

Structure of DNA Polymerase δ from *Saccharomyces cerevisiae**

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DNA polymerase δ (Pol δ) from *Saccharomyces cerevisiae* consists of three subunits, Pol3 (125 kDa), Pol31 (55 kDa), and Pol32 (40 kDa), present at a 1:1:1 stoichiometry in purified preparations. Previously, based on gel filtration studies of Pol δ , we suggested that the enzyme may be a dimer of catalytic cores, with dimerization mediated by the Pol32 subunit (Burgers, P. M., and Gerik, K. J. (1998) *J. Biol. Chem.* 273, 19756–19762). We now report on extensive gel filtration, glycerol gradient sedimentation, and analytical equilibrium centrifugation studies of Pol δ and of several subassemblies of Pol δ . The hydrodynamic parameters of these assemblies indicate that (i) Pol32 is a rod-shaped protein with a frictional ratio $ff_0 = 2.22$; (ii) any complex containing Pol32 also has an extremely asymmetric shape; (iii) the results of these studies are independent of concentration (varied between 0.1–20 μM); (iv) all complexes are monomeric under the conditions studied (up to 20 μM). Moreover, a two-hybrid analysis of the Pol32 subunit did not detect a Pol32–Pol32 interaction *in vivo*. Therefore, we conclude that the assembly structure of Pol δ is that of a monomer.

DNA polymerase δ (Pol δ)¹ has been proposed to replicate both the leading and lagging strands of the eukaryotic replication fork. This proposal was initially based on the results of *in vitro* DNA replication studies of the viral simian virus 40 genome, which is efficiently replicated by two DNA polymerases: Pol α functioning as a DNA primase for the initiation of Okazaki fragment synthesis and Pol δ to elongate Okazaki fragments and to carry out leading strand DNA synthesis (reviewed in Refs. 1–3). Pol ϵ , the third DNA polymerase required for DNA replication, may serve its essential function unrelated to elongation because a deletion of the catalytic polymerase domain of yeast Pol ϵ is viable (4, 5). However, opposing opinions exist that assign a role for bulk DNA replication by Pol ϵ in

wild-type yeast cells. These are based in part on the lethality of point mutants in the active site of the *POL2* gene and on strand-specific mutator phenotypes of proofreading-deficient Pol ϵ versus proofreading-deficient Pol δ mutants (5–7).

Saccharomyces cerevisiae Pol δ consists of three subunits: Pol3 (125 kDa), Pol31 (55 kDa), and Pol32 (40 kDa). The *POL3* and *POL31* (*HYS2*) genes are essential, but the *POL32* gene is dispensable, although deletion mutants show defects in DNA replication, DNA repair, and mutagenesis (8–11). The enzyme from *Schizosaccharomyces pombe* consists of four subunits: Pol3, Cdc1 (the ortholog of Pol31), Cdc27 (the ortholog of Pol32), and Cdm1 (12–15). The genes for the three large subunits are essential, but a deletion of the *Cdm1*⁺ gene, a homologue of which has not been identified in *S. cerevisiae*, shows no phenotype in the absence of mutations in the *Cdc1*⁺ gene (16). Human orthologs of the third and fourth subunit have been identified (17–19).

Recently we developed overproduction strains for *S. cerevisiae* Pol δ (20). Initial gel filtration analysis of the purified three-subunit Pol δ yielded a Stokes radius consistent with that of a globular complex of 500–600 kDa, suggesting that Pol δ may be a dimer of catalytic cores with dimerization mediated by the Pol32 subunit. We now report extensive *in vitro* studies, which in contrast indicate that Pol δ when purified from yeast is a monomer with an extremely elongated shape. Additional *in vivo* studies fail to detect any interaction indicative of a dimeric Pol δ .

EXPERIMENTAL PROCEDURES

Enzymes—Pol δ , Pol δ^* were purified from the yeast overproduction strain BJ2168 (*MATa*, *ura3-52*, *trp1-289*, *leu2-3,112*, *prb1-1122*, *prc1-407*, *pep4-3*), containing plasmids pBL336 (*TRP1 GAL1-POL3*), pBL338 (*LEU2 GAL1-POL31*) plus pBL340 (*URA3 GAL10-POL32*) for Pol δ , and pBL336 plus pBL338 for Pol δ^* . Cell growth and induction was carried out as described, as was the purification of Pol δ and Pol δ^* (20).

The Pol31–32 complex was purified as a side product from the Pol δ overproduction. Pol3 was overexpressed at substantially lower levels than Pol31 and Pol32, and the latter two subunits eluted as a distinct complex from a MonoQ column at ~350 mM NaCl and from a MonoS column at ~300 mM NaCl using the same chromatographic conditions that were applied for the purification of Pol δ .

The Pol32 subunit, containing an amino-terminal tag with seven histidines, was purified from an *Escherichia coli* BL21 (DE3) containing plasmid pBL370 (11). To obtain soluble His₇-Pol32 protein, the strain was grown and induced at 17 °C, and the induction time was extended to 16 h. His₇-Pol32 was isolated from the *E. coli* cell lysate by ammonium sulfate fractionation, phosphocellulose column chromatography, and nickel-agarose chromatography per the manufacturer's instructions (Qiagen). The protein was eluted from the beads at 150 mM imidazole.

Gel Filtration Analysis—A Superose 12 gel filtration column was equilibrated at 4 °C in buffer A (40 mM Hepes pH 7.4, 10% glycerol, 1 mM EDTA, 0.005% Igepal-40, 200 mM NaCl, 5 mM dithiothreitol, 5 mM sodium bisulfite, 2 μM leupeptin, and 2 μM pepstatin A). The excluded volume ($V_0 = 8.2$ ml) and included volume ($V_i = 19.8$ ml) of the column were determined by injection of blue dextran and adenosine, respectively. After this, the elution volume (V_e) was measured for several

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¹ The abbreviations used are: Pol δ , DNA polymerase δ ; Pol δ^* , Pol δ lacking Pol32; Pol31–32, complex of the Pol31 and Pol32 subunits; PCNA, proliferating cell nuclear antigen.

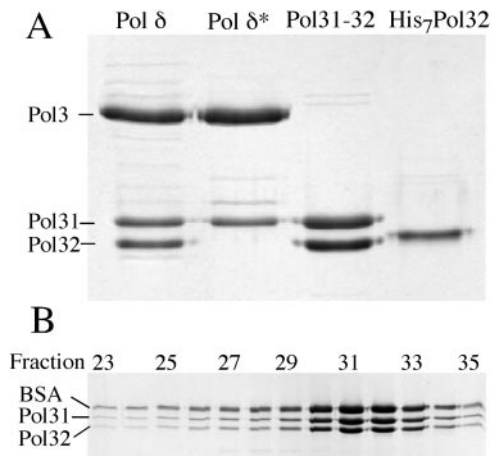


FIG. 1. *A*, complexes used in this study. Pol δ , Pol δ^* , Pol31-32, and His₇Pol32 as indicated were analyzed by 11% SDS-polyacrylamide gel electrophoresis and stained with colloidal Coomassie Blue. Note that the His₇ tag gave a slight shift in mobility compared with unmodified Pol32p. *B*, determination of the sedimentation coefficient of the Pol31-32 complex. Pol31-32 was sedimented on a 10–35% glycerol gradient as described under “Experimental Procedures.” Bovine serum albumin was included as an internal standard. Fractions were analyzed by 11% SDS-polyacrylamide gel electrophoresis and stained with colloidal Coomassie Blue.

standard proteins with a known Stokes radius (21). For each protein, K_{av} was calculated from Equation 1, and a linear relationship was obtained by plotting $(-\log K_{av})^{1/2}$ against the Stokes radius (22).

$$K_{av} = (V_e - V_o)/(V_i - V_o) \quad (\text{Eq. 1})$$

The standard proteins used were: carbonic anhydrase ($V_e = 15.2$ ml), chicken ovalbumin ($V_e = 14.2$ ml), bovine serum albumin ($V_e = 13.4$ ml), yeast alcohol dehydrogenase ($V_e = 12.7$ ml), catalase ($V_e = 12.5$ ml), apoferritin ($V_e = 11.1$ ml), *E. coli* β -galactosidase ($V_e = 11.1$ ml), and thyroglobulin ($V_e = 9.47$ ml).

Glycerol Gradient Centrifugation—5–100 μ g of the different pure protein complexes together with bovine serum albumin as an internal control, in a total volume of 100 μ l, were layered on top of a 4.5-ml 10–35% glycerol gradient in buffer A. The samples were spun for 16 h at 50,000 rpm in an SW65 rotor at 4 $^{\circ}$ C. Fractions were collected from the bottom of the tube, and aliquots were analyzed by SDS-polyacrylamide gel electrophoresis. Whole cell extracts from yeast strain BJ2168 were prepared as described, and 100 μ l of the cleared lysate were loaded onto the glycerol gradient (23). The elution positions of Pol3, Pol31, and Pol32 were determined by Western blot analysis. Standard proteins with known sedimentation coefficients were used to calibrate the gradients: carbonic anhydrase (2.8 S), chicken ovalbumin (3.6 S), bovine serum albumin (4.3 S), yeast alcohol dehydrogenase (7.4 S), catalase (11.3 S), *E. coli* β -galactosidase (15.9 S), and apoferritin (17.6 S) (21).

Equilibrium Sedimentation Analysis—Pol δ in buffer B (buffer A with 5 mM 2-mercaptoethanol instead of dithiothreitol) at three different initial concentrations, 1.8 μ M (as monomer), 0.8 μ M, and 0.23 μ M, was spun at 10 $^{\circ}$ C for 24 h at 10,000 rpm in a Beckman XL-A centrifuge until equilibrium had been reached, followed by a 24-h spin at 13,000 rpm. Pol31-32 was examined similarly at initial concentrations of 4.6 μ M, 2.1 μ M, and 0.7 μ M. Absorbance measurements were carried out at 280 nm. Molecular mass was calculated from the data using the manufacturer’s software.

Two-hybrid Analysis—The analysis was carried out as described previously (11). Plasmid pBL390-10 (lexA-Pol32-C Δ 7) carries a POL32 allele lacking the terminal seven amino acids, thereby removing the PCNA-binding domain. This plasmid will be described.²

RESULTS AND DISCUSSION

In addition to the three subunit Pol δ , we investigated the hydrodynamic parameters of three additional species that could be obtained in stable form: a complex of the catalytic subunit Pol3 with the second subunit Pol31, which we have called Pol δ^* and which is structurally analogous to tradition-

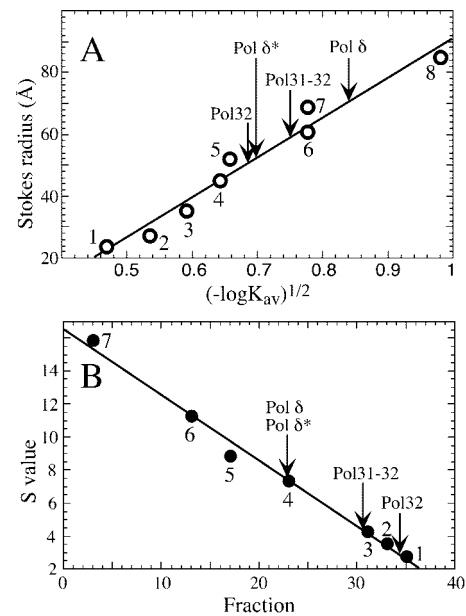


FIG. 2. **Determination of hydrodynamic parameters.** *A*, gel filtration. The calibration curve for the Superose 12 column was established with carbonic anhydrase (data point 1, 23.9 Å), chicken ovalbumin (data point 2, 27.3 Å), bovine serum albumin (data point 3, 35.5 Å), yeast alcohol dehydrogenase (data point 4, 45 Å), catalase (data point 5, 52 Å), apoferritin (data point 6, 61 Å), *E. coli* β -galactosidase (data point 7, 69 Å), and thyroglobulin (data point 8, 85 Å). The elution positions of the complexes are as indicated, and the estimated Stokes radii are given in Table I. *B*, glycerol gradient centrifugation. Gradient calibration was carried out with carbonic anhydrase (data point 1, 2.8 S), chicken ovalbumin (data point 2, 3.6 S), bovine serum albumin (data point 3, 4.3 S), yeast alcohol dehydrogenase (data point 4, 7.4 S), β -amylase (data point 5, 11.3 S), *E. coli* β -galactosidase (data point 6, 15.9 S), and apoferritin (data point 7, 17.6 S). The sedimentation positions of the complexes are as indicated, and the estimated sedimentation coefficients are given in Table I. For details, see “Experimental Procedures.”

ally purified mammalian Pol δ (24, 25); a complex of the two small subunits (Pol31-32); and the individual third subunit (Pol32). The first three complexes were obtained from overproduction in yeast, but the Pol32 subunit was overproduced in *E. coli*. Previously, this subunit could not be obtained in soluble form from *E. coli* lysates and had to be obtained by renaturation from urea-solubilized precipitates (11). However, addition of an N-terminal His₇ tag and lowering of the expression temperature in *E. coli* to 17 $^{\circ}$ C allowed us to isolate the protein in soluble form (see “Experimental Procedures”). The His₇ tag-containing protein fully complements the defects of a yeast POL32 deletion strain.² The subunits in the Pol δ , Pol δ^* , and Pol31-32 complexes are present in equimolar ratios (Fig. 1A, Ref. 20).

Gel Filtration and Glycerol Gradient Centrifugation—For spherical molecules, the molecular mass of a species can be calculated from a combination of the measured Stokes radius and sedimentation coefficient using Equation 2 (22),

$$M = 6\pi\eta N a s / (1 - \bar{v}\rho) \quad (\text{Eq. 2})$$

in which M is the molecular mass, a is the Stokes radius, s is the sedimentation coefficient, \bar{v} is the partial specific volume, η is the viscosity of medium, ρ is the density of medium, and N is Avogadro’s number.

Although this formula does not hold for non-spherical species, in practice a good approximation of the molecular mass can often be obtained because the Stokes radius of an elongated molecule is larger than that of a spherical molecule with the same molecular mass, whereas the sedimentation coefficient of the elongated species is smaller, and the two opposite devia-

² E. Johansson, A. L. Pautz, and P. M. J. Burgers, unpublished data.

TABLE I
Molecular weight of Pol δ and subcomplexes

M_r values listed as $\times 10^{-3}$ and Stokes radii in \AA . Theoretical M_r values were from the polypeptide sequences. M_r from S and \AA obtained from Equation 2; f/f_0 obtained from Equation 3; M_r from equilibrium sedimentation obtained from Equation 4. Indicated errors in the calculated M_r reflect measurement errors only. See text for details.

| Subunits | Theoretical M_r for | | S value | Stokes radius | M_r from S and \AA | f/f_0 | M_r from equil. sed. |
|----------------------------|-----------------------|-------|---------------|---------------|-------------------------------|---------|------------------------|
| | monomer | dimer | | | | | |
| Pol3-31-32 (Pol δ) | 220 | 440 | 7.4 ± 0.4 | 70.5 ± 2 | 219 ± 20 | 1.78 | 196 ± 10 |
| Pol3-31 (Pol δ^*) | 180 | 360 | 7.4 ± 0.4 | 52 ± 1 | 162 ± 15 | 1.39 | n.d. ^a |
| Pol31-32 | 96 | 191 | 4.3 ± 0.4 | 59 ± 1 | 106 ± 15 | 1.96 | 104 ± 5 |
| His ₇ Pol32 | 41 | 82 | 2.8 ± 0.4 | 50.5 ± 1 | 59 ± 12 | 2.22 | n.d. |

^a Not determined.

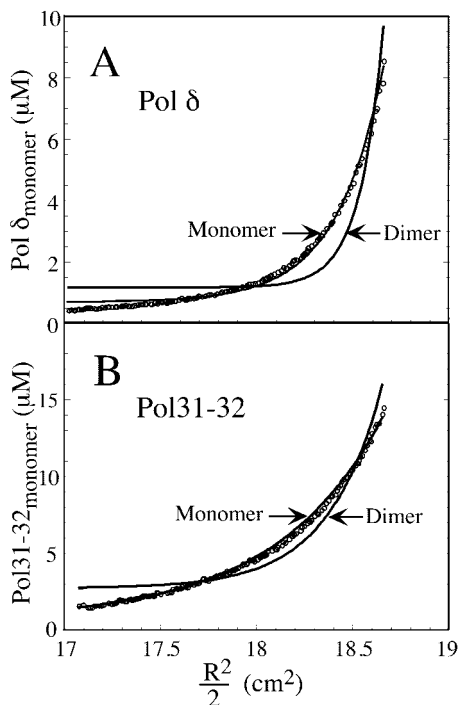


FIG. 3. Equilibrium sedimentation of Pol δ (A) and Pol31-32 (B). The theoretical curves for monomer and dimer species are calculated based upon the polypeptide sequences.

tions from ideality tend to cancel when the product is taken in Equation 2. The frictional coefficient f/f_0 can be determined from the molecular mass and the Stokes radius as seen in Equation 3.

$$f/f_0 = a/(3\bar{v}M/4\pi N)^{1/3} \quad (\text{Eq. 3})$$

A Superose 12 gel filtration column was calibrated by injecting proteins with known Stokes radii, and a calibration plot of $(-\log K_{av})^{1/2}$ against the Stokes radius was obtained (see "Experimental Procedures") (Fig. 2A). After this we injected Pol δ , Pol δ^* , Pol31-32, and His₇Pol32 over the column. The measured elution volume yielded the $(-\log K_{av})^{1/2}$, and we could determine the Stokes radii of the different complexes by interpolation (Fig. 2A). The results for Pol δ and Pol δ^* are comparable with those obtained previously with a Superose 6 gel filtration column (20). Three species (Pol δ , Pol31-32, and His₇Pol32) yielded much larger Stokes radii than anticipated from the molecular masses calculated from the gene sequences, suggesting the presence of either multimeric or extremely elongated species (Fig. 2A, Table I). In contrast the Stokes radius of Pol δ^* suggested that it may exist as a simple heterodimer. Each complex was systematically analyzed at two or three different concentrations starting at 0.5–2 mg/ml at time of injection down to 5–10 $\mu\text{g/ml}$ at time of injection. For no species did we observe a concentration-dependent change in elution

volume. For instance, in one series of experiments Pol δ was initially injected onto the column at a concentration of 10 μM (monomer concentration). The peak fractions were reinjected, and this procedure was repeated two more times until the concentration of Pol δ was 20 nM and the subunits could only be detected using Western blot analysis. The elution volume of Pol δ remained constant through this reiterative procedure (data not shown).

The same complexes were also subjected to velocity sedimentation on a 10–35% glycerol gradient. Proteins with known sedimentation coefficients were included as internal standards, e.g. bovine serum albumin for Pol31-32 (Fig. 1B). Sedimentation coefficients were obtained by interpolation on the standard curve (Fig. 2B). Again, for each species, sedimentation was carried out at varying protein concentrations (from 0.05–1 mg/ml), representing at least a 10-fold difference in concentration for each species, without observing a concentration-dependent change in S value (data not shown). Finally, a whole cell lysate from log phase wild-type yeast cells was also analyzed on a glycerol gradient. Pol3, Pol31, and Pol32 polypeptides, identified by Western blot analysis, co-sedimented on the glycerol gradient with a sedimentation coefficient of 7.4 S, identical to that obtained for purified Pol δ from the overproduction strain (data not shown).

The molecular mass of each complex was calculated from the Stokes radius and the S value. The estimated molecular masses of Pol δ , Pol δ^* , and Pol31-32 are consistent with these complexes being monomeric in structure, i.e. composed of one subunit each per complex (Table I). However, the estimated molecular mass of His₇Pol32 was 59, halfway between that of a 41 monomer and an 82 dimer. Given the additional fact that both the Stokes radius and the S value of this protein are independent of concentration, it must be clear that the shape of this protein is such that its molecular mass cannot be determined using these hydrodynamic parameters and Equation 2. The calculated frictional ratio of His₇Pol32 (assuming that it is a monomer) is 2.22, indicating that the protein is extremely elongated. Even if we assume Pol32 to be dimeric, the frictional ratio would still be 1.78, suggesting a very elongated shape. In addition, all larger complexes containing Pol32 are also much more elongated than those without; e.g. compare Pol δ^* with Pol δ . Consequently, we turned to equilibrium sedimentation analysis to determine molecular masses more accurately, because our molecular mass estimation of Pol δ and Pol31-32 using Equation 2 must be considered unreliable in view of the presence of Pol32 in these complexes.

Equilibrium Sedimentation—In this technique the distribution of a molecule across the cell is dependent on its molecular mass but not on its shape. Therefore, equilibrium sedimentation is an ideal technique for molecular mass determinations of non-spherical complexes in Equation 4,

$$C(r) = C(r_0)e^{\omega^2 M(1 - \bar{v}\rho)(r^2 - r_0^2)/2RT} \quad (\text{Eq. 4})$$

in which $C(r)$ and $C(r_0)$ are the concentrations at radius r and

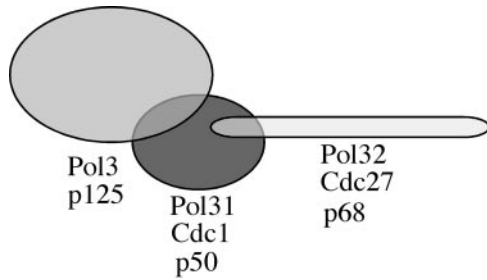


FIG. 4. **A model of monomeric DNA polymerase δ .** Subunit-subunit interactions of the yeast enzyme follow from previous studies and are similar to those for *S. pombe* and human Pol δ (11, 13, 15, 18, 27).

a reference point r_0 , respectively, ω is the radial speed, and R and T are the gas constant and the temperature.

Unfortunately, this analysis could only be carried out with Pol δ and Pol31–32, because Pol δ^* and His₇Pol32 aggregated significantly during the extended period of centrifugation necessary to reach equilibrium. For Pol δ , the same distribution curve was obtained at each of three concentrations examined, 1.8 μM (as monomer), 0.8 μM , and 0.23 μM . Therefore, a global analysis of the data at all three concentrations was carried out, assuming the presence of a single species, which yielded a molecular mass of 196. No better fit was obtained when we assumed the presence of a monomer-dimer equilibrium. Fig. 3A shows the data points for the highest concentration of Pol δ together with the expected curves for a monomer or a dimer of Pol δ . We also carried out sedimentation studies of Pol δ in buffer B containing 5 mM MgCl₂ and 125 mM NaCl with essentially identical results, suggesting that dimerization is not induced under conditions when Pol δ is active for DNA synthesis (data not shown). The data for Pol31–32 were also obtained at three different concentrations: 4.6 μM , 2.1 μM , and 0.7 μM . A global fit yielded a molecular mass of 104 kDa, very close to the theoretical molecular mass of 96. If we assume a monomer-dimer equilibrium and fix the M of the monomer at that of the theoretical value of 96, we obtain a dimerization constant of $\sim 300 \mu\text{M}$ from the global fit, but this fit is not statistically better than the fit for a monomeric species. Therefore, based upon these sedimentation data we conclude that both Pol31–32 and Pol δ , purified from yeast overproduction strains, are monomeric in solution.

Is Pol32 a Dimer Inside the Cell?—We have previously carried out an analysis of subunit-subunit interactions of Pol δ using the two-hybrid system (11). These studies, together with biochemical studies, indicate that Pol3 interacts primarily with Pol31, which in turn binds Pol32. In addition, both two-hybrid studies and biochemical experiments showed an interaction between Pol32 and the replication clamp PCNA (11, 26). During these studies, however, we also noted the presence of a small but significant interaction signal when we carried out a Pol32-Pol32 two-hybrid analysis and suggested that the observed signal was indicative of dimerization of Pol32 and therefore supportive of the model that Pol δ is dimeric *in vivo*. Recently, we carried out a deletion study of the *POL32* gene.² The PCNA-binding motif in Pol32 is localized to the extreme carboxyl terminus of Pol32, as was observed for the *S. pombe* ortholog Cdc27 (27). Deletion of the carboxyl-terminal seven amino acids of Pol32 yields a mutant (Pol32-C Δ 7) that fails to interact with PCNA *in vitro* as well as in a two-hybrid analysis. The truncated protein is chromatographically identical (including gel filtration analysis) to full-length Pol32. When we tested this truncated form of Pol32 in a two-hybrid analysis (Pol32-C Δ 7 was fused to the *lexA* DNA-binding domain and full-length Pol32 to the Gal4 activation domain) no signal indicative of

interaction was observed. We interpret these results to mean that the positive signal observed in the wild-type Pol32-Pol32 two-hybrid assay was actually due to a bridging interaction by PCNA (*lexA*-pol32-PCNA-Pol32-Gal4), which was eliminated using the PCNA interaction-defective Pol32-C Δ 7. Therefore, we can detect no *in vivo* interaction suggestive of Pol32 dimerization.

Comparison with Pol δ from Other Organisms—A biochemical characterization of *S. pombe* Pol δ and of several subcomplexes was recently published (15). The results of this study are very similar to ours. The gel filtration analysis of the Cdc27 protein suggested that this protein is either multimeric or an extremely elongated species. Further analysis by gel filtration of the Pol3-Cdc1-Cdm1 complex and the Pol3-Cdc1-Cdm1-Cdc27 complex suggested that the four-subunit complex is a dimer of the heterotetramer and, moreover, that the Cdc27 subunit may play an important role contributing to the dimerization of Pol δ . Interestingly, very large complexes ranging from 260–500 kDa, as estimated by gel filtration analysis, were also detected in highly purified Pol δ preparations containing the p68 subunit (the human ortholog of Pol32) (28). Considering our finding that the Pol32 subunit is highly elongated and that Cdc27 and p68 are the orthologs of Pol32, we suggest that these subunits may also be very elongated and yield abnormally large estimates of the molecular mass of the analogous preparations of Pol δ when analyzed by gel filtration.

Conclusions—Our studies indicate that Pol δ isolated from an *S. cerevisiae* overproduction strain has a monomeric structure in solution. An analysis of the size of Pol δ in extracts from wild-type yeast cells gave the same result. Although Pol3 and Pol31 may be fairly globular based upon the analysis of Pol δ^* , the presence of Pol32 in the complex gives the entire complex a very elongated shape (Fig. 4). These findings do not address the structure of this enzyme at the replication fork. If Pol δ replicates both the leading and the lagging strand of the fork, putative dimerization of the two sides of the fork may be mediated by other factors. However, in light of the present results, a reinvestigation of the role of Pol ϵ in bulk DNA replication is justified.

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