Human DNA polymerase α catalytic polypeptide binds ConA and RCA and contains a specific labile site in the N-terminus

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ABSTRACT

The catalytic polypeptide of DNA polymerase α is often observed *in vitro* as a family of phosphopolypeptides predominantly of 180 and 165 kDa derived from a single primary structure. The estimated Mr of this polypeptide deduced from the full-length cDNA is 165 kDa. Immunoblot analysis with polyclonal antibodies against peptides of the N- and C-termini of the deduced primary sequence indicates that the observed family of polypetides from 180 kDa to lower molecular weight results from proteolytic cleavage from the N-terminus. Antibodies against the N-terminal peptide detect only the 180 kDa species suggesting that this higher molecular weight polypeptide may be the result of posttranslational modification of the 165 kDa primary translation product. The catalytic polypeptide is not only phosphorylated but is also found to react with lectins ConA and RCA. N-terminal sequencing of the isolated catalytic polypeptide from human cells and of the recombinant fusion proteins indicates that the often observed 165 kDa polypeptide is the *in vitro* proteolytic cleavage product of the modified 180 kDa protein at the specific site between lys_{123} and lys_{124} within the sequence -RNVKKLAVTKPNN-.

INTRODUCTION

DNA polymerase α purified from species as distinct as unicellular fungi and mammals displays a remarkably similar set of constituent polypeptides (1−7). Each contains a cluster of large polypeptides predominantly 165 to 180 kDa with catalytic function, a 70 kDa protein of unknown function and two polypeptides, 55 and 49 kDa, reported to contain the primase activity (5−10). Peptide mapping indicates that the catalytic polypeptides, 180 and 165 kDa have identical peptide maps (6). This finding suggests that these high molecular weight polypeptides are derivatives of a single primary translation product. The full length cDNA from human cells and a genomic clone from *S. cerevisiae* encoding the catalytic polypeptide have been isolated (11−13). The primary sequence deduced from human or from yeast genetic sequences predicts a molecular mass of 165 kDa (11,13). In this report, we have used polyclonal antisera against two synthetic peptides corresponding to the N- and C-termini of the catalytic polypeptide, lectin binding assays, and amino acid sequence analysis of the N-terminal portion of the protein to resolve the discrepency of the observed and the predicted molecular mass of DNA polymerase α catalytic polypeptide.

MATERIALS AND METHODS

Production of antibodies against synthetic peptides

Two amino acid sequences of 20 residues corresponding to the N- and C-termini of human DNA polymerase α catalytic polypeptide were chosen as peptide antigens based on the criteria described (14). The peptides were synthesized with an Applied Biosystems Automated Peptide Synthesizer (ABI, 430) with an additional cysteine at the C-terminus and then conjugated to myoglobin or ovalbumin according to the method of (15). Two hundred fifty micrograms of each conjugated peptide were used to immunize New Zealand white rabbits according to the described protocol (16). Antisera titer were screened by ELISA assay and further characterized by immunoblotting with human DNA polymerase α purified by monoclonal antibody immunoaffinity chromatography (5,6,17). Antiserum against the N-terminal peptide is designated DPN; while antiserum against the C-terminus is designated DPC.

Detection of polymerase α catalytic polypeptide with antisera and lectins

Immunoaffinity purified DNA polymerase α polypeptides were subjected to gel electrophoresis and transferred to Problott membrane (Applied Biosystems, Inc.) as described (18). Detection of catalytic polypeptide with antisera DPN or DPC was performed according to (16) with goat anti-rabbit IgG conjugated with horseradish peroxidase (Bio-Rad Laboratories) as secondary antibody followed by developing in 3,3'-diaminobenzidine and peroxide as decribed (16). For lectin binding, the membranes were incubated with 10 μg/ml of biotinylated lectins (Vector Laboratories, CA) for 1 to 2 hours and developed with precomplexed avidin-biotin-peroxidase

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according to the manufacturer's instruction (Vector Laboratories, CA). For ConA and LCA bindings, 1 mM Ca^{+2} and Mn^{+2} were added to each incubation, respectively.

Production of recombinant human DNA polymerase α as fusion proteins

Two plasmids, pBC509 and pBC943, that express the N-terminal portion of human DNA polymerase α fused with β-galactosidase protein under control of the T7 RNA polymerase promoter were constructed. Both plasmids were constructed by linker ligating the 5′ NcoI site of E1−19 cDNA clone (11) with EcoRI linkers followed by subsequent digestion with EcoRI and either Sau3AI or BclI. The 509 bp EcoRI-Sau3AI and the 943 bp EcoRI-BclI restriction fragments from E1−19 were separately ligated to the 6.2 Kb BamHI-SalI restriction fragment from pMC874 (19) and inserted into the EcoRI-SalI digested pT7−7 vector (20). Induction of the fusion proteins in *E. coli* was accomplished by a modified procedure of Tabor and Richardson (20) and purified from the cleared cell lysate using p-aminophenyl β-D-thiogalactopyranoside-agarose (APTG, Sigma) as described (21). Purified fusion proteins were subjected to gel electrophoresis and transferred to Problott membrane for either amino acid sequence analysis or immunoblotting with DPN as described above.

N-terminal amino acid sequence analysis

Human DNA polymerase α protein from KB cells (6) and DNA polymerase α-/β-galactosidase fusion protein were subjected to electrophoresis and transferred onto Problott membrane. Protein bands of interest were excised and subjected to automated Edman degradation performed on an Applied Biosystems Model 477A gas phase sequencer with an on-line PTH-amino acid analyser Model 120A, using the NORMAL-1 program (22).

Other methods

The amount of immunoaffinity purified DNA polymerase α protein applied to each gel electrophoresis is estimated in terms of units of polymerase α activity. One unit of polymerase α activity is defined as the amount of enzyme that catalyzes the incorporation of 1 nmole of labeled dNMP into acid-insoluble product in 1 hour at 37°C under standard assay conditions (26). By densitometric scanning of Coomassie stained gels loaded with known units of DNA polymerase α, it was previously estimated that 1 unit of polymerase α activity is equivalent to 5 ng of protein

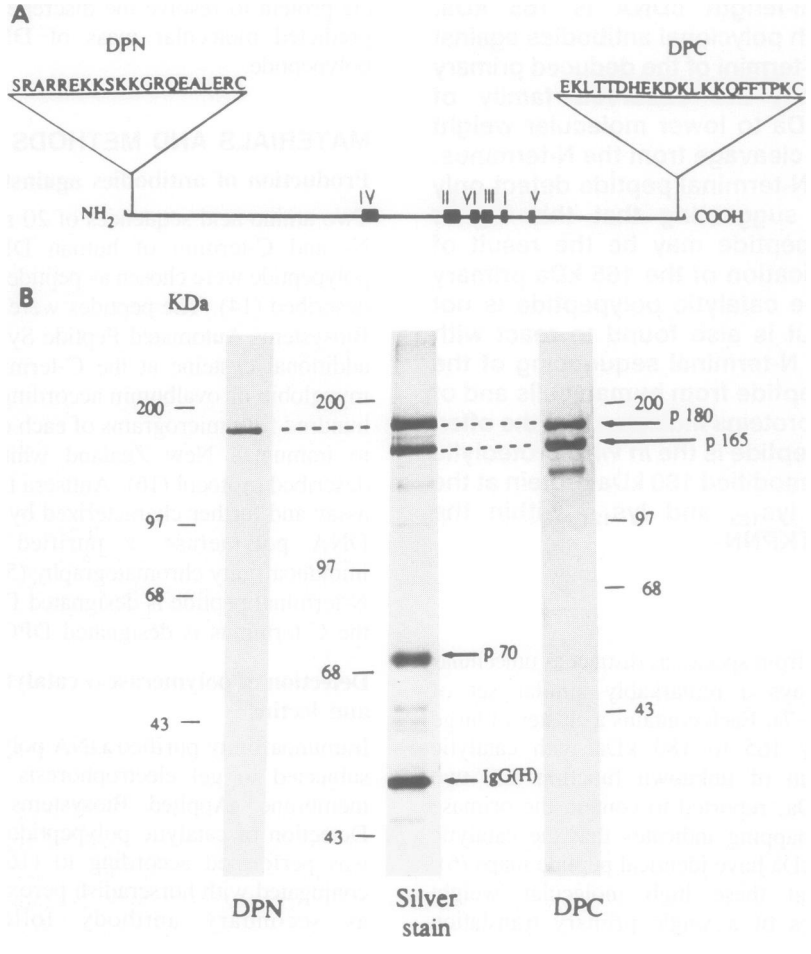

Figure 1. A. Schematic diagram of position of peptide antigens in the predicted primary protein sequence of human DNA polymerase α for antisera, DPN and DPC. DPN was raised against a peptide of 20 amino acids beginning at 19 amino acids from the translation start site. DPC was raised against a 20 residues peptide 37 amino acids upstream from the C-terminus stop codon. The line represents the entire 1462 amino acid sequence derived from the full-length cDNA, the solid boxes depict the six conserved regions (11, 23). B. Immunoblot of DNA polymerase α by antisera DPN and DPC. Sixty units of immunopurified human DNA polymerase α were immunoblotted by antisera DPN or DPC, and used in silver stain for comparison as described in Materials and Methods. Protein markers of 200, 97, 68 and 43 kDa are myosin, phosphorylase b, bovine serum albumin and ovalbumin, respectively.

containing the four subunits of the polymerase α/primase complex.

α-D-mannosidase, α-D-glycosidase and β-D-galactosidase are from Boehringher-Mannhein, Biochemicals and used according to the manufacturer's instruction.

RESULTS

DNA polymerase α is degraded from the N-terminus

DNA polymerase α purified either by conventional biochemical methods or by an immunoaffinity protocol with monoclonal antibody contains: a family of high molecular weight polypeptides of 165 to 180 kDa with catalytic function, a 70 kDa polypeptide of unknown function and two polypetides of 55 and 49 kDa reported to be associated with DNA primase activity, as shown in Figure 1B (4−6,11). Previous studies have demonstrated that the two predominant high molecular weight members of the catalytic polypeptide family have identical peptide maps (6). The molecular mass difference between these two catalytic polypeptides (p180 and p165) can be explained either by differential modification or N- or C-terminal degradation of a single primary translation product. The full length cDNA of human DNA polymerase α catalytic polypeptide encodes a 165 kDa protein (11,23; Pearson, Nasheuer and Wang, submitted). Genomic sequence of the yeast PolI, a homolog of polymerase α, also predicts a protein of 167 kDa (13).

To resolve the difference between the observed and the predicted molecular mass of DNA polymerase α catalytic polypeptide, purified human DNA polymerase α immobilized on membrane was incubated with polyclonal antisera raised against 20 amino acid residue synthetic peptides corresponding to sequences at the N- or C-termini, Figure 1A. The antisera against the N-terminus, DPN, recognized epitopes on a single high molecular weight polypeptide of 180 kDa (p180), while antisera against the C-terminus, DPC, recognized epitopes on both 180 and 165 kDa polypeptides (p180 and p165), Figure 1B. Two smaller polypeptides of this catalytic polypeptide cluster with apparent molecular masses of 140 and 125 kDa which are occasionally observed were also found to contain the DPC epitopes but not DPN epitopes (Fig. 1B). These results indicate that the p180 contains the N-terminal sequence recognized by DPN antisera, whereas p165, p140 and p125 proteins lack this N-terminal epitope. This result in addition to the observation that full-length recombinant human polymerase α when expressed in E. coli yielded a 165 kDa polypeptide (Copeland and Wang, unpublished observation), strongly suggest that the p180 is a modified form of the predicted 165 kDa primary translation product of DNA polymerase α transcript, while p165, as well as p140 and p125 polypeptide, are N-terminally degraded products of the p180.

DNA polymerase α catalytic polypeptide is specifically bound by the lectins ConA and RCA

It is possible that the observed higher molecular mass catalytic polypeptide is due to posttranslational modification. We have previously reported that p180/p165 and p70 of DNA polymerase α enzyme preparations are phosphoproteins (6). The recent findings that some nuclear proteins are glycoproteins (24) prompted us to test whether the catalytic polypeptide can bind lectins. Membranes bearing immunoaffinity purified human DNA polymerase α proteins were treated with eight biotinylated lectin derivatives: concanavalin A (ConA), which primarily recognizes α-D-mannoside and α-D-glucoside residues; wheat germ agglutinin (WGA), which recognizes N-acetylglucosamine residues; dolchos biflorus agglutinin (DBA), specific for α-linked N-acetylgalactosamine; lens culinaris agglutinin (LCA), specific for α-linked fucose residue attached to the N-acetylchitobiose portion of the core oligosaccharide; soybean agglutinin (SBA) which binds to oligosaccharide structures with terminal α- or β-linked N-acetylglucosamine or galactose residues; peanut agglutinin (PNA) which recognizes galactosyl (β-1,3) N-acetylgalactosamine; ricinus communis agglutinin I (RCA) which preferentially binds to oligosaccharides ending in galactose and also binds to N-acetylgalactosamine; and Aulex europeaeus agglutinin (UEA), specific for glycoproteins containing α-linked fucose residues (25). Binding of ConA and RCA to both p180 and p165 was observed, Fig 2A. In the presence of 0.2 M α-D-mannoside, a competitive inhibitor of ConA binding, or 0.2 M α-D-galactoside, a competitive inhibitor of RCA binding, reactivity with ConA or RCA, respectively, was completely abolished, as shown in Fig. 2B, lane 2 and 5. ConA binding could likewise be competitively abolished by α-D-glucoside (data not shown). The inability of ConA or RCA to bind to these polypeptides in the presence of these inhibitors is not caused by removal or absence of the polymerase α protein on the membrane, because these membranes did not lose the ability to bind antisera raised against the C-terminus, DPC, as demonstrated in Fig. 2B, lane 3 and 6. In addition, it is unlikely that the ConA reactivity is due to comigration of another glycoprotein of 180 and 165 kDa sizes, since covalently linked ConA-Sepharose is able to deplete DNA polymerase α activity from a partially purified enzyme fraction (data not shown).

To further define the specificity of the ConA and RCA interaction, purified polymerase α were incubated with three glycosidases, α-mannosidase, α-glucosidase and β-galactosidase prior to ConA or RCA binding. Preincubation with α-glucosidase did not abrogate the ConA binding, whereas preincubation with α-mannosidase completely abolished the binding of ConA (Fig. 3A, lane 2 and 3, respectively). This result suggests but does not rigorously prove that the ConA binding moiety on this polypeptide is α-D-mannoside. Treatment with β-galactosidase abolished binding by lectin RCA, as shown in Fig 3B, lane 2. As with the binding competition experiments, the inability to bind ConA or RCA after α-mannosidase or β-galactosidase treatment was not due to removal or absence of polymerase α proteins from the membrane, because either enzyme treatment did not diminsh the binding of DPC to p180 or p165 (Fig. 3A, lane 4 and Fig. 3B, lane 3). It should be noted that in the polymerase α enzyme fraction only the catalytic polypeptide reacts with ConA and RC-A. The 70 kDa subunit and the two primase related subunits did not bind to any of the eight biotinylated lectins tested.

Proteolytic cleavage occurs at a specific labile site of the N-terminus

p165 is often reproducibly observed in DNA polymerase α preparations from a variety of eukaryotic cell sources. Results of immunoblots with antisera DPN and DPC strongly suggest that p165 is a proteolytic degradation product of p180 (Figure 1). To investigate this proteolytic event in detail, the N-terminus of p165 isolated from KB cells by the immunoaffinity protocol was sequenced and found to be KLAVTKPN-, Table 1. Two fusion proteins each containing overlapping 5′ portions of the polymerase α cDNA fused with the lacZ gene at the 3′ end were expressed from the pT7−7 vector in *E. coli*, purified, and analyzed on SDS gels. For each construct, more than one major

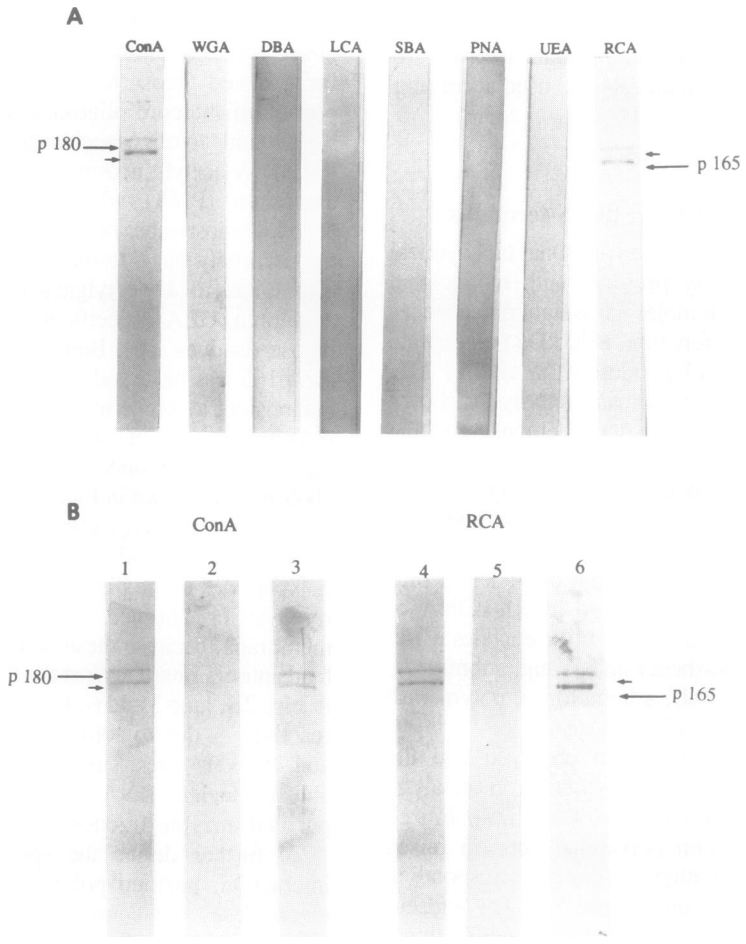

Figure 2. Detection of DNA polymerase α catalytic polypeptide by lectin. A. Immunopurified human DNA polymerase α, 90 units, were probed with biotinylated lectins as described in the Materials and Methods. Lectins used are depicted on top of each Problott membrane strip. B. Competition of ConA and RCA binding. Membranes containing purified DNA polymerase α samples, 90 units each, were incubation with ConA or RCA in the absence of competitive sugar (lane 1 for ConA and lane 4 for RCA), or in the presence of 0.2 M α-methyl-D-mannoside (lane 2) for ConA, or 0.2 M β-D-galactoside (lane 5) for RCA. DNA polymerase α polypeptides were detected by antisera DPC on Problott membranes bearing DNA polymerase α after preincubation with ConA in the presence of α-methyl-D-mannoside (lane 3) or after preincubation with RCA in the presence of β-D-galactoside (lane 6).

Table I

Mapping a Specific Labile Site in the N-Terminal Peptides of Human DNA Polymerase α

Cycle No.	KB DNA pol α p165		pBC 509 p118		pBC 943 p135	
	Residue	pmol	Residue	pmol	Residue	pmol
1	K	2.1	K	182	K	30
2	L	1.9	L	206	L	32
3	A	3.4	A	287	A	31
4	V	2.2	V	201	V	27
5	T	1.9	T	230	T	12
6	K	2.8	K	132	K	27
7	P	2.9	P	168	P	23
8	N	3.1	N	162	N	22
9			N	248	N	28
10			I	142	I	19

Sequence analysis is described in Materials and Methods. Shown are the phenylthiohydantoin (PTH)-amino acids observed at each cycle. Yield is presented in pmoles.

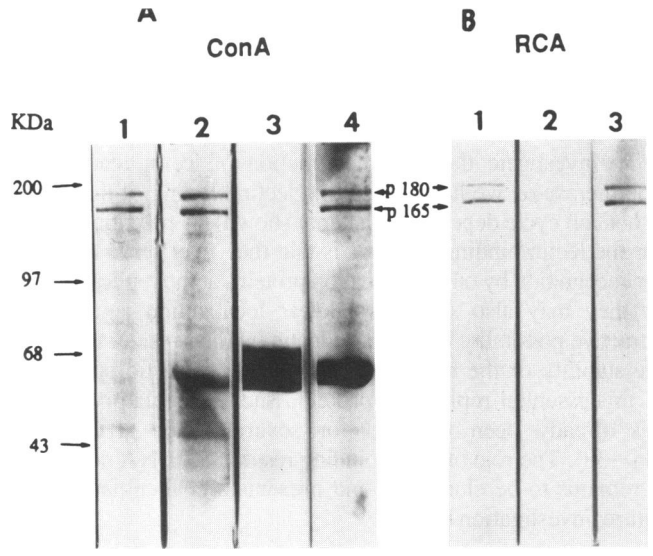

Figure 3. ConA and RCA binding specificity. A.Immunoaffinity purified DNA polymerase α, 120 units, was incubated with no glycosidase (lane 1), 2.5 units of α-glucosidase (lane 2), or 5.0 units of α-mannosidase (lane 3 and lane 4) for 20 hours at 37°C under the conditions recommended by Boehringer Mannheim Biochemicals. These glycosidase treated DNA polymerase α samples were then subjected to SDS gel electrophoresis, transferred to Problot membrane and ConA binding as described in Figure 2. Lane 4 demonstrates the detection of DNA polymerase α polypeptide by antisera DPC after the sample was treated with α-mannosidase and stained with ConA as described in lane 3. The lower molecular weight ConA binding proteins in lane 2, 3 and 4 are α-glucosidase and α-mannosidase which are both glycoproteins and bind to ConA. Depending on the batch of ConA used, minor ConA binding bands in the lower molecular weight range were occasionally observed. B. RCA binding to purified DNA polymerase α (90 units) after incubation with no β-galactosidase (lane 1) and with 2.2 units of β-D-galactosidase (lane 2). Detection of DNA polymerase α polypeptide by antisera DPC after β-galactosidase treatment (lane 3).

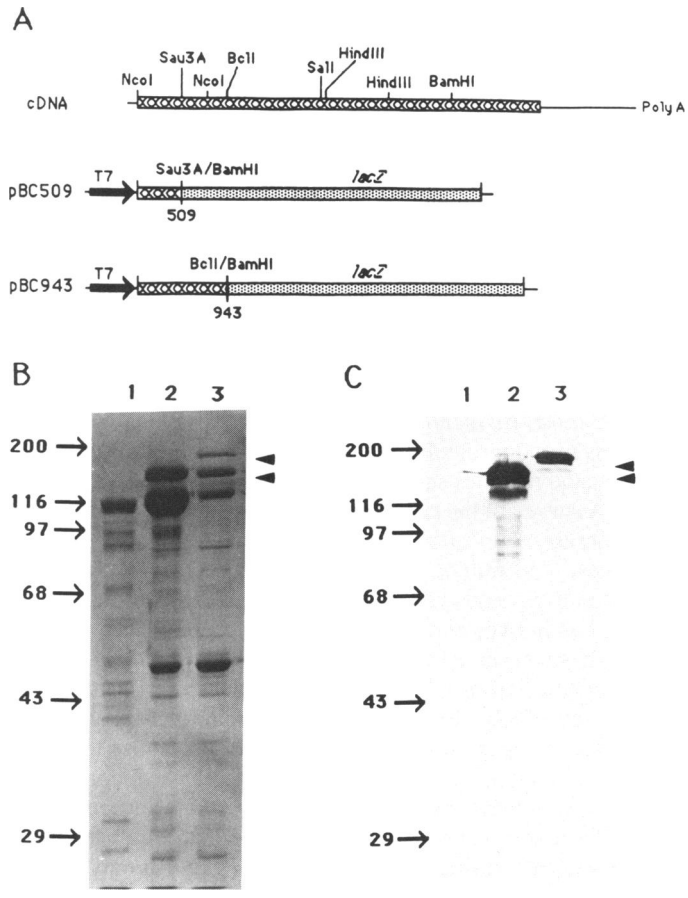

Figure 4. N-terminal peptides of human DNA polymerase α in recombinant fusion proteins. A. Schematic diagram of N-terminal peptide fusion protein constructs. The top diagram is the restriction map of the full-length cDNA of human DNA polymerase α. pBC509 and pBC943 were constructed as described in the Materials and Methods. The heavy arrow is the T7 RNA polymerase promoter, while the N-terminal portion of polymerase α and the lacZ gene are illustrated in different patterns. Number 509 and 943 represent the cDNA nucleotide numbers which encode the portions of N-terminal amino acids of DNA polymerase α in these fusion constructs. B. Recombinant proteins produced from the fusion constructs. Lane 1 demonstrates 5 μg of β-galactosidase, lane 2 and 3 are 25 and 10 μg of affinity purified fusion proteins from pBC504 and pBC943, respectively. DNA polymerase α fusion proteins expressed from each construct are indicated by solid triangles. C. Immunoblot of polymerase α N-terminal fusion proteins by DPN. Lane 1 is β-galactosidase, lane 2 and 3 are 25 and 10 μg of expressed fusion proteins from pBC509 and pBC943, respectively. Locations of the immunoreactive proteins to DPN are indicated by solid triangles.

protein species was isolated by APTG-affinity column, as shown in Figure 4B. Expression of pBC509 yielded two protein bands of 135 and 118 kDa (p135 and p118) as shown in Fig 4B, lane 2, while pBC943 yielded three protein bands of 151, 135 and 118 kDa (p151, p135 and p118), as shown in Fig 4B, lane 3. Immunoblotting with DPN antisera revealed that only the largest proteins from each construct (i.e. p135 from pBC509 and p151 from pBC943) contain the N-terminal epitopes. The smaller proteins, p118 from pBC509 or p135 and p118 from pBC943, do not contain the epitopes recognized by DPN antisera, as shown in Fig. 4C, lane 2 and 3. Interestingly, the 15 kDa molecular mass decrement within each set of fusion proteins closely matches the difference between the observed polymerase α proteins (p180 and p165) isolated from KB cells. Sequence analyses of fusion proteins p135 of pBC934 and p118 of pBC509 revealed N-terminal amino acid sequence identical with that of p165 isolated from KB cells (Table 1). These sequence analyses strongly suggest the presence of a specific labile site between lys_{123} and lys_{124} within the N-terminal domain -RNVKKLAVTKPNN- of human polymerase α catalytic polypeptide. Cleavage of p180 at this labile site generates the often observed p165.

Taken together, the results of anti-peptide antibody studies, lectin binding and N-terminal sequence analyses support the conclusion that posttranslational modification of a 165 kDa primary translation product results in a protein of apparent molecular mass of 180 kDa, and that subsequent proteolytic cleavage of the modified p180 species at a labile site within the N-terminus gives rise to the protein with an apparent molecular mass of 165 kDa which is commonly observed in purified DNA polymerase α.

DISCUSSION

The reproducible observation of the catalytic polypeptide as a family of proteins with apparent molecular mass differences of approximately 15 to 20 kDa has been a perplexing issue. Recently, we have isolated a genomic clone which spans the 5′ end of this gene and 1.62 kb of sequence upstream from the translation start site (Pearson, Nasheuer and Wang, submitted). Characterization of the genomic clone has confirmed that the cDNA previously isolated is indeed full-length (11), and thus that the predicted molecular mass of the protein encoded by the full-

length cDNA is 165 kDa. This knowledge enables us to address the polymerase α structural problem at the molecular level.

We have previously reported the presence of six regions in the primary sequence of DNA polymerase α which are conserved among replicative polymerases of prokaryotes and eukaryotes (11, 23). The six domains have provisionally been designated as the substrate binding domains and a cysteine-rich region towards the C-terminus is predicted to be the DNA binding domain (11,23). These predicted functional domains are all localized towards the C-terminal region of polymerase α protein. Thus, it is reasonable to assume that cleavage at labile site(s) near the N-terminus may not affect the DNA polymerizing activity. This is in agreement with an earlier finding of active DNA polymerase α of molecular mass as low as 66 to 77 kDa (26).

The most frequently observed posttranslational proteolytic process is endoproteolysis at the carboxyl side of a pair of basic amino acid residues such as lys-arg, arg-arg, arg-lys and lys-lys (27). A survey of the predicted primary sequence of human DNA polymerase α reveals the presence of numerous pairs of basic residues. The finding that the cleavage occurred reproducibly between lys_{123} and lys_{124} in proteins purified from human cells as well as in over-expressed recombinant proteins from *E. coli* strongly suggests that the bond between lys_{123} and lys_{124} can be distinguished from the other basic residue pairs in the protein and is selectively cleaved. At present we do not know whether there are endopeptidase(s) that specifically recognize this lys-lys pair *in vivo* or *in vitro*. Alternatively, the physical constraint of the protein folding may cause the lability at this particular lysine pair. The latter seems likely as prompt and careful manipulation of the enzyme during purification often results in only the p180 being present.

Evidence in the past has led to the belief that glycoproteins are primarily restricted to the cell surface and lumenal compartments of the cell. The covalent attachment of most types of glycosyl residues occurs within the lumenal compartments of the endoplasmic reticulum or Golgi apparatus, from which nucleoplasmic and cytoplasmic proteins are excluded. Recently, several reports have described a novel type of glycosylation in which N-acetylglucosamine is O-glycosidically attached to proteins (24,28,29). Remarkably, these O-linked N-acetylglucosamine residues have been found on proteins localized to the cytoplasmic and nucleoplasmic compartments of the cell, including proteins at the faces of nuclear pores, cytoskeletal proteins and transcription factors (24,30). There are also reports of unusual types of O-linked mannose oligosaccharides localized in the cytoplasm (Reviewed in 24). It is interesting that this nuclear replicative polymerase (31) reacts with ConA and RCA which suggests that polymerase α catalytic polypeptide is a glycoprotein.

Data presented in this report suggest that the lectin binding residues are exo-glycosyl residues, since both glycosidases are exoglycosidase and removal of the lectin binding residues does not contribute to the 15 kDa shift (Figure 3A). Attempts have been made to reproduce a report of growing cells in 30 μg/ml of tunicamycin to inhibit glycosylation of cellular proteins which claimed to have a decrease of DNA polymerase α activity (32). Polymerase α protein from tunicamycin-treated cells showed diminished binding to ConA as compared to the proteins from untreated control cells. However, evaluation by antisera staining with DPC or DPN indicated that the tunicamycin-treated cells reproducibly contained much less polymerase α protein per cell (data not shown). It is known that tunicamycin at high concentration such as 30 μg/ml inhibits protein translation (33). Thus, the interpretation in the previous report (32) implicating polymerase α from tunicamycin treated cells having decrease enzymatic activity is possibly due to the decrease of polymerase α protein.

To investigate the possible function of lectin reactivity on polymerase α, we have found that lectin binding of this protein is not cell cycle dependent (data not shown). A potential function for the lectin binding residues is that they may serve as ligands for recognition by other replication proteins at the replication fork, or they may also serve as nuclear localization signals. One attractive possibility is that these lectin binding residues modulate the stability or the nascent folding of the catalytic polypeptide of this essential replicative protein. Such a regulatory function has already been reported for several other glycoproteins (34–38). The role of lectin binding residues of DNA polymerase α remains to be elucidated and presents a challenging task for future investigation.

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