Minireview

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DNA Polymerase α*

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A DNA polymerase is essential for DNA replication. Although this may now seem obvious, the first indication that this is indeed the case did not come until nearly a decade after the discovery of DNA polymerase in *Escherichia coli* (1) when it was found that mutations in gene 43 of bacteriophage T4, one of the genes known to be essential for T4 DNA replication *in vivo*, produced a defective DNA polymerase as measured *in vitro* (2). The necessity of a DNA polymerase for chromosomal DNA replication in eukaryotes was not established until approximately 20 years later (3).

A eukaryotic DNA polymerase activity was first identified in calf thymus tissue in 1958 (4). In the ensuing years it became clear that eukaryotes possess not a single DNA polymerase but, like *E. coli*, are endowed with multiple DNA polymerases, each with a distinctive role in DNA metabolism (5). DNA polymerases α and δ serve in chromosomal DNA replication; they may also be involved in the repair of DNA. DNA polymerase β is believed to function in DNA repair, but there is no direct proof that this is the case. Finally, DNA polymerase γ is located within the cellular mitochondria and is very likely required for mitochondrial DNA replication. Again, there is no direct proof; however, its intracellular location argues strongly for such a role. This minireview will focus primarily on DNA polymerase α (α polymerase or Pol α). Substantial evidence has developed over the past 5 years that supports a primary role for this enzyme in eukaryotic DNA replication, and a considerable amount has been learned about its structure and associated activities.

Pol α Is Required for Chromosomal DNA Replication

When cells are disrupted by the usual aqueous procedures, almost all of the α polymerase is found in the cytoplasm, and indeed most purification procedures begin with cytoplasmic extracts. There is no doubt, however, that Pol α is located in the nucleus, as expected of an enzyme that plays a key role in nuclear DNA replication. In fact, most (90–95%) of the Pol α is nuclear when cells are disrupted in the appropriate nonaqueous media (6). Moreover, immunofluorescent staining of KB cells that had been exposed to a monoclonal antibody directed against Pol α showed all immunoreactive material to be confined to the nucleus; none could be found in the cytoplasm (7).

Many lines of evidence now support a direct role for Pol α in chromosomal DNA replication. (i) The level of Pol α is highest in tissues where there is rapid cell division, *e.g.* in rapidly growing cultured mammalian cells (8) and in lymphocytes that have been stimulated by a mitogen (9), and drops to a low level in quiescent cells (9, 10) where the β polymerase, whose level remains unchanged during rapid cell growth, becomes the predominant activity. (ii) Cellular and SV40 DNA synthesis *in vivo* is strongly inhibited by the antibiotic aphidicolin, a potent inhibitor of the α polymerase *in vitro* (11). (iii) Monoclonal antibodies directed against Pol α inhibit cellular DNA replication in permeabilized nuclei or when directly injected into cultured mammalian cells (12). (iv) Mutant mouse cells that contain a temperature-sensitive Pol α are unable to replicate their DNA at nonpermissive temperatures (13). (v) HeLa cell extracts depleted of Pol α by immunoprecipitation do not support SV40 DNA replication *in vitro*. Replication can be restored specifically by the addition of the appropriate (human or monkey, but not murine) α polymerase (14). (vi) The gene for the polymerase subunit of the yeast (*Saccharomyces cerevisiae*) analogue of Pol α, DNA polymerase I (Pol I), is present in only a single copy and its disruption is lethal (3). Recently the yeast Pol I gene has been identified with cdc 17, one of a group of conditional cell cycle mutants in which DNA replication is blocked at elevated temperatures (15). Thus, Pol α is clearly required for chromosomal DNA replication.

Structure and Catalytic Activities of Pol α

Although DNA polymerase α has been studied extensively over the past 2 decades, elucidation of its subunit structure has been hampered by several factors. First, although Pol α is the most abundant of the four cellular DNA polymerases, at least in rapidly proliferating cells, it is nonetheless a relatively rare protein. Early purification schemes were laborious, involved numerous chromatographic steps, and yielded little protein for structural analysis. Second, the high molecular weight multisubunit enzyme is often fractionated into multiple forms distinguishable by their chromatographic and catalytic properties, even when purified from a single cell type or tissue (16). Third, extensive proteolysis during purification yields active but highly degraded forms of Pol α (17, 18). With the development of rapid purification schemes including the use of immunoaffinity chromatography which minimized proteolysis and permitted the isolation of largely intact enzymes, it became apparent that common structural features exist in Pol α in species as divergent as yeast and man (19, 20). As indicated in Table I, Pol α is composed of four subunits: a large subunit of M_r 180,000 or, more often, a family of polypeptides ranging in size from 140 to 185 kDa; a subunit of M_r about 70,000; and two small subunits with M_r values of approximately 60,000 and 50,000. DNA polymerase activity is associated with the 180-kDa subunit, and DNA primase, which is an integral component of Pol α, is associated with the 60- and 50-kDa subunits (21–23).

A genomic DNA clone of the polymerase subunit of yeast Pol I (3) and a cDNA clone of the analogous subunit of human Pol α (24) have been isolated. Both clones encode polypeptides that share six regions of homology with both viral and bacteriophage DNA polymerases. Two of these regions may form the deoxynucleoside triphosphate binding domain and a third region may represent the DNA binding domain (24). Clearly, there is strong conservation of both subunit structure and amino acid sequence in Pol α.

The availability of the gene for the yeast polymerase subunit has also permitted its functional overproduction and genetic analysis (3, 25). As noted above, the Pol I gene is present in only a single copy and its disruption is lethal, indicating that neither yeast DNA polymerases II or III (see below) can compensate for the loss of Pol I function. Although the structure of the overproduced polypeptide was not examined, the genomic clone was used to identify a 5.4-kilobase mRNA sufficient to encode a polypeptide of 210 kDa. Likewise, RNA blot analyses performed using the human cDNA clone identified a single 5.8-kilobase mRNA in KB cells (24). However, the single open reading frame deduced from the DNA sequences of the human cDNA

TABLE I

Subunit structure of DNA polymerase α

Families of polymerase subunit polypeptides are indicated in parentheses. See Ref. 20 for references to individual enzymes.

Source	Polypeptide composition (kDa)
D. melanogaster embryos	180, 73, 60, 50
Human KB cells	(180, 165, 140, 125), 77, 55, 49
Human HeLa cells	(145, 135, 130), 64, 52, 45
Calf thymus	(180, 170), 70, 65, 60
	(185, 160), 68, 55, 48, 40
Yeast	(180, 140), 74, 58, 48
Monkey CV-1 cells	(176, 158, 133, 118), 62, 57, 53, 30
Mouse hybridoma cells	
Polymerase	185, 68
Primase	56, 46

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and yeast genomic clones predicts recombinant polypeptides of only 165 kDa (24, 25). Further analysis of yeast and human genomic clones combined with amino-terminal sequence analysis of the polymerase subunit family should provide an unambiguous picture of the primary translation product.

The DNA primase activity associated with Pol α from *Drosophila melanogaster* and the yeast Pol I have been examined in detail. When coupled to polymerase, the *Drosophila* primase synthesizes a primer 12–14 nucleotides in length (26). However, the product formed by the primase when it is dissociated from the polymerase is almost exactly twice the unit length, 24–28 nucleotides (27). Similarly, the primase purified from mouse hybridoma cells (28) and the primase associated with the intact yeast Pol I synthesize oligoribonucleotides consisting of multiples of 8–12 nucleotides (29). The ability to synthesize a primer of defined length is therefore inherent in the primase subunits and is not a consequence of coupling to polymerase action. In all cases, however, coupling of DNA polymerase to primase suppresses multimeric primer synthesis and results in attenuation of primer length (27, 29, 30). The mechanism that generates the switch from primer synthesis to DNA chain elongation under normal physiological conditions, *i.e.* in the presence of both ribo- and deoxynucleoside triphosphates, is not known.

The primase associated with the yeast Pol I consists of 58- and 48-kDa subunits (23). Antibody directed against the 48-kDa subunit inhibits primase activity, thus providing direct evidence for the involvement of this subunit in primase function (31). This subunit also contains an ATP-binding site, suggesting that the catalytic site is probably situated within this subunit.[1] The yeast genes encoding the 58- and 48-kDa subunits have been cloned and sequenced, and the predicted molecular weights, 47,623 and 61,500, respectively, are in good agreement with the molecular weights determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (32).[1] In both cases the genes encoding the two primase subunits are present in single copies and are essential for viability.[1]

Although as noted above, DNA primase activity is an intrinsic component of the Pol α (which should more properly be referred to as DNA polymerase-primase), it is devoid of other enzymatic activities normally associated with DNA replication (5). For example, the polymerase-primase from *Drosophila* exhibits neither 3′→5′ nor 5′→3′ exonuclease activity. Similarly, DNA-dependent ATPase (indicative of a DNA helicase), RNase H, or endonuclease activities are not associated with the four-subunit enzyme (33). However, these and other enzymatic activities can be found in association with higher molecular weight DNA polymerase complexes. For example, a 640-kDa polymerase-primase complex has been isolated from synchronized HeLa cells in which Pol α is associated with two primer recognition proteins (see below), a diadenosine 5′,5″-P^1,P^4-tetraphosphate-binding protein and a 3′→5′ exonuclease (34). Similarly, in an early stage of its purification, calf thymus polymerase-primase is associated with a topoisomerase II, a DNA-dependent ATPase, RNase H, and 3′→5′ exonuclease, as indicated by co-chromatography and by cosedimentation at S values >11 (35). In neither instance has a functional association of these activities been demonstrated.

Although the *Drosophila* polymerase-primase is devoid of 3′→5′ exonuclease activity, the four-subunit enzyme contains a cryptic proofreading 3′→5′ exonuclease that is revealed only upon dissociation of the subunits: the potent 3′→5′ exonuclease activity present in the 182-kDa subunit appears to be unmasked specifically upon removal of the 73-kDa subunit (36). Consistent with this finding, the replication fidelity of the isolated 182-kDa subunit is 100-fold greater than that of the four-subunit enzyme (36). Thus, the formation of sub- and superassemblies of Pol α may result in the manifestation of altered enzymatic activities, *e.g.* DNA polymerase, DNA primase, and 3′→5′ exonuclease. Attempts to isolate the 180-kDa polymerase subunit free of the 70-kDa subunit in a mammalian Pol α (that from calf thymus) using procedures that have been effective with the *Drosophila* enzyme have thus far been unsuccessful.[2] It is therefore unclear whether a cryptic 3′→5′ exonuclease is a general feature of DNA polymerase α. As in the case of the polymerase and primase subunits of the yeast Pol I, disruption of the gene encoding the 74-kDa subunit is lethal.[3] On the basis of immunoblotting experiments it has been proposed that the 74-kDa polypeptide is derived, presumably by proteolysis, from a larger 86-kDa polypeptide (37).[3]

[1] P. Plevani, personal communication.
[2] A. MacNicol and I. R. Lehman, unpublished results.
[3] D. Hinkle, personal communication.

Pol α Accessory Factors

Although Pol α is a versatile enzyme with regard to the replication of template primers of high primer density, the four-subunit enzyme is very inefficient in the replication of template primers containing long stretches of single-stranded DNA. The rate of replication of singly-primed φX174 or M13 single-stranded viral DNA or homopolymers with a low density of primers by the human KB (38), HeLa (39), *Drosophila* embryo (40), calf thymus (41), and monkey CV-1 (42) α polymerases is ≤10% of the rate on activated DNA containing short single-stranded stretches. This is very likely a consequence of two factors. First, Pol α has a high affinity for single-stranded DNA and binds nonproductively to regions lacking primers (43). It also exhibits pausing in DNA synthesis at sites predicted to contain stable secondary structures and at a variety of other sites where such structures are unlikely to exist (44, 45). Second, the enzyme under the usual conditions of assay shows a low processivity of nucleotide polymerization (40, 46–48, 90). Thus, polymerase accessory factors should exist that permit the polymerase to overcome these impediments to rapid, efficient DNA replication. Factors that interact with the polymerase have been detected in association with and separate from Pol α. Two factors (C1 and C2) that interact with each other and with Pol α have been purified to near-homogeneity from HeLa cells (39, 48). Two factors with analogous properties have been found in association with the α polymerase from monkey CV-1 cells (42, 48). In both cases, the factors alone bind tightly to single-stranded DNA but when reconstituted with Pol α stimulate its activity on single-stranded DNA templates ≥20-fold. Similar, but less well defined, factors have been identified in *Drosophila* embryos (49) and in mouse FM3A cells (50). A striking feature of the C1 and C2 proteins is their high degree of specificity. Thus, the HeLa C1 and C2 proteins stimulate the HeLa Pol α but not Pol α from CV-1 cells. Similarly the C1 and C2 proteins from CV-1 cells stimulate only the homologous Pol α (48).

Since the C1 and C2 proteins increase utilization of short primers and produce a greater than 20-fold decrease in the K_m for template primers with a low primer density, it is likely that they play some role in primer recognition. The finding that the K_m for template primers with high primer densities is not affected suggests that the stimulation does not result from an increased affinity for primer termini *per se* but from a decrease in nonproductive binding to single-stranded DNA, such that the enzyme is more effective in identifying available 3′-hydroxyl termini. It has therefore been proposed that the C1 and C2 proteins facilitate primer recognition by enabling Pol α to slide along the template until it encounters a primer terminus. An equally plausible mechanism, however, is that they increase the rate of enzyme recycling upon nonproductive binding to single-stranded DNA.

An RNase H from *Drosophila* stimulates DNA synthesis by the homologous Pol α (51), and an analogous situation has been described in yeast (52). In the case of the *Drosophila* RNase H, stimulation is specific for reactions in which priming and chain elongation are coupled and appears to occur by increasing the recycling capacity of the primase, thereby increasing the rate of chain initiation. Stimulation of DNA synthesis by the yeast RNase H appears to proceed by a different mechanism, involving an RNase H-promoted increase in the affinity of Pol I for primer termini (52).

Although single-stranded DNA-binding proteins do not interact directly with DNA polymerase, they do affect the rate of nucleotide polymerization. By analogy to the *E. coli* single-stranded DNA binding protein (SSB),[4] a yeast SSB (SSB-1) stimulates DNA synthesis on templates with low primer densities by increasing substantially the processivity of the yeast Pol I (53–55). Surprisingly, the processivity of the isolated 182-kDa polymerase subunit of the *Drosophila*, Pol α is dramatically increased by the *E. coli* SSB, whereas that of the intact four-subunit enzyme is largely unaffected (56), suggesting that the interaction of an as yet unidentified *Drosophila* SSB may be highly specific.

Recently a mammalian single-stranded DNA-binding protein has been identified as an essential component of a multienzyme system that can promote SV40 DNA replication *in vitro* (57–59). The protein termed replication protein A (RP-A) which has been purified to homogeneity (60–62) consists of three subunits, with molecular weights of 70,000, 32,000 and 13,000, the largest of which binds single-stranded DNA. The

[4] The abbreviations used are: SSB, single-stranded binding protein; PCNA, proliferating cell nuclear antigen.

precise role of this protein in SV40 DNA replication is presently unknown; however, one function is to assist the SV40 T antigen in unwinding SV40 DNA at the origin of replication, a property that it shares with *E. coli* SSB. It almost certainly serves other functions in SV40 replication as well.

Post-translational Modification of Pol α

Enzyme regulation by post-translational modification can provide a mechanism for the positive or negative modulation of enzyme activity or for the differential regulation of one or several functions of a multifunctional enzyme. For example, a phosphorylation-dephosphorylation cycle could regulate the DNA polymerase, primase, or cryptic exonuclease functions. Several groups have investigated the possibility that Pol α might exist as a phosphoenzyme. While the data are preliminary, and in some respects conflicting, evidence supporting this hypothesis has been accumulating.

After immunoprecipitation of Pol α from extracts of KB cells labeled with [^{32}P]orthophosphate, the 180- and 77-kDa subunits appeared as phosphoproteins; the 55- and 49-kDa (primase) subunits remained unlabeled (63). No effects of phosphorylation on polymerase activity could be detected. In a similar study with the Pol α from rat embryonic fibroblasts, a 220-kDa subunit was identified as a phosphoprotein. In this case, polymerase activity was greater than 10-fold reduced upon dephosphorylation, and enzymatic activity could be restored upon incubation with ATP (64). In another study the HeLa cell Pol α was found to be stimulated by protein kinase C in a reaction involving ATP and phospholipid cofactors (65). The 2- to 3-fold increase in activity observed upon incubation with protein kinase C was specific for Pol α; Pol β and Pol γ were unaffected (Pol δ was not examined). The stimulation observed apparently results from a decrease in K_m for the DNA template. At the same time, direct measurement of nucleotide misincorporation with homopolymer templates indicated that the replication fidelity of Pol α isolated from both quiescent and replicating cells was increased severalfold. Although the phosphorylated subunit was not identified, the finding that the effects of phosphorylation were unrelated to the stage of enzyme purification suggests that a polymerase subunit is the target. In view of the increased fidelity of the 182-kDa polymerase subunit of the *Drosophila* Pol α upon removal of the 73-kDa subunit and its enhanced processivity in the presence of *E. coli* SSB, this subunit may be a target for phosphorylation. For example, phosphorylation might lower its affinity for the polymerase subunit thereby activating its 3′→5′ exonuclease activity and enhancing its processivity. Future efforts to demonstrate a positive correlation between phosphorylation at a specific site in the DNA polymerase-primase and the modulation of its catalytic properties will be of great interest, as will be the identification of the site of phosphorylation.

DNA Polymerase δ

Although DNA polymerase δ (Pol δ) has been known for more than a decade (66), interest in the enzyme has heightened with the finding that it may be an essential component in the complex of enzymes that promotes SV40 DNA replication *in vitro* (60–62). Recently, a protein of M_r 36,000 purified from calf thymus that stimulates the activity of the homologous Pol δ on templates with low primer density was shown to be identical to cyclin or proliferating cell nuclear antigen (PCNA) (67, 68), which is identical to a protein purified from HeLa cells that is essential for SV40 DNA replication *in vitro* (69). Pol δ was initially distinguished from Pol α by its 3′→5′ exonuclease activity. While the subunit structure of Pol δ and its relationship, if any, to Pol α are as yet unresolved, several features appear relevant. Both enzymes are sensitive to the drug aphidicolin and are inhibited by *N*-ethylmaleimide. Both are also resistant to dideoxynucleoside triphosphates (70). In contrast, the nucleotide analogue butyl phenyl dGTP (70–72) strongly inhibits Pol α but is only marginally inhibitory to Pol δ. Recent evidence indicates that Pol δ levels parallel those of Pol α in different tissues, lending further support to a role for Pol δ in chromosomal DNA replication (73). Moreover, both Pol α and Pol δ are required for the synthesis and maturation of nascent DNA in permeabilized cells (74).

DNA polymerase δ has been isolated in two forms distinguished primarily by their response to PCNA. One form shows a low degree of processivity, but upon interaction with PCNA becomes highly processive. As isolated from calf thymus, this form of Pol δ consists of two subunits of 125 and 48 kDa (75). The yeast equivalent of Pol δ, Pol III, is also composed of two subunits with nearly the same molecular mass, 125 and 55 kDa, and shows a comparable response to PCNA (76, 77). A third PCNA-responsive enzyme purified from human placenta consists of only a single 170-kDa polypeptide (78). The second form of Pol δ is moderately processive in the absence of PCNA and is not significantly affected by PCNA. One example of this form of Pol δ, isolated from calf thymus, contains five polypeptides and, unlike the other forms of the enzyme, has an associated DNA primase activity (79). A second example of a PCNA-independent Pol δ is the enzyme isolated from HeLa cells that consists of only a single polypeptide of M_r 205,000 (80). Finally, a PCNA-independent Pol δ has been purified from rabbit bone marrow and consists of a single polypeptide of M_r 122,000 (81). As is obvious from the foregoing, there is still considerable uncertainty regarding the structure of the δ polymerase and its interaction with PCNA. This issue has been further complicated by the very recent finding of Hurwitz and his colleagues (82) that PCNA may act in SV40 DNA replication not by interacting directly with Pol δ but by counteracting the effect of an inhibitory factor.

As noted above, Pol δ is clearly distinguishable from Pol α by four criteria: (i) it consists of only one or two subunits; (ii) it possesses a proofreading 3′→5′ exonuclease; (iii) it is relatively insensitive to the nucleotide analogue, butyl phenyl dGTP; and (iv) with one exception it is devoid of primase activity. However, the 182-kDa polymerase subunit of the *Drosophila* Pol α exhibits all of these features; by these criteria it is indistinguishable from Pol δ (83). On the other hand, several reports (78, 84, 85) have documented the lack of immunological cross-reactivity between the two enzymes from mammalian sources, indicating that Pol δ is a unique entity, unrelated structurally to the α polymerase. Furthermore, the very recent finding that the yeast Pol III is the product of the cdc2 gene, whereas Pol I is encoded by cdc17, demonstrates conclusively that in yeast Pol I and Pol III are distinct enzymes (86).

The absence of primase activity and the high processivity of nucleotide polymerization have led to the suggestion that the δ polymerase functions in leading strand synthesis, and the α polymerase with its associated primase and low processivity functions in lagging strand synthesis (84). The notion of distinct polymerases acting in leading and lagging strand synthesis is an attractive conjecture that accommodates nicely to the inherent asymmetry of the replication fork. Indeed, there is some evidence that the complex multisubunit DNA polymerase III holoenzyme of *E. coli* does exist as an asymmetric dimer (87, 88).

Our understanding of the structure of the eukaryotic DNA polymerases and their role in chromosomal DNA replication is presently at a stage that is not too different from that for prokaryotes in the early 1970s. At that time, the discovery of DNA polymerase III and the development of systems that catalyze bacteriophage DNA replication *in vitro* revolutionized our understanding of DNA replication and the enzymes that are involved in this extraordinarily complex process (87). With the wealth of new information about the structure and function of eukaryotic replicative polymerases and the development of cell-free systems that initiate and sustain the replication of animal virus DNAs (89), it is not too unrealistic to anticipate a similarly rapid advancement of our knowledge of what must be an even more complex problem, the mechanism and control of chromosomal DNA replication in eukaryotes.

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