The Influence of the Cdc27 Subunit on the Properties of the *Schizosaccharomyces pombe* DNA Polymerase δ*

Received for publication, March 25, 2002, and in revised form, July 16, 2002
Published, JBC Papers in Press, July 17, 2002, DOI 10.1074/jbc.M202897200

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**Schizosaccharomyces pombe** DNA polymerase (*pol* δ) contains four subunits, pol 3, Cdc1, Cdc27, and Cdm1. In this report, we examined the role of Cdc27 on the structure and activity of *pol* δ. We show that the four-subunit complex is monomeric in structure, in contrast to the previous report that it was a dimer (Zuo, S., Bermudez, V., Zhang, G., Kelman, Z., and Hurwitz, J. (2000) *J. Biol. Chem.* 275, 5153–5162). This discrepancy between the earlier and recent observations was traced to the marked asymmetric shape of Cdc27. Cdc27 contains two critical domains that govern its role in activating *pol* δ. The N-terminal region (amino acids (aa) 1–160) binds to Cdc1 and its extreme C-terminal end (aa 362–369) interacts with proliferating cell nuclear antigen (PCNA). Mutants of *S. pombe* *pol* δ, containing truncated Cdc27 derivatives deficient in binding to PCNA, supported DNA replication less processively than the wild-type complex. Fusion of a minimal PCNA-binding motif (aa 352–372) to C-terminally truncated Cdc27 derivatives restored processive DNA synthesis in *vitro*. In *vivo*, the introduction of these fused Cdc27 derivatives into *cdc27Δ* cells conferred viability. These data support the model in which Cdc27 plays an essential role in DNA replication by recruiting PCNA to the *pol* δ holoenzyme.

At least three essential DNA polymerases, α, ε, and δ, play critical roles in DNA replication (1–3). All are multisubunit enzymes containing a large catalytic subunit and two to three additional smaller subunits. The precise function of these smaller subunits is of considerable importance because they influence the activity of these enzymes and contribute to the interactions of the polymerase with other replication components such as SV40 T antigen, Dna2 which influences Okazaki fragment processing, and components of the eukaryotic pre-replication complex (4–6).

SV40 viral DNA replication, carried out with highly purified proteins, has defined the role of DNA polymerase α (*pol* α) in the initiation of both leading and lagging strands. The polo-association of primase subunits synthesizes small oligoribonucleotides (10–15 nucleotides long) that are elongated for a short distance (~35 nucleotides) by the large catalytic subunit of *pol* α (1, 7, 8). These short RNA-DNA segments are then elongated by *pol* δ or ε via a series of reactions in which the *pol* α-primase complex is displaced by RFC and PCNA (9, 10). The precise role of *pol* δ and *pol* ε in the synthesis of leading and lagging strands, however, remains unclear. In the SV40 replication pathway, *pol* δ plays a more important role than *pol* ε in supporting SV40 DNA replication (1, 11, 12), whereas various chromosomal DNA replication models, based on biochemical and genetic studies, suggest that lagging and leading strand DNA synthesis may be catalyzed by either *pol* ε and *pol* δ (summarized in Refs. 1 and 3). In budding yeast, deletion of the catalytic activity of *pol* ε is not lethal suggesting that under these conditions *pol* δ is capable of replicating both strands (13, 14).

*pol* δ has been isolated and characterized from *Schizosaccharomyces pombe, Saccharomyces cerevisiae,* and mammals (summarized in Ref. 15). *S. pombe* *pol* δ is a heterotrimer composed of four subunits (4S) of 125 (pol 3), 55 (Cdc1), 45 (Cdc27), and 22 kDa (Cdm1) (16–18). The catalytic subunit, *pol* 3, interacts directly with Cdc1 which in turn binds Cdc27 (16, 18). The mechanism by which Cdm1 is included in this complex is presently unknown. However, a three-subunit complex (3S) containing *pol* 3, Cdc1, and Cdm1 (devoid of Cdc27) has been isolated (17). Direct interactions between Cdm1 and *pol* 3, Cdc1 or Cdc27 have not been detected, although genetic interactions have been reported (16, 18). *pol* 3, Cdc1, and Cdc27 are essential for S-phase completion in *S. pombe,* whereas Cdm1 is non-essential in *vivo* (19). The 4S complex of *S. pombe* *pol* δ is required for maximal processivity during *in vitro* DNA replication, whereas the 3S complex, *pol* 3-Cdc1-Cdm1, is considerably less processive than the 4S complex (17).

In *S. cerevisiae,* purified *pol* δ is a heterotrimer, consisting of 3S, *Pol3p, Pol31p,* and *Pol32p* (20) which are homologues of the *S. pombe* subunits, *pol* 3, Cdc1, and Cdc27, respectively. As in *S. pombe,* *Pol3p* and *Pol31p* are essential, whereas *Pol32p* is not, although *pol32Δ* cells grow poorly and display DNA replication defects (20). In addition, the heterotrimeric *S. cerevisiae* *pol* δ is highly processive, whereas the heterodimer, *Pol3p-Pol31p,* is less processive, in keeping with observations made in *S. pombe.* Whereas in early reports, mammalian *pol* δ was shown to contain two subunits, the p125 catalytic subunit and the 55-kDa Cdc1 homologue (21), later studies (22–24) revealed that mammalian *pol* δ contained four subunits. These included, in addition to p125 and p55, two additional subunits, p66 and p12, with homology to Cdc27 and Cdm1, respectively. Recently, reconstitution of the cloned human *pol* δ 4S complex as well as a 3S complex, p125-p50-p66, was reported (25). The addition of the p12 subunit (homologous to Cdm1) to the 3S complex stimulated the activity of the complex. To date, no homologue of Cdm1 or p12 has been identified in *S. cerevisiae.*

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* This work was supported in part by National Institutes of Health Grant GM 58559 (to J. H.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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1 The abbreviations used are: *pol*, DNA polymerase, RFC, replication factor C; PCNA, proliferating cell nuclear antigen; SSB, single strand DNA-binding protein; aa, amino acid; DTT, dithiothreitol; 4S complex, *S. pombe* DNA polymerase δ containing the subunits *pol* 3, Cdc1, Cdc27, and Cdm1; 3S complex, *S. pombe* DNA polymerase δ containing *pol* 3, Cdc1, and Cdm1 subunits; BSA, bovine serum albumin; Bicine, N,N-bis(2-hydroxyethyl)glycine; GST, glutathione S-transferase.
Three additional proteins are required to maximize the activities associated with pol δ and pol ε. These include replication protein A (to at least remove secondary structure impediments along the primer template DNA and prevent spurious binding of polymerase), RFC (the clamp loader), and PCNA (the sliding clamp). RFC, which interacts with PCNA, loads the clamp onto the 3′-end of a primer template in an ATP-dependent manner. PCNA, a ring-shaped homotrimer, encircles the DNA and acts to tether the pol δ complex to the primed DNA template thus permitting processive DNA synthesis (1). The subunits Cdc27, Pol32p, and mammalian p66 all contain a short PCNA-binding sequence at their C termini, resembling the eight-residue PCNA-binding motif found in p21 (Waf1/Cip1) (18, 20, 22). In addition, the large catalytic subunit of S. cerevisiae, S. pombe, and mammals contains a weak PCNA-binding motif near their N terminus (26, 27). However, pol δ preparations devoid of the strong PCNA-binding subunit (Cdc27, Pol32p, and p66) are considerably less processive (17, 20, 22, 23).

Studies in S. pombe, carried out both in vitro and in vivo, have shown that Cdc27 interacts directly with PCNA (18), and the PCNA-binding motif present at the C terminus of Cdc27 is required for this reaction. Furthermore, this C-terminal motif is essential in vivo suggesting that in its absence the processivity of the 4S S. pombe pol δ complex is probably compromised. In this report, we have examined this problem in vitro using cloned 4S complexes containing C-terminal truncated Cdc27 derivatives. We report that in the absence of the C-terminal PCNA-binding region, pol δ is less processive than the wild-type 4S complex. The fusion of the PCNA-binding motif to a truncated Cdc27 subunit, possessing the Cdc1-bind-}

**MATERIALS AND METHODS**

**Reagents and Enzymes—**Poly(dA)$_{300}$ and oligo(dT)$_{12-18}$ were obtained from Life Sciences (St. Petersburg, FL) and Amersham Biosciences, respectively. Labeled and unlabeled dNTPs were from American Biosciences, Inc. (St. Petersburg, FL) and Amersham Biosciences. S. pombe RFC was purified from S. pombe cells, and recombinant S. pombe PCNA was isolated from Escherichia coli as described previously (17). E. coli SSS was from Amersham Biosciences or a generous gift of Dr. M. O’Donnell (Rockefeller University). Polyclonal antibodies against p125, Cdc1, Cdc27, and Cdm1 were prepared as described previously (17, 18).

**Construction and Purification of Cdc27 Mutants—**Recombinant baculoviruses expressing p125, Cdc1, Cdm1, and full-length Cdc27 were generated using the Bac-to-Bac Baculovirus Expression System (Invitrogen) as described previously (17). Table 1 summarizes the various Cdc27 derivatives described here (see Fig. 1A). These were generated by FCR cloning using pFastBacHb-Cdc27 as template. For N-terminal truncations, a forward primer containing BamHI site S′ to the ATG codon that was in-frame to the region that was amplified, and a pFastBacHb reverse primer was used in the PCR amplification. The PCR products were cloned into the BamHI site of pFastBacHb. For the C-terminus truncations, the pFastBacHb forward primer was used as 5′ primer, and the reverse primer contained the BstXI site introduced in-frame to the region that was amplified. The PCR products were cloned into the BamHI and XbaI site of pFastBacHb. The Cdc27 construct that resulted in the D6M deletion mutant (containing amino acids 1–241 fused to amino acids 352–372) was generated by PCR using a mutagenic primer with the sequence, 5′-GATCT-GAAATTATAAGAAAGAATAGCTG-3′. The D7M deletion mutant (aa 1–351) was generated by using a mutagenic primer with the sequence, 5′-GAAAAAGCCTTTCAAGAAGAATGCTG-3′. Recombinant baculoviruses were produced using these pFastBacHb-Cdc27 derivatives according to manufacturer’s instructions (Bac-to-Bac Baculovirus Expression Systems, Invitrogen).

**Preparation of Recombinant pol δ Complexes—**Monolayer high five insect cells were grown at 27 °C to near 80% confluence in Grace’s medium supplemented with 10% fetal bovine serum. High five cells (5 × 10$^8$ cells/ml) were infected with recombinant baculoviruses encoding a single pol δ subunit or infected with multiple viruses to produce various pol δ complexes, as described (17). Infected cells were incubated at 27 °C for 64–72 h and then centrifuged at 2,500 rpm (1,800 × g) for 15 min at 4 °C as described previously (17). Various subunits and complexes of S. pombe pol δ were purified from infected cells as described previously (17).

**Preparation of the Structure of Various S. pombe pol δ Complexes and Cdc27 Derivatives—**The Stokes radii of Cdc27 and pol δ were determined from their elution profile from a Superdex 200 PC 3.2/30 column equilibrated with 25 mM Tris-HCl, 0.75% glycerol, 0.2 mM NaCl, and 1 mM DTT. The column was developed at a flow rate of 0.5 ml/min and fractions (50 μl) were collected. The Stokes radius of each protein was calculated using the following equation:

\[
R_s = \frac{N_A}{} \frac{v_p}{} \frac{p}{} \frac{1}{6} \frac{M}{R} \frac{a}{2} \frac{9}{4} \frac{1}{a}
\]

where $N_A$ = Avogadro’s number, $a = $ Stokes radius, $s = $ sedimentation coefficient, $v = $ partial specific volume, and $p = $ density of the medium. The frictional ratio ($f/f_0$), which estimates the deviation of the protein from a globular structure, was determined by the equation $f/f_0 = a/(3s/M4πη)^{1/2}$.
RESULTS

Cdc27 Is a Highly Elongated Protein—The different Cdc27 derivatives used in this study are shown in Fig. 1A. The proteins designated D6M and D7M in Fig. 1A, containing aa 1–241 and 1–160, respectively, were fused in-frame to the C-terminal 21 aa of Cdc27 (352–372). Each protein was expressed in the baculovirus insect cell system and purified. All preparations yielded predominantly a single band after SDS-PAGE analysis (some of which are shown in Fig. 1B), with the exception of the Cdc27-D6M derivative, which yielded three protein bands (Fig. 1B). These three protein bands were immunoreactive to polyclonal antibodies specific for Cdc27, and their migration was not altered by λ phosphatase treatment (data not presented).

We noted previously that Cdc27 (a 45-kDa protein, based on its aa content) migrates aberrantly during SDS-PAGE analysis (like a 54-kDa protein) (17), and a number of the Cdc27 derivatives described in Fig. 1A also migrated slower than predicted. This was true of Cdc27-D1, Cdc27-D2, and Cdc27-DF6. The migration of the truncated derivative D3, as well as further C-terminal truncated proteins, was as expected. These findings suggest that the amino acid sequence between 281 and 372 may contribute to the aberrant mobility of Cdc27.

The quaternary structure of the 4S and 3S S. pombe pol δ complexes as well as the different Cdc27 derivatives were examined. Their Stokes radii (Fig. 1C) and sedimentation values (summarized in Fig. 1D) were determined by their elution properties from a Superdex 200 PC 3.2/30 column and sedimentation in glycerol gradients, respectively. The gel filtration chromatography and glycerol gradient sedimentation analyses were carried out at protein concentrations ranging from 0.5 to 12 μM. No significant concentration-dependent changes were observed in the Stokes radii and sedimentation values. Based on the Monte-Siegal equation, which includes both the sedimentation value and the Stokes radius, the 4S complex had an apparent molecular mass of 210 kDa, which agreed fairly well with the predicted molecular mass of 240 kDa, based on the amino acid content of all four subunits present in stoichiometric amounts (17). The apparent molecular weight of the 3S complex, 161 kDa, was also close to the predicted molecular weight of this complex (195 kDa). The 4S and 3S complexes sedimented in glycerol gradients with the same sedimentation value but differed significantly in their Stokes radii (56.8 and 46.8 Å, respectively). This difference led to the earlier conclusion that the 4S and 3S complexes were dimeric and monomeric, respectively (17).

Previous gel filtration studies with the individual soluble subunits of S. pombe pol δ, Cdm1, Cdc1, and Cdc27 (the pol δ subunit alone was insoluble) indicated that Cdm1 and Cdc1 were monomeric and Cdc27 was tetrameric in structure (17). In light of the above finding, the hydrodynamic properties of Cdc27 were re-investigated. As shown in Fig. 1D, based on both the Stokes radius and sedimentation value, the apparent molecular mass of Cdc27 was 73 kDa, approximately halfway between that predicted of a monomer (45 kDa) and a dimer (91 kDa). The calculated frictional ratio of Cdc27 was 1.85, suggesting a highly elongated protein.

The D1-Cdc27 derivative (aa 1–362) exhibited hydrodynamic properties similar to full-length Cdc27. However, upon further truncation the Stokes radii of the Cdc27 derivatives decreased somewhat, and their properties were more in accord with a monomeric structure (Fig. 1D). All Cdc27 derivatives that interacted with Cdc1 yielded a monomeric heterodimer with little discrepancy between their predicted and apparent molecular weights (Fig. 1D and data not shown). These results indicate that complexes containing Cdc27 and its derivative are highly elongated, a property contributing to their aberrant elution from sizing columns. In keeping with this notion, quaternary structure of the 4S-D6M complex was the same as the 4S complex with full-length Cdc27 (Fig. 1D).

The C-terminal 20 aa of Cdc27 Are Sufficient for PCNA Binding—The interaction of Cdc27 and its derivatives with Cdc1 and PCNA were examined. Cdc1 interacted with all Cdc27 derivatives containing the N-terminal aa 1–160 but not with mutants lacking this region (Fig. 2A), in keeping with previous observations (18). As shown in Fig. 2B, whereas the D6-Cdc27 derivative did not bind PCNA, D6M, a fusion protein between D6 and the 20 aa regained the ability to interact with PCNA.

We also determined the region in Cdc27 required for its interaction with pol δ by co-infecting insect cells with baculoviruses expressing the p125, Cdc1, and Cdm1 subunits and a virus expressing either full-length Hisα-Cdc27, Hisα-FLAG2-D7-Cdc27 (aa 1–160) containing only the Cdc1-binding domain, or Hisα-DF6-Cdc27 (aa 160–372) that included the PCNA-binding motif. Nickel-agarose pull-down experiments demonstrated that both Cdc27 and the D7-Cdc27 formed 4S complexes, whereas DF6 did not interact with the other subunits (data not presented). To determine whether DF6 (C-terminal half of Cdc27) could be included in the pol δ complex in the presence of the N-terminal half of Cdc27, Hisα-FLAG2-D7 and Hisα-DF6 were coexpressed with the three other subunits of pol δ in insect cells, and extracts were subjected to consecutive nickel-agarose and FLAG-agarose pull-downs. DF6 was not included in the pol δ complex even in the presence of the D7-Cdc27 derivative (data not presented). These findings suggest that the interaction of Cdc27 with the pol δ complex is solely through its interaction with Cdc1 and that the C-terminal region of Cdc27 does not interact stably with the other pol δ subunits.

DF1-Cdc27 (aa 41–372) was shown to interact with Cdc1 by co-immunoprecipitation experiments using antibodies specific to Cdc27 or to Cdc1. However, attempts to isolate a 4S complex containing this N-terminal truncated Cdc27 derivative were unsuccessful (data not presented).

Influence of Cdc27 Derivatives on Activities Associated with 3S and 4S Complexes—The influence of Cdc27 and the truncated Cdc27 derivatives on the activation of DNA synthesis by the 3S complex was examined (Fig. 3A). The 4S complex supported extension of singly primed M13 DNA to full-length material (7 kb), whereas, under the conditions used, the 3S pol δ complex did not (compare lanes 1 and 2). The addition of full-length Cdc27 to reactions containing the 3S complex increased the size of DNA products to full-length in keeping with previous observations (17) (Fig. 3, compare lanes 3 and 5). The addition of Cdc27 to reactions containing the 4S complex did not affect the size of products or increase the level of nucleotide incorporation (lane 4). Truncated Cdc27 derivatives, D1, D3, and D6 (as well as D7, data not shown), increased the size of products formed with the 3S complex, in the range of 1 kb, but not to full-length (lanes 9, 11, and 13). DF6-Cdc27, which lacks the Cdc1 interacting region (aa 1–160), did not affect the length of products formed by the 3S complex. These findings suggest that the PCNA interacting region present at the C terminus of Cdc27, which is absent in D1, D3, D6, and D7, contributes to the processivity of the wild-type 4S complex.

Two additional assays were used to examine the effects of Cdc27 and its truncated derivatives. These included the...
FIG. 1. A, schematic of the Cdc27 truncations. The Cdc27 subunit (designated FL) includes the Cdc1-binding domain contained within aa 1–160 and the PCNA motif, QKSIMSF, present at the C-terminal end of Cdc27 (aa 362–369). The Cdc27 derivatives indicated were truncated to the amino acid position numbered. In the case of the D6M and D7M derivatives, the C-terminal region of Cdc27 was linked to the C terminus of the D6 and D7 proteins as indicated. B, SDS-PAGE analysis of purified His-Cdc27 derivatives isolated from baculovirus-infected cells. The following subunits were subjected to 10% SDS-PAGE followed by Coomassie staining: 1st lane, full-length Cdc27 (0.62 µg); 2nd lane, D1-Cdc27 (1.34 µg); 3rd lane, D3-Cdc27 (1.36 µg); 4th lane, D6-Cdc27 (1.46 µg); 5th lane, D6M-Cdc27 (1.5 µg); and 6th lane, DF6-Cdc27 (0.5 µg). C, determination of Stokes radii of Cdc27, Cdc27 derivatives, and various S. pombe pol δ complexes. The Stokes radii of the indicated proteins and complexes were determined by plotting the \((−\log K_w)^{1/2}\) versus Stokes radius (Å). The standard molecular weight markers used and their Stokes radii are as follows:
thyroglobulin (8 Å), ferritin (61 Å), aldolase (48.5 Å), BSA (35.5 Å), and ovalbumin (30.5 Å).

The closed diamonds represent standard protein markers, and open circles indicate the Cdc27p and pol δ complexes. The table includes a summary of Stokes radii, s values, apparent molecular weights, and predicted molecular weights of various pol δ complexes, Cdc27p, and derivatives of Cdc27p. The predicted molecular weight (MW) values were derived from the known peptide sequences, and the apparent molecular weights were calculated using the Monty-Siegel equation as were the frictional coefficient ratios (f/f₀). These equations were described under “Materials and Methods.” The structures of the proteins and complexes were deduced from the apparent molecular weight and the predicted molecular weight. The asterisk adjacent to Cdc1-Cdc27 indicates that this Stokes radius determination was carried out using a Superose 12 column.

S. pombe pol δ PCNA-dependent elongation of poly(dA)₄₀₀₀-oligo(dT)₁₂₋₁₆ with or without RFC. In the latter case, the DNA self-threading property of PCNA (30) was exploited under conditions in which SSB was omitted (its presence inhibits the reaction), and the pH of the reaction was decreased from 7.5 to 6.8 (which increased poly(dT) synthesis 10-fold). In the RFC-independent reaction, poly(dT) synthesis with the 4S complex was unaffected by the addition of Cdc27 or the truncated Cdc27 derivatives (Table I). Similar to results observed in the M13 singly primed elongation reaction, Cdc27 addition markedly stimulated the activity of the 3S complex, whereas the D1, D3, D6, and D7 derivatives of Cdc27 marginally increased the activity of the 3S complex. Slight but reproducible inhibition of the activity of the 3S complex was observed with DF6. Identical results were observed in reactions carried out in the absence of RFC. In the RFC-independent assay (Table I), PCNA omission reduced incorporation with either the 4S or 3S complex to barely detectable levels.

These findings indicate that Cdc27 derivatives that bind Cdc1 but lack the terminal (aa 352–372) PCNA-interaction domain, as well as additional regions at the carboxyl end, interact with the 3S complex and partially increase its activity. The activation is less efficient than that observed with full-length Cdc27 and may reflect a less stable association between PCNA and Cdc27 derivatives that bind PCNA. These effects appear to be governed solely by the PCNA-dependent reaction, poly(dT) synthesis with the 4S complex was unaffected by DNA substrates containing secondary structures because these effects were observed with poly(dA)-oligo(dT) (lacking secondary structure) and singly primed M13 DNA (containing secondary structure). These effects appear to be governed solely by the interactions between PCNA and pol δ and do not involve RFC because identical results were obtained in the RFC-independent elongation reaction.

To determine whether non-processive DNA synthesis carried out by the 3S complex and truncated Cdc27 derivatives was...
because of inefficient interactions, 4S complexes containing C-terminal truncated Cdc27 derivatives were isolated by using the baculovirus expression system. SDS-PAGE analysis of 4S complexes with either full-length Cdc27, the truncated D6 or D6M derivative, and the 3S complex are shown in Fig. 4. The multiple protein bands observed in the D6M-Cdc27p region of the purified 4S complex were similar to the bands observed with the isolated D6M-Cdc27p (see Fig. 1B). The purity of these complexes varied between 80 and 90%, based on Coomassie staining. Complexes containing Cdc27 derivative D1, D3, or D7 were of comparable purity (data not presented). The elongation of a singly primed M13 DNA by these preparations was examined (Fig. 5). At the highest protein concentration used, 4S complexes containing the D6 or D7 subunit were as effective as wild-type pol δ. At lower enzyme levels, 4S complexes containing D6 or D7 elongated DNA chains less efficiently than the wild-type complex. Similar findings were noted with 4S complexes containing D1 or D3-Cdc27 (data not presented). In the
absence of RFC or PCNA, all 4S complexes (as well as the 3S complex) were inactive (Ref. 17 and data not presented). These findings were in complete accord with the in vitro reconstitution experiment (Fig. 3) that strongly suggested a critical role for the PCNA-binding domain in Cdc27 in DNA synthesis.

Fusion of the PCNA-binding Domain to the Interacting Region of Cdc27 Generates a Fully Functional Cdc27 Derivative Both in Vitro and in Vivo—We next examined whether a fusion of the PCNA-binding domain of Cdc27 to D6-Cdc27 (leading to D6M-Cdc27) supported efficient DNA replication. As shown in Fig. 6A, D6M-Cdc27 resembled Cdc27 in enhancing the activity of the 3S complex in the singly primed M13 assay. The 4S complex containing the D6M-Cdc27 subunit was more processive than the D6-Cdc27 4S complex (Fig. 6B, compare lanes 6 and 7 with lanes 10 and 11). Furthermore, the activity of D6M-Cdc27 4S complex, like the wild-type 4S complex, was more active at low PCNA levels than the 4S complex with the D6-Cdc27 subunit (Fig. 6B, compare lanes 4 and 12 with lane 8 and compare lanes 5 and 13 with lane 9). These observations demonstrate that D6M-Cdc27 is fully active in supporting pol δ-catalyzed DNA synthesis in vitro. Moreover, they indicate that the region spanning aa 242–351 in Cdc27 is not required for pol δ activity in vitro.

We showed previously (17) that the in vitro interactions between the 3S complex and Cdc27 did not form a stoichiometric 4S complex. However, immunoprecipitation experiments indicated that substoichiometric levels of Cdc27 associated with the 3S complex and permitted the detection of pol δ polymerase activity in immunoComplexes formed with antibodies against Cdc27. As shown in Table II, immunoprecipitated full-length Cdc27 or D6M-Cdc27 4S complexes supported DNA synthesis with equal facility.

To test whether the truncated Cdc27 proteins were functional in S. pombe, each mutant allele was cloned into the fission yeast expression vectors pREP3X and pREP41X, downstream of the nmt promoter. The resulting plasmids were then used to transform a cdc27Δ/cdc27::ura4Δ diploid strain. Transformant colonies were then sporulated, and the spores were plated onto minimal medium plates with or without thiamine, and the plates were incubated at 32 °C until colonies formed. The colonies were then analyzed to confirm their genotype.
were examined by quantitative Western blot analysis in wild-type cells expressing either alone or with 3 pmol of Cdc27p or 3.1 pmol of the D6M subunit for 30 min at 4°C, as described previously (17). Anti-Cdc27 (1:100) was added, where indicated, followed by the addition of protein A agarose beads (5 μl). After 30 min at 4°C (with frequent shaking), the beads were collected by centrifugation and washed three times with 1 ml of buffer containing 25 mM Tris-HCl, pH 7.5, 0.2 M NaCl, 1 mM EDTA, 1 mM DTT, 0.25% Nonidet P-40, and 1% BSA, twice with the identical buffer devoid of BSA, and once with the buffer lacking both NaCl and BSA. Material bound to the beads was assayed for its ability to support DNA replication in the poly(dA)-oligo(dT) assay in the presence of RFC and PCNA.

The recovery of the input pol δ activity associated with the beads was as follows: 4 subunit complex, 5%; 3 S complex + Cdc27, 2.3%; 3 S complex + D6M, 4%.

Cells expressing any of the C-terminally truncated cdc27 alleles were viable in the absence of thiamine, irrespective of whether expression was from the nmt1 or nmt41 promoter (Fig. 7A and data not shown). In the presence of thiamine, cells expressing cdc27-D7 from an attenuated nmt41 promoter were inviable (Fig. 7A, left). The remaining alleles varied in their ability to support colony formation in the presence of thiamine, with cdc27-D2, D3, and D6 being particularly poor and cdc27-D5 somewhat better (Fig. 7A). In all cases, however, cells grown on thiamine-containing plates were highly elongated, and colony formation was slow, and the colonies formed contained many dead cells (data not shown). With the exception of cdc27-DF1, none of the N-terminal truncated cdc27 alleles supported growth either in the presence or absence of thiamine; no viable cdc27Δ haploids expressing these mutants were recovered following spore germination (data not shown). It should be noted that DFI-Cdc27p (aa 41–372) retains the ability to interact with Cdc1p (data not shown). Similar to the C-terminal truncated cdc27, cells rescued by cdc27-DF1 were highly elongated.

We then examined the rescue of cdc27Δ by cdc27 derivatives that expressed the Cdc1-binding domain fused to the PCNA-binding motif. Expression of either cdc27-D7M (aa 1–160 fused to aa 352–372) or cdc27-D6M (aa 1–241 fused to aa 352–372) from either a wild-type (nmt1) or attenuated nmt promoter supported growth of cdc27Δ cells in the presence or absence of thiamine (Fig. 7B). However, the two alleles were distinguishable. Growth of cells expressing cdc27-D7M was better than cells expressing cdc27-D6M, especially when the attenuated nmt promoter (nmt41) was used, despite the fact that a greater portion of the gene is deleted in cdc27-D7M.

The levels of expression of D6, D6M, D7, and D7M proteins were examined by quantitative Western blot analysis in wild-type cells expressing the attenuated nmt41 promoter (in the absence and presence of thiamine). No significant differences were noted however, and as expected, the level of each protein expressed in the absence of thiamine was 30–40-fold higher (data not presented). By assuming similar expression in cdc27Δ cells, these findings suggest that the different biological effects of the truncated derivatives observed under repressed conditions (Fig. 7, A and B) were not due to their levels of expression.

It was reported previously (18) that expression of various C-terminal truncated Cdc27p derivatives containing aa 1–352, 1–169, or 1–159 (using pREP3x plasmids), in the presence or absence of thiamine, did not rescue cdc27Δ cells. The results described in Fig. 7 are partly in accord with these findings. Expression of the truncated derivative under repressive conditions (in the presence of thiamine) did not rescue cells deleted of cdc27. However, the results presented here indicated that overexpression of these derivatives (in the absence of thiamine) rescued cdc27Δ cells. The reasons for this discrepancy are presently unclear.

**DISCUSSION**

Structure of DNA Polymerase δ—The results presented here indicate that the baculovirus-expressed 4S S. pombe pol δ complex is monomeric. This solution structure is also true of the 4S complex purified from S. pombe cells (data not presented). We reported previously that these complexes were dimeric based on their elution profile from a sizing column. We now realize that this difference is due to the elongated shape of the 4S complex caused by the highly elongated structure of the Cdc27 subunit. We have also noted that Ddc1-Cdc27 is a monomer of a heterodimer based on gel filtration chromatography and glycerol gradient sedimentation, in contrast to our earlier report that it was a dimer of heterodimer. Consistent with our earlier report, the 3S complex is monomeric in structure. Identical observations have been made with the S. cerevisiae pol δ (28).

Extensive analyses using gel filtration chromatography, glycerol gradient sedimentation, and analytical equilibrium sedimentation suggest that S. cerevisiae pol δ is monomeric in solution. The previous report (20) of its dimeric structure, based on its gel filtration property, was due to the marked asymmetric structure of the Pol32p subunit (28). Interestingly, we have shown that human pol δ preparations containing the p66 subunit exhibit aberrant gel filtration properties on sizing columns (22–24), making it likely that the p66 subunit, the homologue of Cdc27 and Pol32p, is highly elongated as well.

Although S. pombe pol δ is a monomer in solution, its structure in S. pombe cells remains uncertain. It is possible that pol δ could dimerize at the replication fork. In support of such a notion, a truncated Cdc27 derivative (aa 1–159), which neither bound PCNA nor rescued cdc27Δ cells, complemented cdc27-P11 which contained a Cdc27 point mutation in the Cdc1-binding domain (G51Q) that blocked its interaction with Cdc1 at restrictive temperatures (18), suggesting that pol δ may be dimeric in cells (18). In view of the finding that Cdc27 and 4S S. pombe pol δ are monomeric in solution, additional factors would be required to stabilize a putative pol δ dimeric complex.

The Role of Cdc27 in the Activation of the 4S Complex—Cdc27 has two functional domains that are essential for its activity. The N terminus is required for its interaction with Cdc1, and the C terminus is important for its binding to PCNA (18). Both in vivo (18) and in vitro experiments (data not presented) showed that these two domains do not function in trans, suggesting that there is no appreciable interaction between the N- and C-terminal halves of Cdc27.

The 4S complexes containing Cdc27 derivatives lacking the PCNA-binding motif were more processive than the 3S complex devoid of Cdc27 but were less processive than the wild-type 4S complex. The quaternary structure of 4S complexes with truncated Cdc27 subunits was identical to wild-type pol δ (indicated only for 4S-D6M pol δ in Fig. 1D and observed with the other derivatives as well (data not shown)). Thus, the presence of any Cdc27 derivative leads to structural alterations of the 3S complex and an increase in processivity of the 4S complex. On the other hand, fusion of the PCNA-binding motif to C-terminally truncated derivatives of Cdc27 restored the processivity of
these preparations and increased their activity at low PCNA concentrations to that observed with the wild-type 4S complex. Both the 3S complex and the 4S complexes containing Cdc27 derivatives without the PCNA-binding domain required PCNA for activity suggesting the presence of another PCNA-binding site most likely in the large catalytic subunit. Previous studies (23, 26, 27) with the p125 subunit of human pol /H9254 indicated the presence of a short region close to the N terminus that interacted with PCNA. However, our attempts to detect direct interactions between /S. pombe /PCNA and the large subunit of the /S. pombe pol /H9254 (in the 3S complex) by coimmunoprecipitation were unsuccessful. Furthermore, stable interactions between the wild-type 4S complex and PCNA were not detected by immunoprecipitation or gel filtration experiments (data not shown). Direct interactions between the wild-type 4S complex and PCNA were not detected by immunoprecipitation or gel filtration experiments (data not shown). The latter complex was as processive in supporting DNA replication as the wild-type 4S complex. Interestingly, /cdc27Δ cells synthesize bulk DNA which, however, is defective and leads to aberrant chromosome structures. These findings, to some degree, are similar to the synthesis of DNA observed in vitro with the 3S complex, which exhibited diminished processivity compared with the wild-type 4S complex. /In vivo, decreased processivity could result in incomplete DNA products.

Overexpression of all Cdc27 derivatives devoid of the PCNA-binding motif rescued /cdc27Δ cells. One possible explanation for this unexpected result could be that high levels of any Cdc27 derivative lead to an increase in the cellular concentration of the pol δ complex capable of compensating for a defective polymerase. As shown in Fig. 5, high levels of all 4S complexes fully elongated singly primed M13 DNA. Similarly, high levels of the 3S complex were also capable of extensive elongation of such templates (17). It should be noted that in vivo, removal of thiamine can lead to a marked increase in expression of proteins under the control of the nmt41 promoter (30–100-fold) (31).

In /S. cerevisiae, DNA replication occurs in cells devoid of the large catalytic subunit of pol ε, provided the C-terminal region of this protein is expressed (13, 14). Such cells, although viable, are defective. These findings suggest that pol δ may carry out some functions normally performed by pol ε. /S. pombe cdc1Δ, as
well as cdc27Δ cells, are not viable, but synthesize bulk DNA which is defective (18). Speculatively, this synthesis may be carried out solely by pol ε and would require this enzyme to carry out functions normally executed by pol δ. Such observations suggest plasticity in the action of these replicative polymerases at the fork and that pol δ can carry out a more effective role as the only replicative polymerase than pol ε. More information concerning the role of each of these enzymes during fork progression is needed.

The extreme C-terminal location of the PCNA-binding domain of Cdc27 and Pol32p has been noted for a number of DNA polymerases, such as pol κ and pol ε (32, 33). Putative C-terminal PCNA-binding sequences are present in the archaeal pol b family of proteins (34). Parallel findings have been made for the clamp-binding sequence in T4 DNA polymerase (35) and C-terminal sequences in members of the eubacterial pol B, pol C, and Din B1 family of proteins (34). The crystal structure of the T4 phage-related RB69 DNA polymerase and the T4 clamp (35) suggests that the extended C terminus of the polymerase (with the clamp-binding site) leads to a flexible structure, providing the polymerase-clamp complex with some freedom of movement. Interactions between clamps and the C-terminal ends of polymerases have variable effects on the processivity of the polymerase-clamp complex (i.e., leading to a marked increase with replicative polymerases and little increase in processivity with error-prone polymerases). The influence of the location of the clamp-binding sequence on the processivity of the clamp-polymerase complex clearly warrants further investigation.

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