme. The product obtained with RF-C also revealed a bimodal distribution, but in this case, most of the product did not increase in length. This suggests that RF-C may increase the frequency of initiation at the primer as well as having a small effect on processivity.

Polymerase δ elongated the primer to ~30 bases in this assay (Figure 3, lane 5). RF-A, PCNA and RF-C individually increased the incorporation ~2- to 4-fold, but the products were markedly different. RF-A had no effect on the processivity. PCNA increased the processivity from ~30 bases to 100−400 bases (essentially full length of the template). Since RF-C increased the product length to only 40−80 bases, but stimulated incorporation better than PCNA, it probably increased both the processivity and initiation frequency by pol δ. RF-C, therefore, seems to affect pol α and pol δ on this template in a similar way. Moreover, RF-A and RF-C appeared to increase the initiation frequency of pol δ on this primed template, whereas PCNA and RF-C increased the processivity, but the effect of PCNA on processivity was greater than the effect of RF-C.

Combinations of the replication factors were also tested in these experiments (data not shown). The incorporation by pol α in the presence of both RF-A and RF-C was additive, as was the case for pol α activity on primed-ssM13 DNA. Combinations of PCNA and RF-A or PCNA and RF-C also increased DNA synthesis by pol δ over and above

the level obtained with each factor alone, but the combination of all three factors with pol δ did not further increase DNA synthesis on poly(dA)/oligo(dT) as it did on primed-ssM13 DNA. This could be due either to the short poly(dA) template and the relatively high ratio of primer to template DNA or, alternatively, it could be due to the lack of secondary structure in the homopolymer template.

Specificity between replication factors and DNA polymerases

RF-A is a eukaryotic single-stranded DNA binding protein that has been shown to function in DNA replication (Wobbe *et al.*, 1987; Fairman and Stillman, 1988; Wold and Kelly, 1988). To determine whether or not the stimulation of RF-A is mediated by specific protein−protein interactions, we tested the ability of the *Escherichia coli* single-strand DNA binding protein (SSB) to substitute for RF-A as an accessory factor for both pol α and pol δ. Figure 4(A) shows the product of DNA synthesis on primed-ssM13 DNA by pol α in the presence of the *E.coli* SSB. Interestingly, instead of stimulating pol α, even low levels of *E.coli* SSB dramatically inhibited its activity. Figure 4(B) shows the products of DNA synthesis by pol δ in the presence of PCNA and RF-C and increasing amounts of *E.coli* SSB. In this case, *E.coli* SSB did not stimulate pol δ activity, although RF-A did stimulate the DNA synthesis >10-fold under the same conditions.

When *E.coli* SSB was added instead of RF-A in reactions containing poly(dA)/oligo(dT), it again had no effect on DNA synthesis with pol α and pol δ (data not shown). Therefore, *E.coli* SSB did not substitute for RF-A under any circumstances. Conversely, none of the eukaryotic replication factors affected DNA synthesis by *E.coli* DNA polymerase I on primed-ssM13 DNA (data not shown). Thus, the stimulation of pol α and pol δ by these replication factors is specific and is probably mediated by protein−protein interactions between the eukaryotic DNA polymerases and the replication factors.

Discussion

Relationship between replication factors and other stimulatory factors for pol α

Various factors have been identified that stimulate pol α in assays using primed single strand DNA templates (reviewed in Fry and Loeb, 1986). Some of them affect DNA synthesis by the purified polymerases in a similar way as RF-A or RF-C. However, their physical properties, including elution profiles from several chromatographic columns or their mol. wts, make them clearly distinguishable from RF-A and RF-C. Surprisingly, with the notable exception of PCNA, none of these previously recognized stimulatory factors has been shown to function in DNA replication, in contrast to RF-A, RF-C and PCNA which are essential for DNA replication. The mechanism of stimulation of polymerase activity by each of these replication factors parallels their function in the SV40 DNA replication system, although a new role for RF-A in elongation is suggested by these results.

A role for RF-A during elongation

Single-strand DNA binding proteins are required at various stages of DNA replication. For example, RF-A combines with T antigen to unwind duplex DNA at the SV40 origin and is therefore involved in initiation of DNA replication. Although this is an important function prior to actual DNA synthesis, the origin unwinding function can also be provided by the *E.coli* SSB (Dean *et al.*, 1987; Wold *et al.*, 1987). RF-A, however, was predicted to contribute an additional function other than supporting origin unwinding, since *E.coli* SSB could not substitute for RF-A for complete SV40 DNA replication *in vitro*.

In *E.coli*, bacteriophage and eukaryotic virus systems, single-strand DNA binding proteins (*E.coli* SSB, T4 gene 32 protein, T7 gene 2.5 protein, adenovirus DBP, herpes simplex virus ICP8) are essential components for DNA replication (for review see Kornberg, 1980; Stillman, 1989). Such single-strand DNA binding proteins stimulate the homologous DNA polymerases through specific protein−protein interactions (Huang *et al.*, 1981; Fay *et al.*, 1982; Chiou *et al.*, 1985; Cha and Alberts, 1988; Huber *et al.*, 1988). Results presented in this report demonstrate that the eukaryotic single-stranded DNA binding protein RF-A performs a similar function. RF-A alone stimulates pol α on both primed-ssM13 DNA and on poly(dA)/oligo(dT), and increases its processivity >5-fold. The products of this reaction are short, of the order of the length of Okazaki fragments, which further suggests that pol α, unlike pol δ, can intrinsically measure the extent of DNA synthesis following each initiation event. *Escherichia coli* SSB could not substitute for this function and, conversely, RF-A had no effect on *E.coli* DNA polymerase I on primed-ssM13 DNA. These results suggest that RF-A and pol α interact directly and explain why *E.coli* SSB could not substitute for RF-A for complete SV40 DNA replication.

The effect of RF-A on pol δ was more complicated. RF-A had no effect on the processivity of pol δ on poly(dA)/oligo(dT), but did co-operate with RF-C and PCNA to stimulate DNA synthesis on primed-ssM13. The fact that the three proteins did not co-operate to stimulate pol δ activity on poly(dA)/oligo(dT) suggests that RF-A is required to eliminate DNA synthesis barriers on the template (e.g. secondary structures or specific sequences). It has been reported that *Drosophila* pol α stops at secondary structures in natural DNA templates (Kaguni and Clayton, 1982). It is also possible that RF-A interacts with either RF-C, PCNA or both to stimulate RF-C/PCNA-dependent pol δ activity by direct protein−protein interactions.

A role for RF-A in both initiation and elongation of DNA replication is intriguing. RF-A and T antigen co-operate to unwind the origin of replication, which places RF-A at a prime location to interact with the DNA polymerase and primase that presumably synthesize the first nascent strands at the origin. We suggest that the polymerase α−primase complex functions in this step and then moves from the origin with the replication fork to become the lagging strand polymerase. Thus the first nascent strands at the origin are predicted to be the first Okazaki fragments for lagging strand replication.

The role of RF-C during elongation

RF-C is essential for leading strand DNA synthesis during SV40 DNA replication *in vitro*. The results described here show that in the presence of RF-A and PCNA, DNA synthesis with pol δ on primed-ssM13 DNA was absolutely dependent on RF-C. This observation explains why similar replication products were obtained when RF-C and PCNA

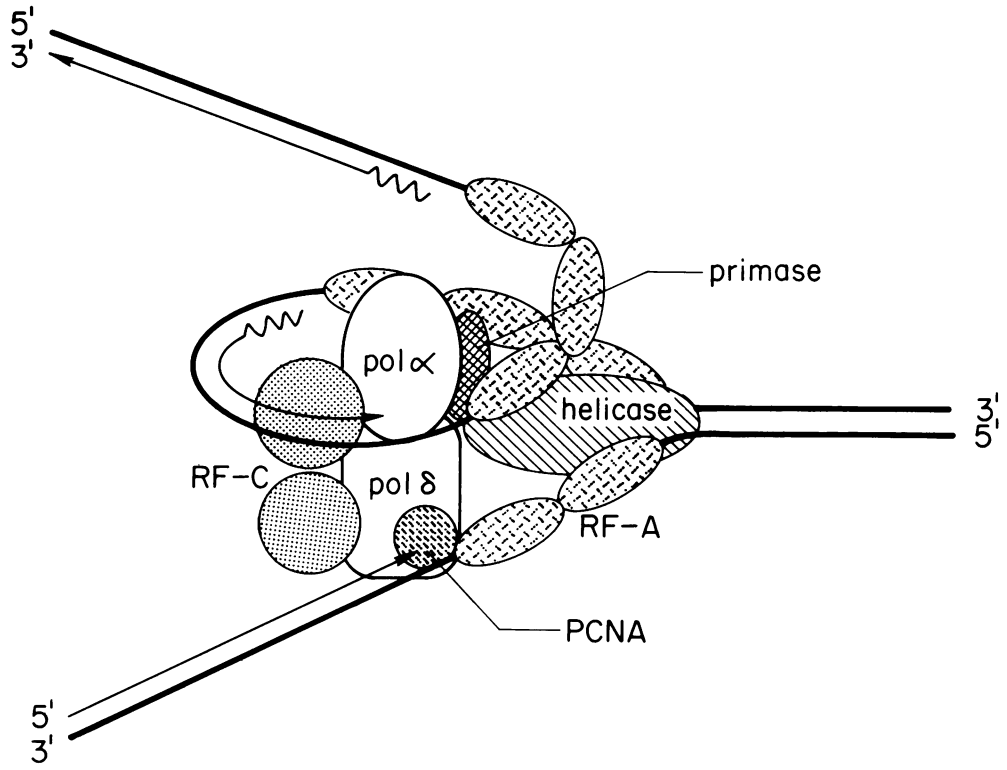

Fig. 5. Proposed model for the multi-protein complex (replisome) at a eukaryotic replication fork showing replication factors RF-A, PCNA, RF-C and the two asymmetrically placed α and δ polymerases synthesizing the lagging and leading strands respectively (see text for detail). During SV40 DNA replication, the major DNA helicase at the fork may be SV40 TAg (see Stillman, 1989, for discussion).

were separately omitted from SV40 DNA replication reactions (Prelich and Stillman, 1988; Tsurimoto and Stillman, 1989).

The experiment using poly(dA)/oligo(dT) as a template suggests that RF-C affected both the processivity of DNA synthesis and the frequency of primer recognition for both pol α and pol δ. RF-C could increase the affinity of the DNA polymerases for a primer annealed to the template polymerases. Indeed, RF-C specifically binds to single-strand DNAs annealed to short primer DNA (unpublished observation). If RF-C specifically binds to the 3′ ends of a primer on the template, we can predict a function for RF-C similar to the DNA polymerase accessory proteins, genes 44/62 and 45 encoded proteins in the bacteriophage T4 system. This complex recognizes the 3′ end of the primer and stimulates the rate and processivity of T4 phage DNA polymerase in an ATP-dependent fashion (Huang *et al.*, 1981). Further analysis of the interactions between RF-C and DNA polymerases and template DNA will be necessary to prove whether RF-C has a function analogous to the T4 DNA polymerase accessory proteins.

Eukaryotic replication complex with two DNA polymerases and multiple replication factors

In general, a replication fork moves in one direction on a double-stranded template containing antiparallel DNA strands, synthesizing both of the complementary DNA strands at the same time. Since DNA polymerases can synthesize DNA only in the 5′−3′ direction, synthesis of the DNA strands must be asymmetric and as a consequence, synthesis of the leading strand proceeds continuously and synthesis of the lagging strand proceeds discontinuously (Ogawa and Okazaki, 1982). To explain how such different modes of DNA synthesis are co-ordinated, Alberts (Sinha *et al.*, 1980; Alberts *et al.*, 1982) and Kornberg (1982) have proposed that a dimer of the replicative DNA polymerase exists at the fork, with one polymerase subunit responsible for leading and the other for lagging strand DNA synthesis. To accomplish this task, they further propose that the lagging strand template is looped around so that the polymerase copying that strand can move in the same direction as the leading strand polymerase. A recent modification is the 'asymmetric dimer hypothesis' proposed for *E.coli* DNA polymerase III (McHenry, 1988; Maki and Kornberg, 1988), in which two DNA polymerase complexes have a different subunit composition and are therefore functionally distinguishable.

In addition to the replicative DNA polymerase, pol α, several lines of evidence strongly implicate pol δ (or its yeast homolog, pol III) in eukaryotic DNA replication (Prelich and Stillman, 1988; So and Downey, 1988; Boulet *et al.*, 1989; Sitney *et al.*, 1989). Although several models could explain the requirement for two replicative DNA polymerases (Blow, 1989), we propose to apply the asymmetric dimer hypothesis to eukaryotic DNA replication, where pol α and pol δ would form a complex at a replication fork and synthesize lagging and leading DNA strands co-ordinately. Taking into account the results described in this report, we further propose that the multiple replication factors RF-A, RF-C and PCNA also contribute to the asymmetric replicative complex of proteins at the replication fork (Figure 5). Since all three replication factors were required to stimulate pol δ on the primed-ssM13 DNA and the products were relatively long, it is likely that pol δ

functions as the leading strand polymerase. This observation further suggests that communication between proteins at a replication fork is not limited to an interaction between the replication factors and the DNA polymerases, but also between the replication factors themselves. The fact that two of these replication factors also influence the function of pol α suggests that protein−protein interactions between these factors may co-ordinate replication by pol α and pol δ. This is consistent with the requirement for both RF-C and PCNA in the co-ordinated synthesis of both strands during SV40 DNA replication *in vitro* (Prelich and Stillman, 1988; Tsurimoto and Stillman, 1989). A similar multi-protein network has been described in the T4 replication system (Cha and Alberts, 1988). Therefore the assembly of a multi-protein complex (replisome) at a replication fork following initiation of DNA replication at the origin must be dictated by a variety of specific protein−protein interactions, and could be controlled by the availability of these replication factors at the site of replication within the nucleus.

One surprising result is the inability of either DNA polymerase to synthesize the full-length product (7.9 kb) on primed-ssM13 DNA, even in the presence of the replication factors. Furthermore, the product length obtained with pol δ in the presence of three factors was reduced to ~1 kb if 5-fold lower amounts of pol δ were used, compared to the experiment described in Figure 2 (lane 12) (data not shown). Therefore, the processivity of pol δ on this template is not sufficient to synthesize a long stretch of DNA following a single priming event. In contrast, prokaryotic DNA polymerases can synthesize DNA strands of >10 kb following a single priming event. SV40 DNA replication *in vitro* was reconstituted with five purified cellular replication factors and one partially purified fraction IIA (Tsurimoto *et al.*, 1989). Since all of the replicative DNA polymerase activities were supplied by this fraction, pol α and pol δ must be present there. When DNA synthesis by fraction IIA on primed-ssM13 DNA was investigated, full-length products were obtained (unpublished observations), suggesting that additional factors in fraction IIA are missing in the purified DNA polymerase preparation. It is clear that further investigation of the SV40 system will shed light on the mechanism of DNA replication in eukaryotic cells.

Materials and methods

Template DNAs

Single-stranded mp18 DNA was prepared from *E.coli* cells infected with the phage as described previously (Prelich and Stillman, 1988). It was annealed with the 17 base sequencing primer at a primer to template ratio of 3:1 in 10 mM Tris−HCl, pH 7.4, 1 mM EDTA (TE) and 100 mM NaCl and used as a single-stranded M13 DNA primed at a unique site (primed-ssM13 DNA). Poly(dA) (Pharmacia; 400 nucleotides) and oligo(dT) (Pharmacia; 12−18 nucleotides) were annealed in TE with 50 mM NaCl (20:1 in nucleotide), and use as poly(dA)/oligo(dT).

Purification of DNA polymerases α and δ

Pol α was purified from 293 cell cytoplasmic extract (S100) by immuno-affinity column chromatography with an anti-pol α monoclonal antibody (SJK 273-71)−Sepharose according to Murakami *et al.* (1986). The purified pol α has a specific activity of 3×10^3 U/mg protein. Pol δ was purified from calf thymus (90 g) by five steps (DEAE−cellulose, phenyl−Sepharose, phosphocellulose, hydroxylapatite and ssDNA−cellulose chromatography) as previously published (Lee *et al.*, 1984), and had a specific activity of 6.8×10^3 U/mg protein. This pol δ was stimulated by PCNA 3- to 6-fold (using assay conditions described below) with poly(dA)/oligo(dT) template, and was insensitive to the inhibition by anti-pol α monoclonal antibodies (SJK 132-20 and SJK 287-38; Tanaka *et al.*, 1982). One unit of DNA polymerase activity was defined as the incorporation of 1 nmol of dTMP at 37°C in 1 h with 0.04 mM poly(dA)/oligo(dT) in 25 μl of reaction mixture as described below.

Purification of the replication factors

RF-A and PCNA were purified from fraction I prepared from a human 293 cell cytoplasmic extract with slight modifications of the original methods (Prelich *et al.*, 1987a; Fairman and Stillman, 1988). The second step (DEAE−cellulose) was substituted by Q-Sepharose column chromatography, and RF-A and PCNA were eluted from the column by a linear gradient of NaCl from 0.1 to 0.6 M in buffer A (25 mM Tris−HCl, pH 8.0, 1 mM EDTA, 0.1 mM PMSF, 0.01% NP-40, 10% glycerol). RF-A eluted in fractions centered at 0.22 M NaCl and was further purified by ssDNA−cellulose column chromatography as described previously (Fairman and Stillman, 1988). PCNA eluted in fractions centered at 0.38 M NaCl and was further purified by hydroxylapatite and phenyl−Sepharose chromatography as described previously (Tan *et al.*, 1986; Prelich *et al.*, 1987a). Final concentrations of RF-A and PCNA were 0.6 mg/ml and 0.25 mg/ml respectively. RF-C was purified by a published procedure (Tsurimoto and Stillman, 1989) from a 293 cell nuclear extract and the ssDNA−cellulose fraction eluted with 0.66 M NaCl (60 μg/ml RF-C) was stored and used without dialysis. Therefore, when the effect of RF-C was tested, NaCl concentrations in the control reactions were kept constant.

DNA synthesis reactions and product analysis

A standard reaction mixture for the DNA polymerase assay with primed-ssM13 DNA (25 μl) was based on SV40 replication reaction conditions (Li and Kelly, 1985; Stillman and Gluzman, 1985) and contained 100 ng of the template DNA, 30 mM HEPES, pH 8.0, 7 mM $MgCl_2$, 0.5 mM dithiothreitol, 0.1 mg/ml bovine serum albumin and 0.05 mM dNTP with [α-^{32}P]dATP (1500−2000 c.p.m./pmol). For the assay with poly(dA)/oligo(dT), the same reaction mixture was used except that 0.08 mM poly(dA)/oligo(dT) and 0.05 mM [α-^{32}P]dTTP (1500−2000 c.p.m./pmol) were used. Since the RF-C preparation used in this study contains 0.66 M NaCl, final concentrations of NaCl in reactions (26 or 40 mM as indicated in each experiment) were used. The reaction mixture was pre-incubated at 0°C for 10 min after the addition of the replication factors, and the DNA synthesis was started by addition of the indicated amount of DNA polymerase at time 0, and then incubated at 37°C for the indicated times. When poly(dA)/oligo(dT) was used as a template, enzyme concentrations were adjusted so that <1 mol of nucleotides was incorporated/mol of primer to ensure that product lengths reflected a single binding event by DNA polymerase. The reaction was terminated with 10 mM Na_2EDTA and acid-insoluble label was measured. For product analysis, precursor nucleotide with a higher specific activity (10 000 c.p.m./pmol) was used, and the reaction was terminated with 25 μl of proteinase K solution (0.2 mg/ml proteinase K, 20 mM Na_2EDTA, 2% SDS) and incubated for an additional 30 min, and DNA in the sample was extracted with phenol/chloroform (1:1), precipitated with ethanol, dissolved in 10 μl of TE and an aliquot was subjected to alkaline agarose gel electrophoresis (Maniatis *et al.*, 1982). After the electrophoresis, the gel was fixed with 10% methanol and 10% acetic acid, dried down on Whatman 3MM paper, and autoradiographed.

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Note added in proof

We have recently demonstrated that RF-C is a DNA-dependent ATPase and primer binding protein, and that PCNA stimulates the ATPase activity of RF-C. These functions are analogous to the functions of the bacteriophage T4 genes 44/62 and gene 45 encoded proteins (T.Tsurimoto and B.Stillman, unpublished).