

Functions of replication factor C and proliferating-cell nuclear antigen: Functional similarity of DNA polymerase accessory proteins from human cells and bacteriophage T4

(simian virus 40 DNA replication/DNA binding/ATPase/evolution)

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ABSTRACT The proliferating-cell nuclear antigen (PCNA) and the replication factors A and C (RF-A and RF-C) are cellular proteins essential for complete elongation of DNA during synthesis from the simian virus 40 origin of DNA replication *in vitro*. All three cooperate to stimulate processive DNA synthesis by DNA polymerase δ on a primed single-stranded M13 template DNA and as such can be categorized as DNA polymerase accessory proteins. Biochemical analyses with highly purified RF-C and PCNA have demonstrated functions that are completely analogous to the functions of bacteriophage T4 DNA polymerase accessory proteins. A primer-template-specific DNA binding activity and a DNA-dependent ATPase activity copurified with the multisubunit protein RF-C and are similar to the functions of the phage T4 gene 44/62 protein complex. Furthermore, PCNA stimulated the RF-C ATPase activity and is, therefore, analogous to the phage T4 gene 45 protein, which stimulates the ATPase function of the gene 44/62 protein complex. Indeed, some primary sequence similarities between human PCNA and the phage T4 gene 45 protein could be detected. These results demonstrate a striking conservation of the DNA replication apparatus in human cells and bacteriophage T4.

At a DNA replication fork, appropriate assembly of accessory proteins and DNA polymerase (pol) is required to form an active holoenzyme (1, 2). Little was known about either the mechanism of eukaryotic DNA replication or the accessory proteins that interact with eukaryotic pols; however, recent studies on simian virus 40 (SV40) DNA replication *in vitro* have provided insights into DNA replication in mammalian cells (3). Although DNA polymerase α (pol α) was long thought to be a primary replicative pol in eukaryotes, the involvement of DNA polymerase δ (pol δ) in DNA replication was suggested only recently by studies with the SV40 system (4, 5) and has been demonstrated by genetic studies in yeast (6, 7). These studies suggest that a dimeric pol complex containing pol α , pol δ , and accessory proteins is responsible for coordinated replication of both the leading and lagging strands at the replication fork (3, 8). Pol δ contains a 3'-5' exonuclease activity and can synthesize DNA processively on poly(dA)-oligo(dT) primer-template DNA in the presence of one accessory protein, proliferating-cell nuclear antigen (PCNA) (9, 10). Further studies demonstrated that pol δ can synthesize DNA processively and efficiently on a primed M13 single-stranded DNA (ssDNA) only in the presence of three replication factors, replication factor A (RF-A), PCNA, and replication factor C (RF-C) (8). RF-A is a multisubunit ssDNA binding protein that is required for both initiation and elongation of DNA replication *in vitro* and functions as a stimulatory factor for pol α and pol δ (8, 11–15). This latter function may

be analogous to the activities of a number of viral, phage, and bacterial ssDNA binding proteins that stimulate their homologous pols (2, 3, 16). RF-C is required, along with PCNA, only for the elongation stage of SV40 DNA replication and appears to be required for the coordinated synthesis of leading and lagging strands (5, 17). Several properties of RF-C, for example, its function as a pol accessory protein and its moderate affinity for ssDNA bound to cellulose (17), suggested to us that it may be involved in template-primer recognition or as a molecular clamp holding the pol onto the template DNA. Analogous functions have been suggested for prokaryotic pol accessory proteins (1, 2, 16).

Studies on *Escherichia coli* and its phages have elucidated the general mechanisms for the initiation and elongation of DNA replication. Particularly relevant to this discussion are the studies on the bacteriophage T4 DNA replication proteins required at the replication fork [for review, see Cha and Alberts (18)]. The phage pol, encoded by gene 43, synthesizes the leading and lagging strands at a replication fork, forming a dimeric pol complex. Pol accessory proteins, encoded by genes 44 and 62, function as a DNA-dependent ATPase and primer-recognition protein complex. This DNA-dependent ATPase activity is stimulated by another protein, encoded by gene 45, and together the gene 44/62 and gene 45 proteins complex and cooperate to stimulate the processivity of pol, forming a pol holoenzyme. In addition to these proteins, the helix-destabilizing protein (gene 32, ssDNA binding protein) and the "primosome" proteins, encoded by genes 41 and 61 (DNA helicase and primase), also function at the replication fork to unwind the parental duplex DNA, form primers for Okazaki fragment synthesis on the lagging strand, and augment pol function. We have further characterized two human cell replication factors, RF-C and PCNA, and demonstrate that they are strikingly similar to proteins encoded by the phage T4 genes 44/62 and 45, respectively.

MATERIALS AND METHODS

Replication Factors, RF-A, PCNA, and RF-C, and Pol δ . RF-A was purified from a human 293 cell cytoplasmic extract as described (15) and a ssDNA-cellulose fraction (650 $\mu\text{g/ml}$) was used. Two sources of PCNA were used in this study. PCNA (200 $\mu\text{g/ml}$) purified from human 293 cells by a published procedure (4) was used in Figs. 2 and 4. PCNA that had the same specific activity as PCNA from human cells was produced in *E. coli* harboring a plasmid carrying the human PCNA cDNA sequence (19) under the control of bacteriophage T7 promoter. The *E. coli*-produced PCNA was purified

Abbreviations: pol, DNA polymerase; pol α , DNA polymerase α ; pol δ , DNA polymerase δ ; pol III, DNA polymerase III; RF-A, replication factor A; RF-C, replication factor C; PCNA, proliferating-cell nuclear antigen; SV40, simian virus 40; ATP[S], adenosine 5'-[γ -thio]triphosphate; ssDNA, single-stranded DNA.

to homogeneity by four steps (K. Fien and B.S., unpublished results) and used in Fig. 3. RF-C was purified from human 293 cell nuclear extracts through four steps (ssDNA-cellulose fraction; 60 or 40 μg of RF-C per ml) or five steps (glycerol gradient fraction; 8 μg of RF-C per ml) as published (17). Pol δ was purified from calf thymus (90 g) by five steps as described (8, 20) and the ssDNA-cellulose fraction with a specific activity of 6.8×10^3 units/mg was used. One unit of pol activity was defined as the incorporation of 1 nmol of dTMP at 37°C in 1 hr under conditions described in ref. 8.

DNA Synthesis Reaction on a Primed M13 ssDNA with Pol δ . The reaction mixture (25 μl) containing 30 mM Hepes (pH 7.8), 30 mM NaCl, 7 mM MgCl_2 , 0.5 mM dithiothreitol, bovine serum albumin (0.1 mg/ml), all four dNTPs (each at 0.05 mM) with [α - ^{32}P]dATP (2000 cpm/pmol), 100 ng of M13 ssDNA (M13mp18) primed with a 3-fold molar excess of a unique 17-base sequencing primer (primer 1211 from New England Biolabs), 200 ng of PCNA, 1 μg of RF-A and 0.27–0.54 unit of pol δ was incubated at 37°C for 30 min, and acid-insoluble radioactivity was determined.

ATPase Assay. The assay for RF-C ATPase was essentially the same as described (21). The reaction mixture (25 μl) containing 50 mM of Tris-HCl (pH 7.9), 0.1 M NaCl, 1 mM dithiothreitol, 2 mM MgCl_2 , bovine serum albumin (100 $\mu\text{g}/\text{ml}$), 0.1 mM [γ - ^{32}P]ATP, and the indicated DNAs was incubated at 37°C for 30 min.

RESULTS

RF-C Binds Specifically to a Primer–Template DNA. RF-C and PCNA cooperate with RF-A to stimulate the putative leading-strand pol, pol δ (8). As noted above, this suggested that they may be similar to prokaryotic pol accessory proteins. Since RF-C bound to DNA, the DNA binding activity of RF-C was studied by nitrocellulose filter binding assays with ^{32}P -labeled poly(dA) [a (template) ssDNA] or poly(dA)-oligo(dT) (a primer–template DNA). As shown in Fig. 1A, RF-C bound to poly(dA)-oligo(dT) but not to poly(dA). The binding specificity of RF-C was demonstrated by testing the ability of various unlabeled DNAs to act as competitors for RF-C binding to ^{32}P -labeled poly(dA)-oligo(dT) (Fig. 1B). Under these conditions, RF-C bound to ssDNA carrying primers but not to ssDNAs or double-stranded DNAs or to an RNA-DNA template–primer [poly(A)-oligo(dT)]. This primer–template-specific DNA binding activity cosedimented in a glycerol gradient with two other activities of RF-C; the ability to stimulate SV40 DNA replication *in vitro* and the stimulation of pol δ on a primed M13 ssDNA in the presence of RF-A and PCNA (Fig. 2). As reported (17), the latter two activities cosedimented with a multisubunit protein containing polypeptides with apparent molecular masses of 140, 41, and 37 kDa. This analysis suggests that the primer–template binding activity is due to RF-C and is not a contaminant in the purified preparation.

RF-C Is an ATPase Stimulated by a Primer–Template DNA and PCNA. Several functions for prokaryotic pol accessory proteins have been reported (2, 23–28) and, among these, the well-characterized bacteriophage T4 gene 44/62 protein complex has a specific primer–template DNA binding activity (23, 25). Moreover, this protein complex also contains a DNA-dependent ATPase activity that is stimulated by the presence of 3' ends on the DNA (27, 28). Thus, we tested the possibility that RF-C might have a similar ATPase activity. Fractions from the glycerol gradient that exhibited the specific primer–template binding activity also contained an ATPase activity when assayed in the presence of poly(dA)-oligo(dT) (Fig. 2B), indicating that RF-C also functions as an ATPase. As shown in Fig. 3A, highly purified RF-C has a low level of DNA-independent ATPase activity, but this activity is stimulated severalfold by either ssDNAs or double-stranded DNAs

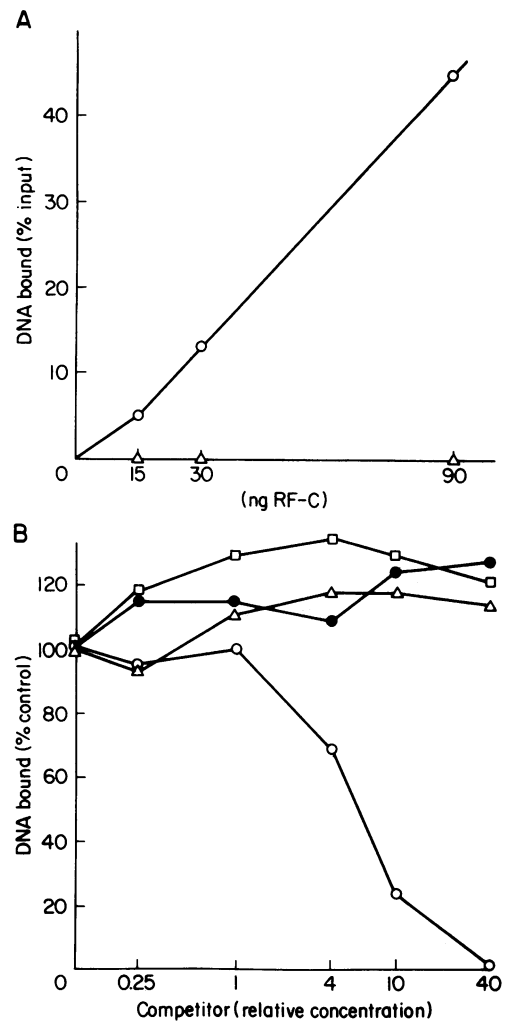


FIG. 1. DNA binding properties of RF-C. (A) Either ^{32}P -labeled poly(dA) (5 ng per reaction mixture, Δ) or ^{32}P -labeled poly(dA) $_{400}$ -oligo(dT) $_{12}$ (1:4 molar ratio, 5 ng per reaction mixture, \circ) were incubated with various amounts of RF-C (ssDNA-cellulose fraction, 60 $\mu\text{g}/\text{ml}$) at 0°C in a 25- μl reaction mixture containing the same buffer as used in the pol δ assay, but with 40 mM NaCl for 30 min. The mixture was then filtered through alkali-washed nitrocellulose (22), the protein-bound DNA was trapped on the filter, and its radioactivity was measured. The 100% value of input cpm was 6000 cpm. (B) Competition of the binding by various DNAs or RNAs was tested. All of the reaction mixtures contained ^{32}P -labeled poly(dA)-oligo(dT) and 60 ng of RF-C as shown in A. The mixtures were incubated with the indicated molar amounts (based upon nucleotide) of competitors at 0°C for 30 min and then filtered onto nitrocellulose. The 100% value of the control with no competitor corresponds to 20% binding of the input label. The competitors were poly(dA) (Δ), poly(dA)-oligo(dT) (\circ), poly(A)-oligo(dT) (\bullet , 1:4 molar ratio), or double-strand adenovirus type 2 DNA digested with *HincII* (\square).

[poly(dA) or adenovirus DNA digested with *HincII*, respectively] and to a greater extent by the presence of primers on the ssDNA [poly(dA)-oligo(dT)]. Therefore, RF-C is a DNA-dependent ATPase but is not absolutely dependent upon primers bound to the template DNA. A strikingly similar dependence on DNA has been observed for the ATPase activity of the phage T4 genes 44/62 protein complex (27).

In addition to the stimulatory effect of DNAs, the phage T4 gene 44/62 protein complex ATPase activity is also stimulated by another accessory protein, the gene 45-encoded protein (26–28). Since the phage T4 gene 45 protein and PCNA do not bind directly to DNA but can form a complex with their respective pols (2, 9), we predicted that PCNA

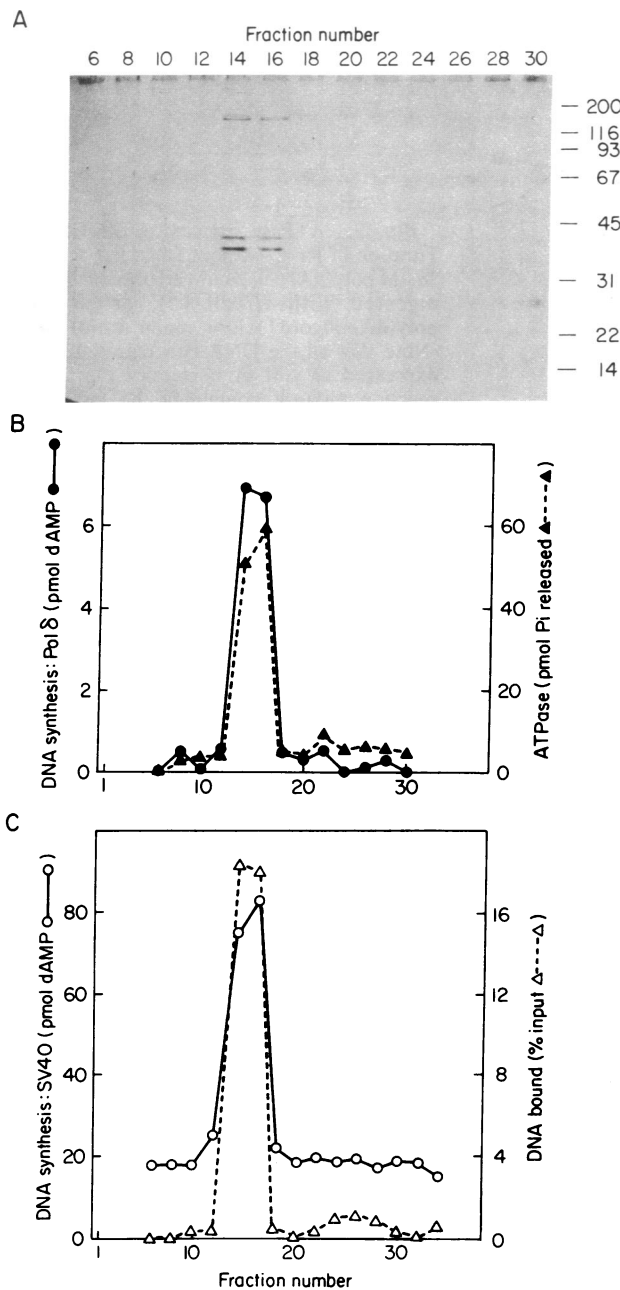


FIG. 2. Cosedimentation of primer-template DNA binding and DNA-dependent ATPase activities with RF-C in a glycerol gradient. A ssDNA-cellulose fraction of RF-C (100 μ l; 40 μ g/ml of protein) was loaded onto a 5-ml 15–35% (vol/vol) glycerol gradient in buffer A containing 0.1 M NaCl as described (17). After centrifugation in a SW 50.1 rotor at 49,000 rpm at 4°C for 24 hr, 38 fractions were collected from the bottom. The positions of marker proteins, which were run in a parallel gradient were catalase (11 S) at fraction 9, alcohol dehydrogenase (7.4 S) at fraction 16, bovine serum albumin (4.3 S) at fraction 24, ovalbumin (3.7 S) at fraction 26, and cytochrome C (1.9 S) at fraction 32. (A) A 10- μ l aliquot of every second fraction was separated in an NaDodSO₄/polyacrylamide gel (12.5%) and proteins were stained with silver. Molecular mass markers (kDa) are shown on the right. (B) Stimulation of pol δ on a primed M13 ssDNA in the presence of RF-A and PCNA, and DNA-dependent ATPase activity measured with 26 μ M poly(dA)-oligo(dT) (1:4 molar ratio), were determined using 4 μ l of each fraction. DNA-independent ATPase activity was also measured in fractions 14 and 16 and was \approx 10% of the level obtained with DNA present (data not shown). (C) Complementation assay for RF-C in SV40 DNA replication *in vitro* and DNA binding to a primer-template. The RF-C complementation was done in a 50- μ l reaction mixture containing 350 μ g of fraction 1*, 1.7 μ g of SV40 tumor antigen, 200 ng of topoisomer-

would be analogous to the phage T4 gene 45 protein and would stimulate the RF-C DNA-dependent ATPase. This was tested by addition of various amounts of purified PCNA into the RF-C ATPase reaction in the presence of primer-template DNA. The DNA-dependent ATPase activity of RF-C was stimulated up to 4-fold in the presence of saturating amounts of PCNA (Fig. 3B). In the absence of RF-C, highly purified PCNA revealed a background level of ATPase, so that it is possible that this minor ATPase observed in the PCNA preparation is greatly stimulated by interaction with RF-C. But the PCNA-stimulated ATPase activity displayed the same specificity for the various DNAs as the RF-C ATPase activity shown in Fig. 3A (data not shown), suggesting that most of the ATPase activity observed is intrinsic to the RF-C polypeptides. Therefore, this result demonstrates an interaction between RF-C and PCNA similar to that observed between the phage T4 gene 44/62 complex and the gene 45 protein.

Hydrolysis of ATP Is Required for Processive DNA Synthesis by Pol δ . In the phage T4 DNA replication system, hydrolysis of ATP or dATP by the accessory proteins is required for the formation of a stable initiation complex containing accessory proteins and pol bound to the 3' end of the DNA primer (2, 23, 29). If RF-C and PCNA are functionally analogous to the phage T4 gene 44/62 protein complex and gene 45 protein, respectively, stimulation of pol δ activity on primed M13 ssDNA by RF-C and PCNA should require hydrolysis of ATP. As reported (8), DNA synthesis by pol δ on primed M13 ssDNA was stimulated about 2-fold by RF-C and PCNA compared to incorporation by pol δ alone. If 1 mM ATP was added to this reaction, the DNA synthesis was further stimulated about 3-fold (Fig. 4). The ATP-independent processive DNA synthesis by pol δ in the presence of RF-C and PCNA may have been due to the utilization of dATP instead of ATP, as has been reported in phage T4 system (2). When all three replication proteins, RF-A, RF-C, and PCNA, were added to reactions containing pol δ and primed M13 ssDNA, addition of ATP had no effect (Fig. 4). This is probably due to maximal stimulation of pol δ by the replication accessory proteins and dATP. We have demonstrated that under these conditions, DNA synthesis by pol δ is very processive (8). However, ATP (or dATP) hydrolysis is required for this stimulation of pol δ because the addition of 1 mM adenosine 5'-[γ -thio]triphosphate (ATP[S]), a non-hydrolyzable analogue of ATP, to the reaction completely abolished the stimulation of processive DNA synthesis by the replication accessory proteins (Fig. 4). ATP[S] did not inhibit the small amount of synthesis obtained with pol δ plus PCNA, suggesting that the effect of ATP[S] was RF-C-dependent and not the result of competitive inhibition of dATP incorporation. Therefore, these results demonstrated that processive DNA synthesis by pol δ on primed M13 ssDNA requires three accessory proteins and hydrolysis of ATP (or dATP), as has been reported in phage T4 system.

DISCUSSION

These biochemical analyses clearly indicated that RF-C and PCNA are functionally analogous to the phage T4 gene 44/62 protein complex and the gene 45 protein, respectively. It was, therefore, of interest to determine whether there were any

ase 1, 90 ng of topoisomerase II, 300 ng of pSVO10, and 8 μ l of each fraction as described (15). After the incubation at 37°C for 1 hr, acid-insoluble cpm were measured. The primer-template DNA binding activity was measured as described in the legend to Fig. 1 with 3 μ l of each fraction, but in the presence of 50 mM NaCl. DNA binding activity using poly(dA) was also tested in parallel with the same fractions; however, no DNA binding was detected (data not shown).

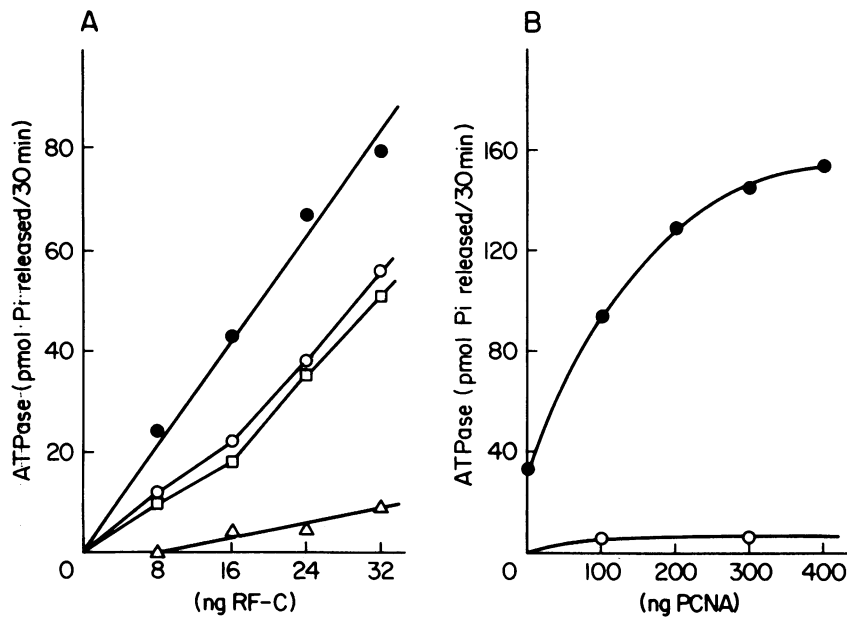


FIG. 3. ATPase activity of RF-C. (A) Titration of RF-C without DNA (Δ) or with $26 \mu\text{M}$ poly(dA) (\square), $26 \mu\text{M}$ adenovirus DNA digested with *HincII* (\circ), or $26 \mu\text{M}$ poly(dA)-oligo(dT) (1:4 molar ratio) (\bullet). (Note that all the DNA concentrations are expressed as μM of nucleotide.) A $25\text{-}\mu\text{l}$ reaction mixture containing the indicated amounts of RF-C was incubated with DNA and [$\gamma\text{-}^{32}\text{P}$]ATP and the released P_i from ATP was determined. (B) Addition of PCNA to RF-C ATPase assay. Increasing amounts of PCNA were incubated in the reaction mixture containing $26 \mu\text{M}$ poly(dA)-oligo(dT) with (\bullet) and without (\circ) 24 ng of RF-C, and the amount of released P_i was measured.

structural similarities between them; however, only a comparison between human PCNA (19) and gene 45 protein (30) is possible at present. A computer search of their primary amino acid sequence revealed limited regional similarities (31–50% similarity) that could be aligned in a linear arrangement, albeit with several gaps in each amino acid sequence (Fig. 5). The fact that these identities span the length of the amino acid sequences and that these proteins are functionally equivalent suggests that the proteins are evolutionarily related and that the identical residues are important for function in both proteins.

The major replicative pol in *E. coli* (pol III) also associates with multiple subunits and requires hydrolysis of ATP for processive DNA synthesis (1, 24, 32). In this case, the $\gamma\delta\delta'$ complex and the β subunit of pol III holoenzyme seem to

function similarly to RF-C and PCNA, respectively. The β subunit was proposed (9) to have a function similar to PCNA, but the amino acid sequence deduced from its coding sequence, the *dnaN* gene, revealed little significant similarity with human PCNA (data not shown). Thus, there appears to be a particular functional and structural relationship between the phage T4 and mammalian cell replication components. Indeed, the T4 gene 45-encoded protein stimulated the RF-C ATPase activity (unpublished results).

As mentioned above, two pols, α and δ , are involved in eukaryotic DNA replication, and in the yeast *Saccharomyces cerevisiae* pols I and III correspond to pols α and δ , respectively (6, 7, 33). Genes coding for human pol α and yeast pols I and III have been isolated. Comparison of their amino acid sequences demonstrated the presence of six conserved regions that may correspond to several functional domains (7, 34). It is striking that bacteriophage T4 pol also belongs to this class of pols by sequence similarity (35). In contrast, the *E. coli* pol III has almost no similarity, suggesting that *E. coli* may have evolved further from the ancestral form. Thus it is likely that during development of eukaryotes an ancestral pol gene of the phage T4 type duplicated and diverged to yield the pol α and pol δ types. This study further suggests that the pol accessory proteins were also conserved during eukaryotic evolution; however, in eukaryotes, the accessory protein complex functions with pol δ , probably because it appears to be the processive leading-strand pol. On the other hand, pol α , which does not appear to interact with PCNA but does interact with RF-C and RF-A, has become partially independent of the accessory proteins (8). Interestingly, pol α has kept the interaction with a DNA primase, presumably because it functions as the lagging-strand pol. Cha and Alberts (36) have demonstrated that the phage T4 primase, encoded by gene 61, is only required for lagging-strand DNA replication and not for leading-strand synthesis.

Fractionation of factors required for SV40 DNA replication *in vitro* has been a powerful method for identifying replication components from eukaryotic cells. Two of them were identified as accessory proteins for pol δ and are undoubtedly involved directly in cellular DNA replication; however, other replication components remain to be purified and characterized.

A remarkable similarity between bacteriophage T4 and eukaryotes has been noted. The group I introns present in some phage T4 mRNAs are related to the group I introns present in some eukaryote RNAs (37). Shub *et al.* (37)

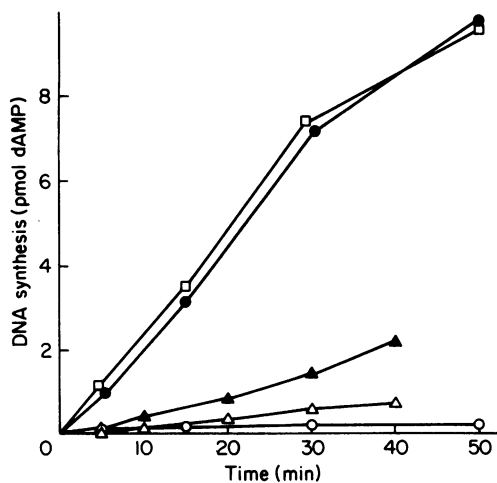


FIG. 4. Time-course of DNA synthesis with pol δ . The reaction mixture ($25 \mu\text{l}$) contained 0.54 unit of pol δ (8), 200 ng of PCNA, 40 ng of RF-C, and, where indicated, 650 ng of RF-A, 1 mM ATP or 1 mM ATP[S]. Samples ($3 \mu\text{l}$) were withdrawn at the indicated times and acid-insoluble radioactivity was measured. Values are shown as the incorporation of dAMP per $25 \mu\text{l}$ of the reaction mixture. Components used were PCNA and RF-C (Δ); PCNA, RF-C, and ATP (Δ); PCNA, RF-C, and RF-A (\square); PCNA, RF-C, RF-A, and ATP (\bullet); PCNA, RF-C, RF-A, and ATP[S] (\circ). Experiments with RF-C and PCNA (with and without ATP) were done separately from other experiments. Experiments with RF-C, PCNA, and ATP[S] or pol δ alone were also done in parallel and yielded almost the same results shown by the open circles (data not shown).

A

HPCNA 1 MFPEARLVQGSILKRVLRALKDILNEACWDISSGGVNLQSMDDSSHVSLVQLTLRSEGFDTYRCDR
 GENE45 1 MKLSKDTTALTKNFATIN--SGIMLKSQGF-----

HPCNA 65 NLAMGVNLTSMKILKCAQNEEDIITLFAEDNADTLALVFEAPNQEKVSYEMKLMIDLVEQLGI
 GENE45 29 -----IMTRAVN--GTTVAEAFNI-----SDVITFDVAIYDL-NGFLGI

HPCNA 129 PEQEYSCVVKMPSGEFARICRDLSHIGDAVVFSCAKDGVKFSASGELGNGNIKLSQTSNVNDEKEE
 GENE45 64 -----LSLVNDA-ELSSQSEDG-----NIKIADARSTI---

HPCNA 193 EAVTIEMNEPVQLTFALRYLNFETKATPLISSTVT-----
 GENE45 90 -----EWRRADEPSTDVAPNKPIPPFPVASAVTEIKAEDLQQLRLSRG

HPCNA 227 LMSADVPLVMEYKIDADM-----HLKYMILAPKIEDEEGS
 GENE45 132 LQIDTIAITVKECKIVINGENKVKVDSALTRVYKSLTLGDYDCEGTFNFIIINMANMKMQPGNYKL

GENE45 196 --LLWAKGKQGAAKFEGEHANYVVALEADSTHDF

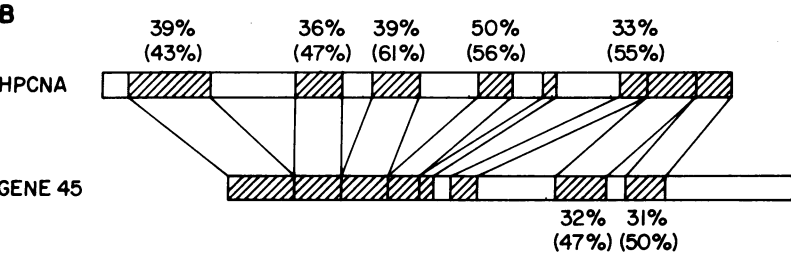


FIG. 5. Comparison of human PCNA (HPCNA) and phage T4 gene 45 protein (GENE45). (A) Amino acid sequences were obtained from refs. 19 and 30. Homologies between human PCNA and gene 45 protein were detected using the ALIGN program (31) and manual inspection. Identical amino acids are boxed and conserved amino acids are indicated with a dot. (B) Regions of homology between the two proteins are indicated and the numbers indicate percent identity and percent conservation (in parentheses) between them with respect to the human PCNA sequence. The single-letter amino acid code is used.

suggested that these group I intron similarities could be due to vertical acquisition of an ancestral form present before the divergence of prokaryotes and eukaryotes or horizontal transmission of introns after the divergence of prokaryotes and eukaryotes. The observation that the DNA replication machinery in phage T4 and human cells are also similar favors vertical acquisition as the mechanism for the evolutionary relatedness. Of course it is possible that phage T4 infected both prokaryotic and eukaryotic cells after their divergence. Although it is interesting that the replication apparatus is highly conserved between human cells and a bacteriophage that infects *E. coli*, ultimately we must also understand how the eukaryotic replication apparatus replicates chromatin, not just DNA, and it is this aspect of DNA replication that may make the eukaryotic replication apparatus unique.

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