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DNA Polymerase α Associated Primase from Rat Liver: Physiological Variations†

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ABSTRACT: A primase activity associated to DNA polymerase α from rat liver is described. Both activities were absent in normal adult rat liver but were concomitantly induced after partial hepatectomy. As previously shown for polymerase α and DNA topoisomerase II, primase activity reached a maximum value 40–43 h after the partial removal of the liver. Primase activity was shown to catalyze dNMP incorporation on unprimed single-stranded DNA template (M13 DNA) in the presence of rNTP. The activity was not detectable on poly(dA) or poly(dG) but was efficient on poly(dT) or poly(dC). However, the reliability of the primase assay in the presence of poly(dC) was dependent upon the degree of purification of the enzyme. The ribo primers were about 10 nucleotides long, and the reaction was completely independent of α-amanitin, a strong inhibitor of RNA polymerases II and III. Primase and polymerase were found tightly associated. A cosedimentation on a 5–20% sucrose gradient was always obtained, independent of the ionic strength. There was also a close coincidence between α-polymerase and primase activities during phosphocellulose, hydroxylapatite, and single-stranded DNA Ultrogel chromatography. It has been previously demonstrated by us and others that primase and α-polymerase are on separated polypeptides. The association of two activities in the replication complex and the conditions allowing their separation are discussed.

The RNA priming of DNA chain synthesis has been demonstrated in polyoma virus (Eliasson & Reichard, 1978), simian virus (De Pamphillis et al., 1979), and mammalian cells (Waqar & Huberman, 1975; Tseng et al., 1979; Kitani et al., 1984). The RNA primers are oligoribodecanucleotides of variable composition, containing a purine ribonucleoside 5′-triphosphate. They are covalently attached to 5′ termini of newly synthesized DNA. In *Escherichia coli*, RNA priming is catalyzed by a specialized RNA polymerase called primase (Kornberg, 1982). Such a primase activity has been also described for bacteriophages T4 and T7 and the ColE1 plasmid (Kornberg, 1982).

More recently, a primase activity has been described in eukaryotic cells of different origins (Conaway & Lehman, 1982; Yagura et al., 1982; Méchali & Harland, 1982; Tseng & Ahlem, 1982; Kaufmann & Hoffman-Falk, 1982; Riedel et al., 1982; Shioda et al., 1982; Hübscher, 1983; Litvak et al., 1984; Philippe et al., 1984; Plevani et al., 1984; Singh & Dumas, 1984; Yamaguchi et al., 1985a). Detailed studies about the specificity of the RNA primer synthesis by the DNA polymerase–DNA primase complex from mammalian cells were recently reported (Hu et al., 1984; Yamaguchi et al., 1985b). The DNA primase was found associated with a subspecies of DNA polymerase α (Yagura et al., 1982; Riedel et al., 1982; Shioda et al., 1982) or with the α-polymerase (Conaway & Lehman, 1982a; Hübscher, 1983; Kaguni et al., 1983a; Wang et al., 1984; Gronostajski et al., 1984). In the latter case, determination of the subunit on which the primase activity resides has led to contradictory results. Hübscher has reported that the high molecular weight (125 000 daltons) subunit of calf thymus DNA polymerase α contained both primase and polymerase activities. On the other hand, primase activity was shown to reside in the 60 000-dalton and/or 50 000-dalton subunit of DNA polymerase from *Drosophila* embryos, whereas the polymerase activity is associated with the high molecular weight (182 000 daltons) subunit (Kaguni et al., 1983b).

It is well-known that there is a clear association between DNA polymerase α activity and mitotic activity, whereas β-polymerase levels did not change. Few data are available about primase activity, except that the levels of primase activity have been correlated positively with DNA synthesis, in spleen and cardiac muscle during postnatal development (Kozu et al., 1982). The finely programmed regeneration of rat liver after its partial removal gives us an attractive system to follow the variation of primase activity during DNA synthesis (Philippe et al., 1984). In this paper, we describe the variation

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of DNA primase activity during liver regeneration, compared to DNA polymerase α and β activities used as internal controls. The relation between DNA primase and DNA polymerase α is also discussed.

Experimental Procedures

Unlabeled deoxyribonucleoside triphosphates were purchased from Boehringer. [^{3}H]dATP and [^{3}H]dTTP were from Amersham [α-^{32}P]ATP and [γ-^{32}P]ATP were from New England Nuclear. Acrylamide, *N,N'*-methylenebis(acrylamide), *N,N,N',N'*-tetramethylenediamine, and ammonium persulfate were obtained from Bio-Rad. Ultrapure urea was from Schwarz/Mann. α-Amanitin was purchased from Boehringer. *Escherichia coli* DNA polymerase I was from P-L Biochemicals. Pancreatic DNase I (grade I) was supplied by Boehringer. Heparin–Sepharose CL-6B, hydroxylapatite HPT, phosphocellulose P11, and single-stranded DNA Ultrogel were respectively from Pharmacia, Bio-Rad, Whatman, and IBF. Resins were prepared according to manufacturer's instructions. Poly(dT), 1000 residues long, was purchased from P-L Biochemicals.

Calf thymus DNA (type I) from Sigma was activated with pancreatic DNAse until it was rendered 12% acid soluble (Fansler & Loeb, 1974). M13 single-stranded DNA was prepared as described by Miller (1972).

Partial Hepatectomies. Two-third partial hepatectomies were performed on 19 male WAG rats. Eighteen hours after partial removal, six rats were killed and then five after 24 h, five after 43 h, and three after 72 h. A pool of three rats was used as control. Postmicrosomal supernatants were prepared for each time as previously described (Mêchali et al., 1980). Percentages of regeneration after 18, 24, 43, and 72 h are respectively 3.8, 43.0, 59.3, and 78.7%. For some experiments, postmicrosomal supernatants were obtained from 25 male WAG rats killed 40 h after partial hepatectomies.

DNA Polymerase Assay (Mêchali et al., 1980). Assays using activated calf thymus DNA (350 μg/mL) were at 37 °C in 50 mM tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl), pH 8.1, 8 mM $MgCl_2$, 5 mM KCl, 5 mM 2-mercaptoethanol, 15% glycerol, 450 μg/mL of bovine serum albumin, and 100 μM each of dATP, dGTP, dCTP, and [^{3}H]dTTP (50–300 cpm/pmol), in a total volume of 50 μL. One unit of DNA polymerase α activity was defined as the amount of DNA polymerase required to convert 1 nmol of total nucleotide into acid-insoluble product in 1 h at 37 °C.

DNA Primase Assay (Conaway & Lehman, 1982). Reaction mixtures (25 μL) were 50 mM Tris-HCl, pH 8.5, 10 mM $MgCl_2$, 4 mM dithiothreitol containing bovine serum albumin at 200 μg/mL, and 200 pmol of single-stranded DNA [M13 DNA, poly(dT), poly(dC), poly(dA), or poly(dG)]. With poly(dT) as template, the mixtures contained [^{3}H]dATP (100 M; 300 cpm/pmol) and ATP (1 mM). *E. coli* DNA polymerase I (0.63 unit) was included where indicated. Incubation was at 30 °C, and reactions were terminated by addition of 1 mL of cold 10% trichloroacetic acid and 1% PP_i as described (Conaway & Lehman, 1982). One unit of DNA primase activity is the amount that catalyzes the incorporation of 1 nmol of dATP into acid-insoluble material in 60 min at 30 °C.

Protein Determinations. Protein was determined as described (Bradford, 1976); bovine serum albumin was the standard protein.

Sucrose Gradients. Sedimentation was performed on preformed 5–20% (w/w) sucrose gradients, containing 50 mM Tris-HCl, pH 7.5, 1–2 mM mercaptoethanol, 1 mM $MgCl_2$, and 250 mM KCl. In some experiments, the KCl concentration was from 0 to 500 mM as indicated in the legends to the figures.

Table I: Requirements for DNA Synthesis with Unprimed Single-Stranded Templates[a]

templates	reaction mixture	dNTP incorporated (pmol)
M13	complete	22.00
	without ATP	5.3
	+α-amanitin (2 mg/mL)	22.25
poly(dT)	complete	15.85
	without ATP	1.2
	+α-amanitin (2 mg/mL)	16.60
poly(dC)	complete	24.95
	without GTP	7.85
poly(dG)	complete	0.0
	without CTP	0.0
poly(dA)	complete	0.0
	without UTP	0.0

[a] Reaction mixtures were formulated as described under Experimental procedures, with 10 μL of a dialyzed ammonium sulfate precipitated cytosol prepared from 40 h regenerating rat liver (about 0.10 mg of proteins). When M13 was the template, the four rNTPs were present (1 mM each) in the reaction mixture. For synthetic polynucleotides, only the complementary ribonucleotide was added at the final concentration of 1 mM.

Gel Electrophoresis. Gel electrophoresis was carried out in 8 or 20% polyacrylamide slab gels containing 7.0 M urea in 90 mM Tris–borate, pH 8.3, and 2.5 mM ethylenediaminetetraacetic acid (EDTA). Samples to be analyzed were ethanol-precipitated after addition of yeast tRNA as carrier and loaded under the conditions described for DNA sequencing (Maxam & Gilbert, 1980). Following electrophoresis, the gels were autoradiographed with RX film (Fuji) at −80 °C.

Results

Regenerating Rat Liver Contains DNA Primase Activity. (A) DNA Synthesis Is Dependent on ATP or GTP and α-Amanitin-Resistant (Table I). The postmicrosomal supernatant from 40-h regenerating rat liver exhibits the ability to catalyze dNMP incorporation on unprimed single-stranded DNA templates in the presence of rNTP (Table I). Primase activity was not detectable on poly(dA) or poly(dG) templates. In the presence of M13 DNA as template, the removal of ATP decreased the incorporation by a factor 4, suggesting that ATP should be the first nucleotide incorporated, as previously shown by other groups (Conaway & Lehman, 1982; Wang et al., 1984). Addition of a high concentration of α-amanitin, a strong inhibitor of RNA polymerases II and III, did not affect the reaction.

The highest incorporation was obtained with poly(dC) as template. However, in the absence of GTP, a positive signal was also observed. To a lower extent, the same observation was made when M13 or poly(dT) was used. This finding led us to check the reliability of the primase assay with different templates along the purification of the α-polymerase.

(B) Reliability of Primase Assay with Different Templates. DNA polymerase α was purified from regenerating rat liver, following the procedure used on *Drosophila melanogaster* embryos (Kaguni et al., 1983a). With samples corresponding to different steps of the purification, primase activities have been determined in the presence of poly(dT), poly(dC), or M13 as template (Table II). Whatever the purification step, the highest incorporation was obtained with poly(dC) as template. However, with this template the dGTP incorporation was independent of the presence of GTP except for the S100 fraction. A similar observation was made many years ago in our laboratory (De Recondo et al., 1973). With M13, a ribo

Table II: Reliability of Primase Assay with Various Templates[a]

template	enzymatic fractions	reaction mixture	dNTP incorporated (pmol)
poly(dT)	S100	complete	1.35
		−ATP	0.0
	phosphocellulose	complete	6.16
		−ATP	0.0
	hydroxylapatite	complete	87.6
		−ATP	0.82
poly(dC)	S100	complete	3.76
		−GTP	0.9
	phosphocellulose	complete	18.26
		−GTP	16.90
	hydroxylapatite	complete	150.66
		−GTP	135.65
M13	S100	complete	2.79
		−rNTP	1.17
	phosphocellulose	complete	2.63
		−rNTP	0.0
	hydroxylapatite	complete	48.75
		−rNTP	5.34

[a] Reaction mixtures were formulated as described under Experimental Procedures. Ten microliters of each each fraction was used, corresponding to 0.8 unit of DNA polymerase α for the S100, 2.9 units for the phosphocellulose fraction, and 15.9 units for the hydroxylapatite fraction of a DNA polymerase α purification.

independence was observed, only with the S100 fraction, probably due to the presence of nuclease in this fraction. When polymerase I was added in the reaction mixture, the sensitivity of the different assays was greater (Conaway & Lehman, 1982), but their reliabilities were similar. So, taking into account both the level of incorporation and the ribo dependence of the reaction, the best template for the primase detection during the purification procedure appears to be the poly(dT). The following results have been obtained with poly(dT) as a template.

(*C*) *Analysis of Products Synthesized in the Coupled DNA Primase–Polymerase α Reaction.* Postmicrosomal supernatant was prepared 40 h after partial hepatectomy. After dialysis against buffer A (50 mM Tris-HCl, pH 8.5, 5 mM KCl, 5 mM 2-mercaptoethanol, 0.1 mM EDTA, and 5% glycerol), a sample was incubated with poly(dT) and [α^{32}P]ATP either with or without dATP. After denaturation, reaction products were subjected to electrophoresis in 8% (with dATP) or 20% (without dATP) polyacrylamide–urea slab gels. The products synthesized in the presence of dATP (Figure 1, panel A) were longer than those synthetized in its absence (Figure 1, panel B). The polynucleotide synthesized in the presence of dATP ranged from 70 to 90 nucleotides in length but was no longer detectable after an alkali treatment (Figure 1, panel A, right lane). After treatment of the product with DNase I, more than 90% of the labeled polynucleotides had the same mobility as a decanucleotide (not shown). When the primase reaction was carried out with [γ^{32}P]ATP (Figure 1, panel B, lane 1), the ^{32}P of [γ^{32}P]ATP was incorporated into the RNA product, showing that the 5′ end of the product remained phosphorylated. Those results suggest that both dATP and ATP were incorporated in the same polynucleotide and than the oligoriboadenylate chain provides a 3′-hydroxyl-terminated primer, which supports extensive DNA synthesis by polymerase α.

Primase Activity Is Positively Correlated with DNA Synthesis. Postmicrosomal supernatants were prepared 0, 18, 24, 43, and 72 h after partial hepatectomy. They were dialyzed extensively against buffer A in order to eliminate endogeneous nucleotides and short pieces of RNA and DNA. Total DNA polymerase and DNA primase activities were assayed on each supernatant. Both activities reached a maximum 43 h after

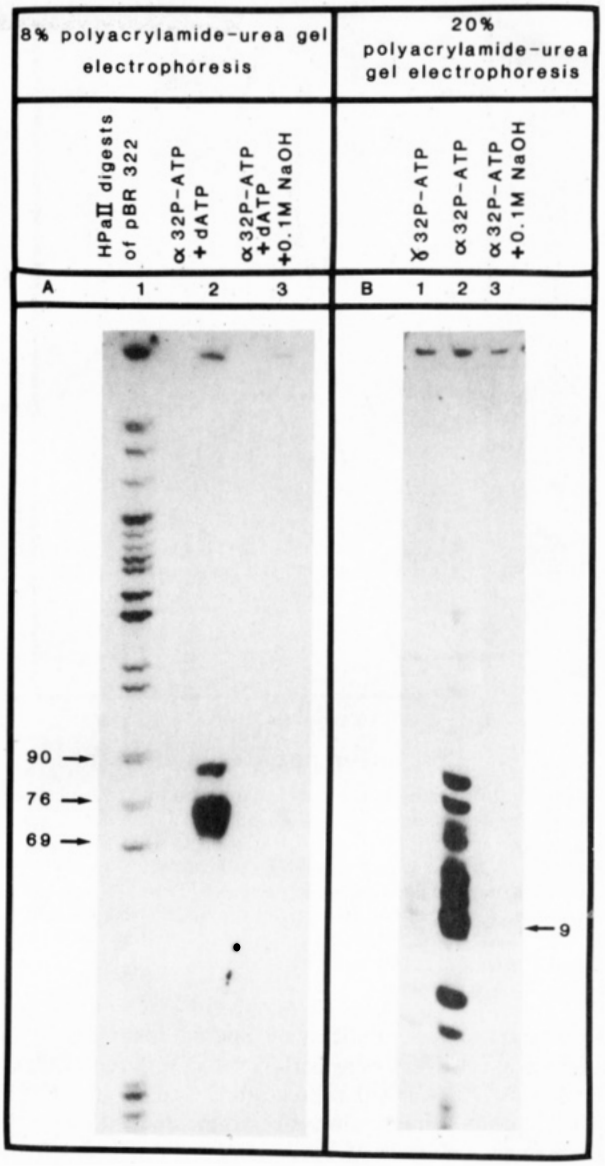

FIGURE 1: Polyacrylamide gel electrophoresis of products synthesized with poly(dT) as template. DNA synthesis and polyacrylamide–7 M urea gel electrophoresis were carried out as described under Experimental Procedures. (Panel A) 8% polyacrylamide–urea gel electrophoresis of the products synthesized in the presence of dATP and [α-^{32}P]ATP. *Hpa*II digests of pBr 322 were used as markers. Treatment with 0.1 M NaOH for 4 h at 60 °C. (Panel B) 20% polyacrylamide–urea gel electrophoresis of the products synthesized without dATP, either with [α-^{32}P]ATP or [γ-^{32}P]ATP. Alkaline treatment was as described precedently. Arrow indicates the mobility of a nonanucleotide.

partial hepatectomy. For DNA polymerase activity, only a 7-fold increase was observed because of the presence of DNA polymerase β, an enzyme that is present at a constant level during rat liver regeneration (Chang & Bollum, 1972). In order to discriminate between α- and β- polymerases, each extract was centrifuged on a 5–20% linear sucrose gradient. DNA polymerase α and primase when present were sedimenting at 7–9 S whereas β-polymerase was found around 3–4 S.

The DNA polymerase β activity level was approximately constant, as predicted. DNA polymerase α and DNA primase activities were both induced by the regeneration process. They reached a maximum value 43 h after partial hepatectomy (Figure 2).

Relation between DNA Primase and DNA Polymerase α. (*A*) *Velocity Gradients at Different Ionic Strength.* Centri-

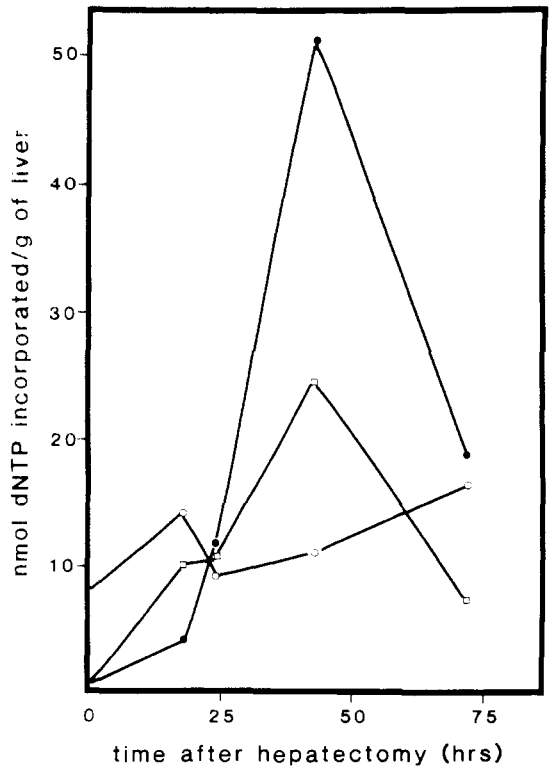

FIGURE 2: Postmicrosomal extracts corresponding to each time of regeneration were sedimented through a 5–20% linear sucrose gradient as described under Experimental Procedures. Odd fractions were assayed for DNA polymerase and DNA primase activities as described under Experimental Procedures. Exogeneous *E. coli* DNA polymerase I was added in the primase assay. Activities of the peak fraction were expressed in dNTP incorporated per gram of liver and plotted vs. the time of regeneration. (●) DNA polymerase α; (○) DNA polymerase β; (□) DNA primase.

fugation on sucrose gradients of supernatants from 40 h regenerating rat livers were performed at different KCl concentrations. At each KCl concentration used (0, 100, 250, and 500 mM), the primase always cosedimented with the DNA polymerase α. It is worth noting that primase and α-polymerase activities presented a different salt sensitivity. At a salt concentration higher than 100 mM KCl, DNA polymerase α activity is quite inhibited as previously shown (Sedwick et al., 1975; Fichot et al., 1979), whereas, primase activity could be detected only when exogenous DNA polymerase I was present in the assay. This result clearly showed that primer synthesis was less inhibited by high salt concentration than DNA synthesis.

(*B*) *Chromatographies under Different Conditions.* Attempts to dissociate primase and polymerase α by chromatography on different matrix were unsuccessful with the rat liver enzymes. In particular, we have also followed the primase activity along the purification procedure of α-polymerase from rat liver according to Kaguni et al. (1983a). At each step of the purification, primase and DNA polymerase α elution profiles were coincident. Under conditions allowing the separation of the mouse primase from α-polymerase on heparin–Sepharose (Tseng & Ahlem, 1982), both activities were eluted at the same position (Philippe et al., 1984).

Discussion

It has been established a long time (Chang & Bollum, 1972) that regenerating rat liver is one of the most convenient systems to study the relation between enzymes and DNA synthesis. In this system, it was clearly shown that the α-polymerase was closely related to DNA synthesis. On the other hand, the level of β-polymerase was constant, independent of the proliferating state of the cells (Chang & Bollum, 1972). More recently, in our laboratory (Duguet et al., 1983), it was shown that DNA topoisomerase type II activity was considerably increased in rat liver nuclei after partial hepatectomy, suggesting a possible involvement of the enzyme in DNA replication. In contrast, the topoisomerase type I level was not significantly changed during this process. We have studied the appearance of the primase as a function of regeneration time of rat liver. We have found that the DNA primase was induced after partial hepatectomy. DNA primase, like DNA polymerase α or topoisomerase II, reached a maximum value and then decreased as a function of time. It appeared that in this tissue the regulations of the three enzymes were quite similar and perhaps under a common control. A correlation between DNA polymerase α and DNA primase activities has also been made during postnatal development of the spleen and cardiac muscle of mice (Kozu et al., 1982).

DNA primase from rat liver was found to work on single-stranded DNA (M13 DNA) and on poly(dT) or poly(dC) but was completely inefficient on poly(dA) or poly(dG). This pyrimidine homopolymer specificity especially for poly(dT) template was also described for primase isolated from various species (Conaway & Lehman, 1982a; Yagura et al., 1983; Nishizawa et al., 1983; Gronostajski et al., 1984; Plevani et al., 1984; Singh & Dumas, 1984; Wang et al., 1984). From this point of view, DNA primase activity from *Xenopus laevis*, which was inefficient with poly(dT), appeared to be an exception because no synthesis was observed (Shioda et al., 1982).

The highest incorporation was obtained with poly(dC) as template, but the reaction did not remain dependent on the presence of GTP along the purification process. This fact was pointed out in our laboratory several years ago (De Recondo et al., 1973) and was not related specifically to rat liver because we have done the same observation on Chinese hamster cells in culture (not shown). However, this did not appeared to be true for all materials (Yagura et al., 1982; Nishizawa et al., 1983; Kozu et al., 1983; Gronostajski et al., 1984), but the GTP dependence of the reaction required a specific protein factor called stimulating factor (Yagura et al., 1982; Kozu et al., 1983).

The sensitivity of the DNA primase and DNA polymerase α to salt concentration was found to be different. In particular, at 150 mM KCl, DNA polymerase α was quite inhibited while primase was still measurable. A different result was reported for the same enzymes isolated from KB cells (Wang et al., 1984). This could be due to the different degrees of purity of the enzyme fractions used in both cases.

The measurement of the sedimentation coefficient of the primase and DNA polymerase α at different ionic strengths (KCl from 0 to 500 mM) showed a close cosedimentation of both enzymes. As found by Hübscher (1983), a cosedimentation was also observed when urea (1.7 M) was used in the gradient instead of KCl (not shown). When the urea concentration was increased (2.8 M), according to Kaguni et al. (1983b), an irreversible inhibition of both activities was obtained (not shown).

Measurement of primase activity during purification of DNA polymerase α by phosphocellulose, hydroxylapatite, and single-stranded DNA ultrogel chromatography showed a coincidence of primase and α-polymerase activities in agreement with the results of Conaway and Lehman (1982). On the contrary, Tseng and Ahlem (1983), using a similar purification procedure, obtained a clear separation between primase and

α-polymerase activities. This difference may be due to some proteolysis of components of the replication complex, which makes easier the dissociation of the subunits in the case of the mouse (Tseng & Ahlem, 1983) compared to our case or to *Drosophila* (Conaway & Lehman, 1982). Chromatography on heparin–Sepharose did not allow any separation either between primase and α-polymerase from rat liver or from Balb/C-3T3 *ts2* cells at the permissive temperature. But, a clear separation was obtained with extracts prepared from the *ts2* mutants at the nonpermissive temperature (Philippe et al., 1984). It is unclear at the moment if this result is directly related to the mutation or to a higher level of proteolysis at the nonpermissive temperature.

Our suggestion is that α-polymerase and primase, which are similarly induced in proliferating cells, are tightly associated with other polypeptides of unknown function in a replication complex. The subunits are very closely linked, but proteolysis can occur very soon during the purification procedure, as shown by Sauer and Lehman (1982), affecting more or less one polypeptide or another. Under these conditions, the dissociation of the replication complex can occur, and subspecies of α-polymerase can also appear. At the present time, several authors (Tseng & Ahlem, 1982, 1983; Kaguni et al., 1983; Philippe et al., 1984; Plevani et al., 1984) have shown that, under appropriate conditions, primase and α-polymerase could be separated from each other, demonstrating that the activities are on separated subunits.

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