

ATP Utilization by Yeast Replication Factor C

II. MULTIPLE STEPWISE ATP BINDING EVENTS ARE REQUIRED TO LOAD PROLIFERATING CELL NUCLEAR ANTIGEN ONTO PRIMED DNA*

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Binding of adenosine (3-thiotriphosphate) (ATP γ S), a nonhydrolyzable analog of ATP, to replication factor C with a N-terminal truncation (Δ 2–273) of the Rfc1 subunit (RFC) was studied by filter binding. RFC alone bound 1.8 ATP γ S molecules. However, when either PCNA or primer-template DNA were also present 2.6 or 2.7 ATP γ S molecules, respectively, were bound. When both PCNA and DNA were present 3.6 ATP γ S molecules were bound per RFC. Order of addition experiments using surface plasmon resonance indicate that RFC forms an ATP-mediated binary complex with PCNA prior to formation of a ternary DNA-PCNA-RFC complex. An ATP-mediated complex between RFC and DNA was not competent for binding PCNA, and the RFC-DNA complex dissociated with hydrolysis of ATP. Based on these experiments a model is proposed in which: (i) RFC binds two ATPs (RFC-ATP $_2$); (ii) this complex binds PCNA (PCNA-RFC-ATP $_2$), which goes through a conformational change to reveal a binding site for one additional ATP (PCNA-RFC-ATP $_3$); (iii) this complex can bind DNA to yield DNA-PCNA-RFC-ATP $_3$; (iv) a conformational change in the latter complex reveals a fourth binding site for ATP; and (v) the DNA-PCNA-RFC-ATP $_4$ complex is finally competent for completion of PCNA loading and release of RFC upon hydrolysis of ATP.

Studies of the role of ATP in clamp loading by the eukaryotic clamp loader replication factor C (RFC)¹ have lagged considerably behind similar studies in the T4 and *Escherichia coli* systems. In the phage T4 system, four ATP molecules bind to the clamp loader gp44/62 (1, 2). This activated complex interacts with the replication clamp gp45, resulting in the hydrolysis of two ATP molecules. Upon contacting DNA, the clamp is loaded, the remaining two ATP molecules bound to the gp44/62 complex are hydrolyzed, and the clamp loader is released from the DNA-gp45 complex. In *E. coli*, a similar mechanism prevails, involving the binding of only two molecules of ATP per clamp loader complex, which after loading of the clamp hydro-

lyze sequentially with release of the clamp loader from the DNA (3, 4).

Studies of the role of ATP in the function of eukaryotic RFC have established, first, that RFC forms a strong complex with the replication clamp PCNA when either ATP or the nonhydrolyzable analog ATP γ S is present (5, 6). Second, a derivative of yeast RFC² with a N-terminal truncation of the Rfc1 subunit, deleting the nonessential ligase homology domain, binds primer-template DNA only in the presence of ATP γ S (6). Although the studies with human RFC are less straightforward because of the presence of the ligase homology domain, which also binds DNA (7, 8), it appears that ATP γ S supports DNA binding more strongly than does ATP (9, 10). Finally, although ATP hydrolysis is required for effective loading of PCNA by RFC, ATP γ S also promotes loading of PCNA although in an inactive form (6, 11–14). However, under some conditions, particularly by removal of excess ATP γ S through gel filtration, this loaded PCNA can function, albeit inefficiently, as a processivity factor for DNA polymerase δ (11, 12) (Scheme I).

Because RFC can form distinct ATP-dependent complexes with PCNA as well as with DNA, the question arises which of these two complexes is an intermediate on the clamp loading pathway as outlined in Scheme I. Current models of eukaryotic clamp loading favor a pathway via steps 2 and 4, assigning a function to a RFC-DNA complex prior to its interaction with PCNA (15). However, our observation that ATP does not allow stable binding of RFC to DNA, presumably because its hydrolysis rapidly dissociates the RFC-DNA complex, makes it less likely that the latter complex functions as an intermediate in the loading of PCNA. Rather, a complex between PCNA and RFC may be the first step in clamp loading proceeding via steps 1 and 3, which is analogous to the prokaryotic clamp loading systems.

In this paper we determine how many ATP molecules participate in clamp loading and at what step in the overall pathway they enter the complex. Furthermore, we show that clamp loading is a sequential ordered process in which PCNA binding to RFC precedes that of DNA.

EXPERIMENTAL PROCEDURES

Enzymes, DNA, and Buffers—Pol δ was purified as described (16). All other enzymes were as described in the first paper. (6). The 80-mer template was designated V6 (5'-T₃₀CTCCCTTCTTCTCCCTCTCCTTCCCT₂₁-Biotin), and the 30-mer primer was designated C12 (5'-AGGGAAGGGAGAGGGAGGAGAAGAAGGGAG). [³⁵S]ATP γ S was obtained from Amersham Pharmacia Biotech, and cold ATP γ S was from Roche Molecular Biochemicals. The radioactive ATP γ S (500 μ Ci) was mixed with an about 10-fold molar excess of cold ATP γ S and purified

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¹ The abbreviations used are: RFC, replication factor C; RPA, replication protein A; PCNA, proliferating cell nuclear antigen; ATP γ S, adenosine (3-thiotriphosphate); SPR, surface plasmon resonance; Pol, polymerase; dAMP-PNP, 2'-deoxyadenyl-5'-yl-imidodiphosphate.

² The truncation derivative of replication factor C containing Rfc1- Δ (aa3–272) has been used in this biochemical study. For ease of reading, this complex has been simply designated as RFC.

over a 0.5-ml MonoQ column using a 5-ml gradient of 50–500 mM NaCl in 30 mM Hepes-NaOH, pH 7.5, 5 mM dithiothreitol. The concentration of ATP γ S in the peak fractions was determined spectrophotometrically, and the aliquots were stored at -70°C . A thin layer chromatographic analysis of the peak fractions indicated a radiochemical purity of $>98\%$. Cold ATP γ S was purified and stored similarly. Buffer A contained 30 mM Hepes-NaOH, pH 7.5, 0.5 mM EDTA, 10% glycerol, 10 mM magnesium acetate, 5 mM dithiothreitol, 0.1% ampholytes 3.5–10, and 0.01% Nonidet P40. Buffer B contained buffer A with 0.2 mg/ml bovine serum albumin. Salt concentrations of the buffers (in mM NaCl) are indicated with a subscript.

Nitrocellulose Filter Binding—Whatman nitrocellulose filters (0.2 μm) were treated for 10 min with 0.4 M KOH and then equilibrated in buffer A₇₅ (without dithiothreitol). Binding reactions were carried out in buffer B₇₅ in a final volume of 20 μl containing 0.2 μM RFC and increasing concentrations of [³⁵S]ATP γ S. PCNA (0.3 μM as trimers), DNA (0.3 μM V6, C12, or V6/C12), and RPA (0.6 μM) were added to the binding reaction where indicated. After an incubation at 0°C for 45 s, a 15- μl aliquot was filtered at ~ 50 $\mu\text{l}/\text{min}$ over a nitrocellulose filter, and the filter was washed at ~ 0.5 ml/min with 150 μl of buffer A₇₅. After air drying of the filter, a water-miscible counting fluid was added, and the samples were counted in a scintillation counter.

Several precautionary measures were taken, and several controls were carried out to ensure that the binding experiments properly measured stoichiometry of binding: (i) RFC was filtered over a 0.1 μM filter prior to use in order to remove aggregated inactive material (17); (ii) ATP γ S and [³⁵S]ATP γ S were purified to radiochemical and chromatographic homogeneity prior to use (see above); (iii) control filter binding assays were carried out with RFC, and the material flowing through the nitrocellulose filter was analyzed by a Western analysis to quantitate RFC passing through the filter; over 98% of the RFC remained on the filter (data not shown); (iv) different conditions were assayed to establish optimal binding of ATP γ S to RFC; nucleotide binding was insensitive to salt concentrations between 50 and 125 mM NaCl and to incubation times between 30 s and 120 s prior to filtration; binding was also independent of the pH of the binding buffer between 7.3 and 8.1, but decreased binding was observed at pH 6.8 and below; and (v) by far the largest decrease in signal was observed during the wash steps; this signal loss was unrelated to background binding of ATP γ S to the filter which was determined in separate experiments and which was in general 1–5% of the signal; by carrying out an increasing number of wash steps, the loss of signal per wash could be determined. A single wash step reduced the RFC-ATP γ S or RFC-PCNA-ATP γ S signal by 17% and reduced the RFC-DNA-ATP γ S and RFC-PCNA-DNA-ATP γ S signal by 11%; therefore, a single wash was carried out and, after background subtraction, the data were corrected for the loss of signal because of this wash step.

ATPase Assays—20- μl assays were performed in buffer B₇₅ containing 20 ng of RFC (0.1 pmol), PCNA (0.2 pmol of trimers), 100 ng of multiply primed single-stranded mp18 DNA (~ 5 primers/circle, ~ 0.2 pmol of primer-template termini), 850 ng of *E. coli* single-stranded binding protein, and a range of [α -³²P]ATP concentrations for determining K_m and k_{cat} . The reactions were incubated at 30°C , and after 2, 4, and 6 min, 5- μl aliquots were removed, quenched with 2.5 μl of 50 mM EDTA, 1% SDS, and 25 mM cold ATP and ADP, and processed for analysis (6). Initial rates were calculated from each time course and plotted against the ATP concentration.

Two-stage Replication Assays—RFC (0.2 pmol), PCNA (1 pmol), and Pol δ (0.5 pmol) were incubated in a final volume of 4 μl in buffer B₇₅ containing increasing concentrations of ATP from 0 to 1 mM. After 60 s at 0°C , a 2- μl aliquot was added to a 98- μl replication reaction equilibrated at 30°C and containing 40 mM Tris-HCl, pH 7.8, 8 mM MgAc₂, 0.2 mg/ml of bovine serum albumin, 1 mM dithiothreitol, 100 μM each of dCTP and dGTP, 50 μM of dAMP-PNP, 25 μM of [³H]dTTP (1000 cpm/pmol dTTP), 0.2 pmol of singly primed single-stranded mp18 DNA (the 36-mer primer is complementary to nucleotides 6330–6295), 2 μg of *E. coli* single-stranded binding protein, 75 mM NaCl, and either no ATP or increasing concentrations of ATP. After 60 s at 30°C , the reaction was stopped, and the acid-precipitable radioactivity was determined.

In a second set of assays, RFC (0.1 pmol) and Pol δ (0.2 pmol) were preincubated with 0.2 pmol of singly primed single-stranded mp18 DNA, *E. coli* single-stranded binding protein (2 μg), and 100 μM dCTP in a final volume of 12 μl in buffer B₇₅ containing increasing concentrations of ATP from 0 to 1 mM. The dCTP was added to prevent exonucleolytic degradation of the primer by the 3'-5'-exonuclease activity of Pol δ . After 60 s at 0°C , a 10- μl aliquot was added to a 90- μl replication reaction equilibrated at 30°C and containing 40 mM Tris-

HCl, pH 7.8, 8 mM MgAc₂, 0.2 mg/ml of bovine serum albumin, 1 mM dithiothreitol, 100 μM each dCTP and dGTP, 50 μM dAMP-PNP, 25 μM [³H]dTTP (1000 cpm/pmol dTTP), 1 pmol of PCNA, 75 mM NaCl, and either no ATP or increasing concentrations of ATP. After 60 s at 30°C , the reaction was stopped, and the acid-precipitable radioactivity was determined.

To assess whether ATP γ S could substitute for ATP in either the first or the second stage of the reaction, the first set of assays was repeated with the following modifications. RFC and PCNA were preincubated with 20 μM of ATP as described above and then diluted 50-fold into a replication reaction containing primed mp18 DNA and 0.25 pmol of Pol δ and either 100 μM ATP or 100 μM ATP γ S. DNA synthesis after 60 s was quantitated. Next, RFC and PCNA were preincubated with 10 μM of ATP, 10 μM of ATP γ S, or no nucleotide as described above and then diluted 50-fold into a replication reaction containing primed mp18 DNA, 0.25 pmol of Pol δ , and 100 μM ATP. DNA synthesis after 30 s was quantitated. In both assays, dAMP-PNP was used as a dATP analog that is readily incorporated into DNA by Pol δ but that, because of the β - γ -imido bond, is unable to load PCNA (11).

Bi-molecular Interaction Analysis—SPR was performed in a BIAcore X apparatus. Buffer B₁₂₅ was the running buffer used in the analysis. The DNA and PCNA chips used in these studies are described in the previous paper (6). Because of the limited lifetime of the chips, several chips had to be made that had slightly different loading densities. As a result, minor quantitative differences in binding were observed from chip to chip, and, therefore, the absolute responses (as resonance units) cannot be compared from experiment to experiment. However, a single series of experiments was always carried out with the same chip.

RESULTS

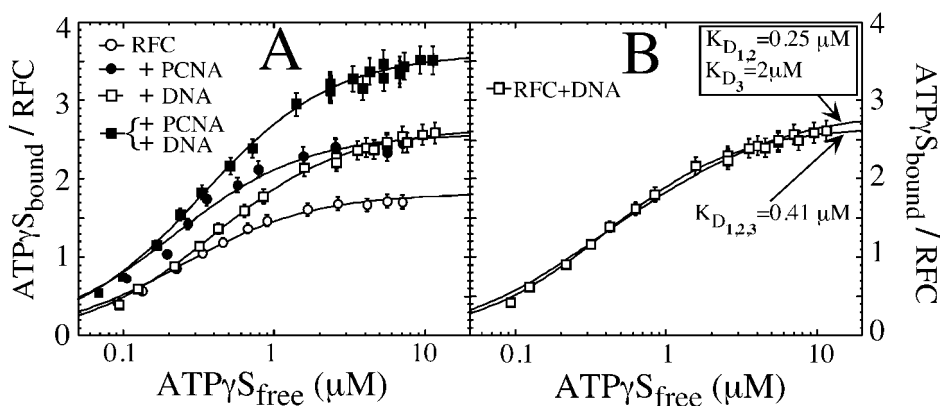
Binding of ATP to RFC Is Modulated by PCNA and DNA—ATP γ S is an ATP analog that has been very useful in biochemical studies because its binding affinity to ATPases is similar to that of ATP, but frequently hydrolysis of the analog is not observed (for a review see Ref. 18). Control studies showed that ATP γ S is not hydrolyzed by RFC with or without DNA and/or PCNA present (data not shown). Early studies have shown that ATP γ S is a potent inhibitor of both the ATPase activity of RFC and the productive loading of PCNA by RFC, suggesting efficient binding of ATP γ S to RFC (11, 12, 19, 20). In addition, previous studies that measured the binding affinities of ATP and ATP γ S for RFC in an indirect fashion showed no significant differences between the two nucleotides (5). However, implicit in our studies remains the assumption that fundamentally contrasting results with ATP and ATP γ S as cofactors derive in essence from the inability of ATP γ S to undergo hydrolysis.

The binding data of ATP γ S to RFC could be fitted to a Langmuir binding curve with a single K_D value of 0.25 μM and a stoichiometry at saturation of 1.78 molecules of ATP γ S bound per molecule of RFC (Fig. 1A). We interpret this to indicate that two molecules of ATP γ S can bind to RFC alone and that a small fraction of the RFC may be inactive and fail to bind the nucleotide. Surprisingly, in the presence of PCNA, three molecules of ATP γ S (actual value of 2.58) were bound with a very similar K_D value of 0.23 μM . Similarly, three ATP γ S molecules (actual value of 2.64) were bound to RFC when primer-template DNA was present; however, the K_D value was significantly higher at 0.41 μM . Finally, four molecules of ATP γ S (actual value of 3.60) bound RFC with a K_D of 0.35 μM when both PCNA and DNA were present. Assuming 11% of RFC to be inactive, calculated occupancy values at saturation of 1.78 (RFC alone), 2.58 (RFC+PCNA), 2.64 (RFC+DNA), and 3.60 (RFC+PCNA+DNA) molecules of ATP γ S recalculate to a



SCHEME I. Alternate loading pathways for PCNA. No role for ATP is shown.

Fig. 1. Binding of ATP γ S to RFC is regulated by PCNA and DNA. Nitrocellulose filter binding studies were performed as described under "Experimental Procedures." The 80-mer template V6 (5'-T₃₀CTCCCTTCTTCTCCTCCCTCCCTCCCT₂₁) was hybridized to the 30-mer primer C12 (5'-AGGGAAGGGAGAGGG-AGGAGAAGAAGGGAG). *A*, the data were fitted to simple Langmuir isotherms assuming equivalent sites. *B*, binding curves were calculated from the data for ATP γ S binding to RFC + DNA assuming either that all three sites have equivalent K_D values of 0.41 μ M or that two sites have K_D values of 0.25 μ M and the third site has a K_D value of 2 μ M.



2.0:2.9:3.0:4.0 ratio, which we consider highly significant evidence for binding by two, three, three, and four molecules, respectively.

The data in Fig. 1A have been fitted to single Langmuir binding curves with all binding sites identical in affinity for ATP γ S. However, particularly the data for ATP γ S binding to RFC in the presence of DNA fit equally well to a model in which the affinity of the first two sites has been set to 0.25 μ M, which is identical to those measured in the absence of DNA and that of the third site to 2 μ M (Fig. 1B). Similarly, binding of the four ATP γ S molecules to RFC with both PCNA and DNA present could just as reliably be modeled with three high affinity binding sites (0.23 μ M) and one low affinity binding site (2 μ M).

The nature of the DNA effector required to induce binding of an additional molecule of ATP γ S was investigated in more detail. The data were obtained at a single concentration of ATP γ S of 2.5 μ M. Because the ATP γ S concentration was below saturation, the measured stoichiometries were lower than those in Fig. 1. They were recalculated to the nearest integer value using this consideration and those described above (see caption to Table I). The 80-mer oligonucleotide V6, which was used as template, induced binding of a third molecule of ATP γ S, and a fourth molecule if PCNA was also present (Table I, entry 2). However, if this single-stranded DNA was first coated with RPA, no binding of an additional molecule of ATP γ S was induced. Previously, we had shown by SPR that binding of RFC to this 80-mer was observed but that binding was abolished when the DNA was coated with RPA (6). The 80-mer V6 (T₃₀CTCCCTTCTTCTCCTCCCTCCCTCCCTCCCT₂₁) was designed such that significant secondary structure would not be present. Therefore, it appears that binding of RFC to DNA *per se*, regardless of its structure, may trigger a conformational change that allows binding of an additional molecule of ATP γ S. RPA did not affect how primer-template DNA induced binding of ATP γ S (Table I, entries 5 and 6). In agreement with previous results showing that PCNA could be loaded onto forked primer-templates, the presence of a 3' single-stranded extension on the primer did not affect binding of ATP γ S (6).

Quantitative Requirement for ATP to Form Complexes between RFC and PCNA or DNA—Previously we have shown that ATP or ATP γ S stabilizes a complex between RFC and PCNA, and ATP γ S stabilizes a complex between RFC and DNA. SPR has allowed us to monitor the formation of such complexes and their dissociation in real time (6). RFC binding to a PCNA chip is greatly stimulated by ATP or ATP γ S. At saturating ATP concentrations the extent of steady state binding of RFC to a PCNA chip is a sole function of both the RFC concentration and the K_D value of RFC for PCNA. At subsaturating levels of ATP, steady state binding is additionally dependent on the K_D value of ATP for RFC, yielding a more complex relationship. However, the linear initial rate of bind-

ing to the chip is only dependent on the concentration of active complexes, *i.e.* those RFC molecules that have the required number of ATP molecules bound to form a high affinity complex with PCNA on the chip. Therefore, by varying the ATP concentration at a constant concentration of RFC, the apparent K_D for binding of the ATP molecule critical for strong complex formation can be determined from the initial binding rates. Both ATP and ATP γ S yielded similar apparent K_D values of 0.5 μ M (Fig. 2A), which are comparable with those determined previously using PCNA-agarose beads (5).

ATP γ S was required to observe a RFC-DNA complex by SPR when the DNA was attached to the chip and coated with RPA (6). A plot of the linear initial rates of binding of RFC to the DNA chip as a function of the ATP γ S concentration yielded an apparent K_D value of 8 μ M for ATP γ S, indicating that the lowest affinity ATP-binding site required for the formation of a detectable DNA-RFC complex has a K_D value of 8 μ M (Fig. 2B). Whether other higher affinity binding sites for ATP exist does not follow from this indirect analysis but is suggested by the direct nucleotide binding experiments in Fig. 1.

Loading of PCNA by RFC onto the primed DNA chip proceeds efficiently in the presence of ATP γ S, but some final step in this process fails to occur because of the absence of hydrolysis of the bound ATP (6). Remarkably, analysis of the initial rates of PCNA loading by RFC as a function of the ATP γ S concentration yielded an apparent K_D value of 1.5 μ M (Fig. 2B). This apparent K_D value is substantially lower than that required for complex formation between RFC and DNA in the absence of PCNA. Thus, ATP-mediated binding of PCNA to RFC allows complex formation with primed DNA at a lower ATP concentration than DNA-RFC complex formation without PCNA.

ATPase Activities of RFC—Previous attempts to determine the steady state ATPase parameters for yeast RFC were complicated by the extreme lability of the enzyme (19). Therefore, with a more stable enzyme available (17), the ATPase kinetics were reexamined. RFC has a basal ATPase activity that is stimulated 2-fold by PCNA (Fig. 3A). The presence of primed DNA stimulated the ATPase much more dramatically, and a synergistic increase was observed when both PCNA and DNA were present (Fig. 3B). Interestingly, the K_m values for all types of ATPase activities, except for the DNA-stimulated ATPase, were significantly higher than the associated K_D values measured with ATP γ S (Table II). These data may suggest that ATP γ S binds to one or more of the available binding sites in RFC with severalfold higher affinity than ATP does. Alternatively, the simple assumption that $K_m = K_D$ may not hold in this complex system.

Binding of a Final ATP Molecule to the RFC-PCNA-ATP Complex Is Required for Loading onto DNA—The ATP γ S binding studies indicate that in the presence of either PCNA or

TABLE I
Binding of ATP γ S to RFC in the presence of different DNA effectors

All binding assays were carried out as described under "Experimental Procedures," but at a single concentration of 2.5 μ M ATP γ S. The template DNA was V6 and the primer C12 (see legend to Figs. 1 and 2B). The base-paired substrate was V6/C12. The forked DNA was V6/C12T₃ (C12T₃ = 5'-AGGGAAGGGAGAGGGAGGAGAAGAAGGGAGTTT) with a 3-nucleotide 3'-extension. Except for entries 2 and 5, the DNA was coated with a 3-fold molar excess of RPA prior to binding of RFC. Binding experiments were done in triplicate, and the estimated errors were 5%. The number of ATP molecules bound was obtained from the actual data by rounding to the nearest integer after correcting for inactive RFC (11%, see text) and for fractional saturation by ATP γ S at the 2.5 μ M concentration used (a K_D of 0.4 μ M for all sites was assumed).

Entry	DNA	RPA	ATP γ S bound		Number of ATPs bound	
			RFC	RFC + PCNA	RFC	RFC + PCNA
1	None	No	1.6	2.4	2	3
2	Template	No	2.1	3.0	3	4
3	Template	Yes	1.6	2.4	2	3
4	Primer	Yes	1.5	2.4	2	3
5	Base-paired	No	2.3	3.1	3	4
6	Base-paired	Yes	2.4	3.1	3	4
7	Forked	Yes	2.4	3.2	3	4

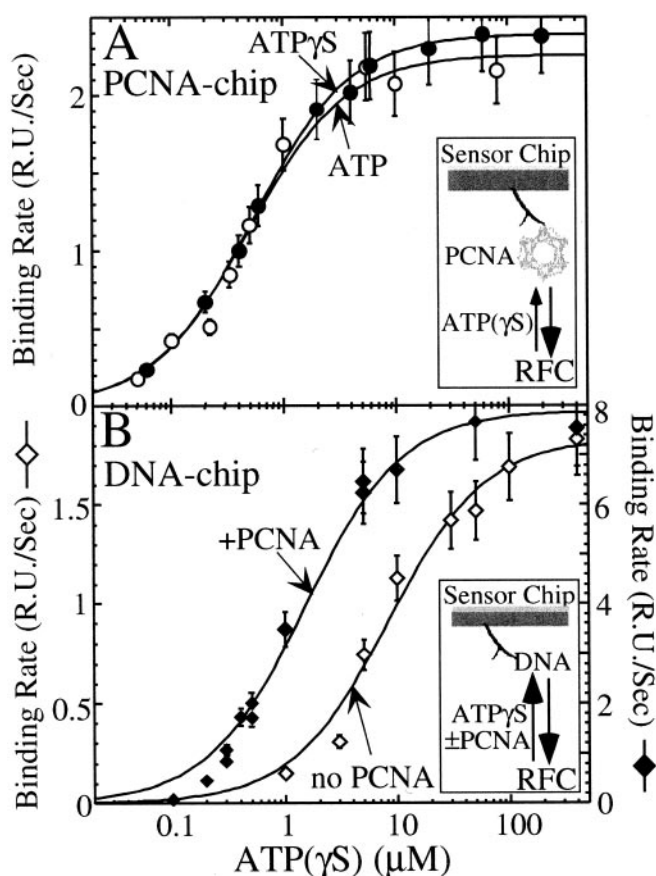


FIG. 2. ATP required for stable complex formation of RFC with PCNA and DNA. A, RFC was flowed across a PCNA chip in the presence of increasing concentrations of ATP or ATP γ S. B, RFC with or without PCNA was flowed across a primed DNA chip in the presence of increasing concentrations of ATP γ S. The data were fitted to a simple Langmuir binding model.

DNA three ATP molecules are bound to RFC, but when both DNA and PCNA are present, a fourth molecule is bound (Fig. 1). Two complimentary sets of experiments were carried out to determine whether the three ATPs in either complex would suffice to complete the loading reaction upon addition of the final required component, *i.e.* DNA to a PCNA·RFC·ATP₃ complex or PCNA to a DNA·RFC·ATP₃ complex (see flow diagrams in Fig. 4). In these assays, PCNA loading was assessed indirectly by measuring processive DNA replication by Pol δ , which is dependent on appropriately loaded PCNA. In the first set of experiments, a control experiment was carried out in which RFC, PCNA, and Pol δ were preincubated together in buffer without ATP and then diluted 50-fold into a replication assay

mix containing DNA and increasing ATP. This assay yielded a $K_{1/2}$ value of 4.5 μ M for ATP in the overall loading reaction (Fig. 4A). In the experiment, RFC, PCNA, and Pol δ were preincubated with increasing ATP concentrations, and this preincubation mixture was diluted 50-fold into a replication reaction containing DNA, but no additional ATP. If the three ATPs prebound to the PCNA·RFC complex would suffice to bind DNA and complete the loading reaction, then an apparent $K_{1/2}$ in the low μ M range should be observed. Yet half-maximal activation was only observed when 210 μ M ATP was present in the preincubation reaction, corresponding to a final concentration of 4.2 μ M ATP after 50-fold dilution of the nucleotide into the assay. The same results were obtained when Pol δ was added to the replication assay rather than the preincubation reaction (data not shown). These results suggest either that complex formation between RFC and DNA has to precede PCNA entry into the complex or that after DNA binding, a fourth ATP molecule has to be bound to the DNA·PCNA·RFC·ATP₃ complex to complete loading.

In the second set of experiments the opposite question was asked, *i.e.* whether a DNA·RFC·ATP₃ complex could complete loading by addition of PCNA without additional ATP. Again, in the control experiment when the preincubation contained no ATP, half-maximal activity required 3.5 μ M ATP in the assay. When ATP was included in the preincubation but not in the assay, half-maximal activity was observed at 42 μ M of ATP, corresponding to a final concentration of 4.2 μ M ATP in the replication assay after the 10-fold dilution of the preincubation reaction (Fig. 4B). These results indicate that regardless of the order of assembly of the loading complex, a fourth and final ATP molecule needs to bind after all other components have assembled, and under the present reaction conditions the $K_{1/2}$ for that fourth ATP is \sim 4 μ M.

ATP γ S did not substitute for ATP in this final binding event. When a preincubation reaction containing a PCNA·RFC·ATP₃ complex was added to DNA in the presence of 100 μ M ATP γ S and Pol δ , DNA replication was inhibited by >95% in comparison with 100 μ M ATP in the assay (Table III, entries 6–8). Once loading was completed in the presence of ATP, the addition of ATP γ S no longer inhibited DNA replication (11).

Similarly, ATP γ S also did not efficiently substitute for ATP in the initial binding event. RFC and PCNA were preincubated with ATP γ S, and this mixture was diluted 50-fold into a replication assay containing DNA and Pol δ . Incubation was continued for 30 s, and DNA synthesis was quantitated (Table III, entries 1–5). DNA synthesis with ATP γ S in the preincubation was substantially less than that obtained with ATP in the preincubation or no nucleotide. We interpret this result to mean that upon dilution of PCNA·RFC·ATP γ S₃ into the assay, loading could only proceed after dissociation of bound ATP γ S

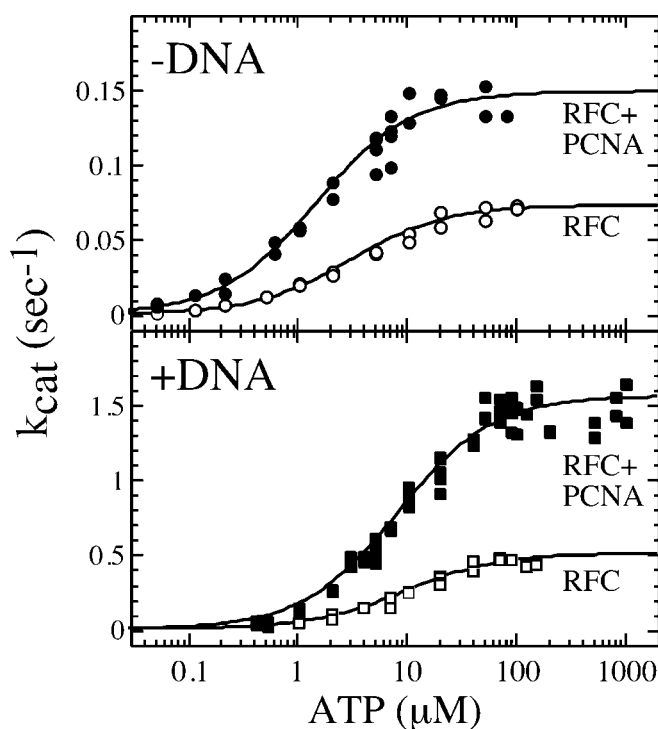


FIG. 3. **The RFC ATPase is stimulated by both PCNA and DNA.** ATPase assays were carried out as described under "Experimental Procedures." A, without DNA with or without PCNA. B, with multiple primed mp18 DNA with or without PCNA. The data were fitted to a one-site Michaelis-Menten model. The K_m values are given in Table II.

and rebinding of ATP to RFC. Therefore, these data show that at least one of the three ATP molecules initially bound must be hydrolyzed and that the fourth ATP bound must be hydrolyzed as well. As will be discussed below (see "Discussion"), these data together with the binding data in Fig. 1 provide evidence for the required binding and hydrolysis of at least two ATP molecules but do not exclude the possibility that binding and/or hydrolysis of one or two ATPs is gratuitous.

Interaction of RFC with PCNA Precedes That with DNA—The sequence of binding events leading up to a complex with PCNA encircling DNA has not been established. Because RFC can form ATP-dependent complexes with either DNA or PCNA, it is possible that assembly occurs either via branch 1 \rightarrow 3 or via branch 2 \rightarrow 4 (Scheme I) or that assembly is random. However, in the previous paper (6) we have shown that a complex between RFC and DNA is only observed with ATP γ S and not with ATP. We have interpreted these observations to mean that a transient complex was formed in the presence of ATP but that hydrolysis of the bound ATP caused complex dissociation. This putative abortive binding cycle of RFC with DNA suggests that branch 2 \rightarrow 4 may be unproductive. To determine the preferred reaction order, we carried out a series of SPR experiments on a DNA chip.

Previous experiments have shown that injection of PCNA together with RFC and ATP γ S over a primer-template DNA chip produces a much larger signal than injection of RFC and ATP γ S without PCNA, suggesting loading of PCNA onto the DNA attached to the chip (Fig. 5, *inset*) (6). Mechanistically these results can be interpreted in two different ways. Loading could proceed by binding of a preformed PCNA-RFC-ATP γ S complex to the DNA chip, but the data are also consistent with a model in which RFC-ATP γ S binds to the DNA chip first, followed by binding of PCNA. If the latter model were correct, one would predict that very high concentrations of PCNA during the injection would be inhibiting because no free RFC

would be available to bind the DNA chip first. However, when 10 nM RFC was injected in the presence of increasing PCNA, a maximum signal was obtained with 10 nM PCNA, and this signal did not change when the PCNA concentration was raised to 100 nM (data not shown).

Although the latter experiment appears to eliminate the possibility of an ordered mechanism via branch 2 \rightarrow 4 (Scheme I), it does not exclude a random mechanism, *i.e.* either branch of Scheme I can lead to a productive loading complex. To address this possibility, the series of experiments in Fig. 5 was carried out. RFC was injected onto a primed DNA chip in the presence of ATP γ S. As soon as the injection of RFC stopped, either the standard buffer B (Fig. 5, *None*) or buffer B containing PCNA and ATP γ S was flowed across the chip. If the RFC bound to the DNA chip is competent to bind PCNA, the response curve can be calculated from the association constant of PCNA to the RFC-DNA chip, the dissociation constant of the RFC-DNA chip complex, and the dissociation constant of the PCNA-RFC-DNA chip complex (see legend to Fig. 5). The theoretical curve calculated from these constants shows a moderate increase followed by a slow decay of the signal. The actual experimental curve (PCNA with ATP γ S) showed a much smaller response than expected for functional loading via branch 2 \rightarrow 4 (Scheme I); however, it was also substantially above the control. A DNA competition experiment was carried out to determine the nature of this increased signal. When primed DNA was included as a trap during the second stage of the experiment, the signal was reduced almost back to that of the negative control. This result suggests that the observed increase in signal without DNA trap was a SPR artifact involving rebinding; after dissociation of RFC from the DNA chip, it formed a complex with ATP γ S with or without PCNA in solution followed by rebinding of the complex to the DNA chip, resulting in the increased signal. In addition, if RFC when bound to the DNA chip were able to bind PCNA from solution, the inclusion of the trap should not have affected the observed signal in the second stage of the binding experiment. Therefore, these experiments suggest that no productive complex can form through a RFC-DNA complex and consequently indicate that loading of PCNA has to proceed via a RFC-PCNA complex (branch 1 \rightarrow 3 in Scheme I).

DISCUSSION

Fig. 6 summarizes our current understanding of the eukaryotic clamp loading system, and Scheme II compares the role of ATP in RFC function with that of the bacterial and phage clamp loaders. Our SPR studies provide evidence that clamp loading is an ordered process in which formation of a RFC-PCNA complex precedes binding to the DNA substrate. This is the same order as observed in the T4 and *E. coli* systems. However, with regard to ATP usage, there are striking differences between RFC and the other systems. The initial binding of two ATP molecules to the *E. coli* γ -complex suffices to drive all subsequent steps in clamp loading, *i.e.* formation of a stable complex with the β -dimer, complex formation with DNA, loading of the β clamp, and, finally, dissociation of the γ -complex with hydrolysis of the initially bound ATP (Scheme II) (3, 4). Although the details of the pathway are slightly different, a similar situation exists for the T4 44/62 clamp loader in that the ATP molecules required for clamp loading are bound in the initial step (1, 2).

In an interesting and striking departure from the prokaryotic scheme, each step on the reaction pathway by the eukaryotic clamp loader is propagated by binding of an additional ATP molecule (Scheme II). Thus, although two ATPs can initially bind to RFC, the remaining two ATP-binding sites either are buried or have an extremely low affinity for ATP. Binding of

TABLE II
 K_D and K_M values (in μM) for ATP and ATP γS in RFC-PCNA and RFC-DNA interactions

Components	Filter binding		SPR		K_m ATPase
	ATP γS		ATP	ATP γS	ATP
RFC	0.25				3
RFC + PCNA	0.23		0.5	0.5	1.5
RFC + DNA	0.41 or $2 \times 0.25 + 1 \times 2$		–	8	9.7
RFC + PCNA + DNA	0.41 or $3 \times 0.23 + 1 \times 2$		–	1.5	8.4

FIG. 4. **Binding of a fourth ATP is required after RFC binds PCNA and DNA.** A, complex of RFC, PCNA, and ATP added to DNA. B, complex of RFC, DNA, and ATP added to PCNA. A flow diagram of the experiment is given above each panel.

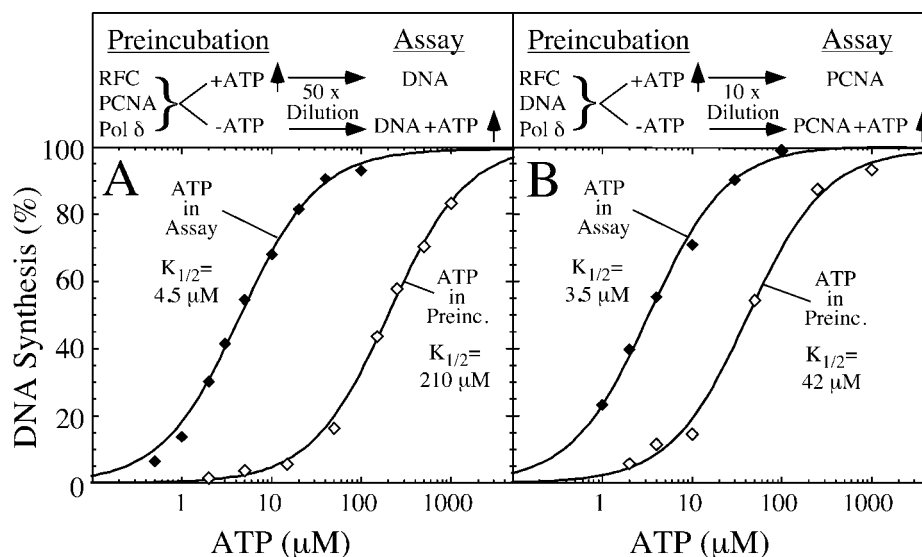


TABLE III
Requirement for hydrolysis by multiple ATPs

Preincubation of RFC with PCNA with or without the indicated nucleotide was followed by a 50-fold dilution into the replication assay containing DNA, Pol δ , and the indicated nucleotide for the indicated time. DNA synthesis (pmol) was measured. The experiment in entries 1–5 was carried out three times and has an error of 10%. The experiment in entries 6–8 was performed twice with an error of 20%. See “Experimental Procedures” for details.

Entry	Preincubation	Assay	s	pmol
1	No nucleotide	No nucleotide	30	1
2	No nucleotide	100 μM ATP	30	64
3	10 μM ATP	100 μM ATP	30	68
4	10 μM ATP	No nucleotide	30	3
5	10 μM ATP γS	100 μM ATP	30	38
6	10 μM ATP	No nucleotide	60	5
7	10 μM ATP	100 μM ATP	60	134
8	10 μM ATP	100 μM ATP γS	60	5

PCNA to RFC $\cdot\text{ATP}_2$ induces a conformational change that makes one additional ATP-binding site available. Upon binding of DNA to the resulting PCNA $\cdot\text{RFC}\cdot\text{ATP}_3$ complex, another conformational change in RFC makes one final ATP-binding site available. This fourth ATP needs to be bound for the loading process to proceed to completion, and ATP γS will not substitute (Fig. 4).

The realization that all steps in the clamp loading pathway are fueled by sequential binding of ATP molecules does not address the function of these ATP binding events. Opening of the PCNA trimer is a necessary prelude to its loading around primer-template DNA. It is likely that the PCNA $\cdot\text{RFC}\cdot\text{ATP}$ complex that we detect as a salt-stable entity is already in the ring-opened form (5). However, whether the initial complex formed between PCNA and RFC $\cdot\text{ATP}_2$ undergoes a conformational change, which allows binding of a third ATP, and it is the binding of this third ATP that drives ring opening or whether the initial PCNA $\cdot\text{RFC}\cdot\text{ATP}_2$ complex itself undergoes ring opening with conformational changes that then allow binding

of the third ATP cannot be determined by these studies (Fig. 6). Because the K_D value for the ATP required for PCNA $\cdot\text{RFC}$ complex formation (Fig. 2A) and the K_D values for the three ATPs involved in this process are all similar in magnitude, the individual ATP binding sites cannot be separated by the techniques used in this study (Table II). On the other hand, the finding that the formation of a stable complex between RFC and DNA requires a relatively high concentration of ATP γS ($K_D = 8 \mu\text{M}$; Fig. 2B) may indicate the involvement of a third ATP molecule in this process. Upon contacting of the DNA by RFC $\cdot\text{ATP}\gamma\text{S}_2$, a transient complex may be formed which allows binding of a third ATP γS in the lower affinity binding site, which in turn stabilizes the RFC $\cdot\text{DNA}$ complex. It would then be the PCNA $\cdot\text{RFC}\cdot\text{ATP}\gamma\text{S}_3$ complex, which is detected by SPR. Although this complex is not a relevant intermediate in the clamp loading pathway, a similar progression may hold for the interaction between PCNA $\cdot\text{RFC}\cdot\text{ATP}\gamma\text{S}_3$ with DNA. The K_D value of 1.5 μM for formation of the stable complex detected by SPR may well reflect binding of the fourth and last ATP γS molecule (Table II).

It should be stressed that our data solely indicate that binding of four ATP molecules is observed during the loading of PCNA. This observation does not necessarily imply that all four ATPs are required for clamp loading. Firm data for required binding and hydrolysis of ATP only exist for the fourth and last molecule of ATP to enter the complex (Fig. 4). The additional experiments in Table III indicate that binding and hydrolysis of at least one additional ATP molecule is required for PCNA loading. Therefore, gratuitous binding of one or two ATPs to RFC may actually occur, and their function may be important for other cellular processes unrelated to PCNA loading. Consequently, a minimal action scheme involving two required ATP molecules cannot be excluded at the moment. Presumably, this would be the one ATP molecule that enters the complex upon binding of PCNA, followed by the one that enters the complex upon binding of DNA.

The ATPase activity of RFC gives some additional insights in

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