# Primary Structure of the Catalytic Subunit of Calf Thymus DNA Polymerase δ: Sequence Similarities with Other DNA Polymerases†,‡

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ABSTRACT: The 125- and 48-kDa subunits of bovine DNA polymerase δ have been isolated by SDS–polyacrylamide gel electrophoresis and demonstrated to be unrelated by partial peptide mapping with *N*-chlorosuccinimide. A 116-kDa polypeptide, usually present in DNA polymerase δ preparations, was shown to be a degraded form of the 125-kDa catalytic subunit. Amino acid sequence data from *Staphylococcus aureus* V8 protease, cyanogen bromide, and trypsin digestion of the 125- and 116-kDa polypeptides were used to design primers for the polymerase chain reaction to determine the nucleotide sequence of a full-length cDNA encoding the catalytic subunit of bovine DNA polymerase δ. The predicted polypeptide is 1106 amino acids in length with a calculated molecular weight of 123 707. This is in agreement with the molecular weight of 125 000 estimated from SDS–polyacrylamide gel electrophoresis. Comparison of the deduced amino acid sequence of the catalytic subunit of bovine DNA polymerase δ with that of its counterpart from *Saccharomyces cerevisiae* showed that the proteins are 44% identical. The catalytic subunit of bovine DNA polymerase δ contains the seven conserved regions found in a number of bacterial, viral, and eukaryotic DNA polymerases. It also contains five additional regions that are highly conserved between bovine and yeast DNA polymerase δ, but these regions share little or no homology with the α polymerases. Four of these additional regions are also highly homologous to the herpes virus family of DNA polymerases, whereas one region is not homologous to any other DNA polymerase that has been sequenced thus far. The polypeptide also contains two C-terminal clusters of cysteine residues postulated to be DNA binding sites or zinc fingers.

Although DNA polymerase α (pol α) has been generally held to be solely responsible for replication of chromosomal DNA in eukaryotic cells, recent evidence suggests that DNA polymerase δ (pol δ) is also required for replication of eukaryotic chromosomes and that pol α and pol δ have distinct roles at the replication fork [for reviews see Challberg and Kelly (1989), Stillman (1989), Burgers (1989), So and Downey (1991), Thommes and Hubscher (1990), and Wang (1991)]. Genetic studies in the budding yeast *Saccharomyces cerevisiae* have shown that yeast pol α (formerly called pol I) and yeast pol δ (formerly called pol III; Burgers et al., 1990) are encoded by essential genes: yeast pol α by *POL1* (formerly called *CDC17*; Johnson et al., 1985) and yeast pol δ by *POL3* (formerly called *CDC2*; Boulet et al., 1989; Sitney et al., 1989; Blank & Loeb, 1991). Temperature-sensitive mutants of either pol α (Budd & Campbell, 1987; Pizzagalli et al., 1988) or pol δ (Conrad & Newlon, 1983) are defective in DNA replication and arrest during the S phase of the cell cycle when grown at a nonpermissive temperature. Recently, a third DNA polymerase, yeast pol ε (pol II), was shown to be essential for cell viability in *S. cerevisiae* (Morrison et al., 1990). Deletion of the gene coding for pol ε (*POL2*) resulted in a terminal morphology characteristic of an S-phase arrest. Accordingly, it has been suggested that yeast pol ε may also be required for chromosomal replication.

Studies on the in vitro replication of plasmids containing the simian virus 40 (SV40) origin of replication using highly purified mammalian replication proteins and SV40 large T antigen have also demonstrated that both pol α and pol δ are required for SV40 DNA replication and, by implication, for cellular DNA replication (Weinberg et al., 1990; Tsurimoto et al., 1990). It was demonstrated that synthesis of both leading and lagging stands is observed only when both pol α and pol δ are present in the replication system. Omission of pol δ or its accessory proteins resulted in the loss of leading strand synthesis, while the synthesis of short lagging strand fragments continued. Thus it is currently believed that pol δ is the leading strand replicase and pol α, with its tightly associated DNA primase, is required for synthesis of the lagging strand as well as for initiation at origins of replication.

Although significant progress has been made in elucidating the role of pol δ in eukaryotic DNA replication, detailed knowledge of the expression of this protein during the cell cycle and identification of the cis- and trans-acting elements which regulate its expression require that the nucleotide sequence of the gene and its upstream regulatory elements be determined. Furthermore, a comparison of the primary structure of the protein with those of other DNA polymerases may facilitate our understanding of the functional domains of the polymerases as well as the relationships among them. As a first step toward these goals, the sequence of a full-length cDNA coding for the catalytic subunit of calf thymus pol δ has been determined.

## Materials and Methods

*Protein Purification and Partial Amino Acid Sequences.* Pol δ was purified to apparent homogeneity from fetal calf thymus as described previously (Lee et al., 1984; Ng et al., 1991). To isolate the individual subunits, the protein was

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reduced and electrophoresed in a 6.5% SDS–polyacrylamide slab gel according to the method of Laemmli (1970) using a Hoeffer SE600 unit. Gel slices containing either the 125-kDa subunit, a degradative form of it (116 kDa), or the 48-kDa subunit were treated with *N*-chlorosuccinimide according to Lischwe and Ochs (1982). The peptides were electrophoresed in a second 8–18% gradient SDS–polyacrylamide gel and stained with silver (Wray et al., 1981). For the preparation of peptides for amino acid sequence determination, gel slices containing both the 125- and 116-kDa polypeptides were digested in situ with either cyanogen bromide (Sigma) (Nikodem & Fresco, 1979) or *Staphylococcus aureus* V8 protease (Boehringer) (Cleveland et al., 1977). The peptides were electrophoresed in a second 16% SDS–polyacrylamide gel according to Schagger and von Jagow (1987) and blotted onto a PVDF membrane (Matsudiara, 1987), and individual peptides were sequenced by automated Edman degradation on an Applied Biosystems 470A protein sequencer. In other experiments the 125- and 116-kDa polypeptides were electroeluted from gel slices, dialyzed, alkylated, and precipitated according to the method of Matsudiara (1989). The polypeptides were then digested with TPCK-treated trypsin (Boehringer) or *S. aureus* V8 protease. The peptides were separated by HPLC on a microbore C8 column (Brownlee Lab) and sequenced as described above.

*Oligonucleotide Synthesis.* Oligonucleotides were synthesized by the phosphoramidite method on an Applied Biosystems 380B DNA synthesizer. The oligonucleotides used for DNA amplification were designed with an *Eco*RI or *Bam*HI restriction site at the 5′ ends to facilitate subsequent cloning. The sequences of the oligonucleotides and the corresponding peptides from which they were derived are shown in Table I. Additional synthetic oligonucleotides with sequences identical to regions of the partial bovine pol δ sequence are also shown in Table I.

*Preparation of cDNA.* Total cellular RNA was prepared from freshly frozen fetal calf thymus (Antech) by the guanidinium isothiocyanate method (Chomczynski & Sacchi, 1987), and poly(A) RNA was twice selected by chromatography on oligo(dT)–cellulose (Sambrook et al., 1989). Poly(A) RNA was reverse transcribed by Moloney murine leukemia virus (M-MLV) reverse transcriptase (BRL) in the presence of an oligo(dT)- containing primer, EDT, as described (Sambrook et al., 1989).

*DNA Amplification.* Amplification was performed in a Perkin-Elmer Cetus thermal cycler, and the reaction (100 μL) contained 10 mM Tris-HCl, pH 9.0, 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% (w/v) gelatin, 0.1% Triton X-100, 200 μM solution of each dNTP, 100 pmol of each of two oligonucleotide primers, 10–100 ng of cDNA, and 2.5 units of Taq polymerase (Promega). The samples were denatured at 94 °C for 1 min, annealed at 55 °C for 2 min, and extended at 72 °C for 3 min for 30 cycles. PCR-amplified DNA fragments were extracted with phenol/chloroform and ethanol precipitated. The DNA was digested with appropriate restriction enzymes to generate cohesive ends and fractionated by electrophoresis in a 1% low melting point agarose gel, and the appropriate DNA bands were excised.

*Cloning of the 5′ End of Pol δ cDNA.* The 5′ end of the pol δ cDNA was cloned by a modified rapid amplification of cDNA ends (RACE) protocol (Frohman et al., 1988). cDNA was synthesized in the presence of the specific primer HR1 (Table I) with M-MLV reverse transcriptase as described above. The poly(A) RNA was hydrolyzed in 0.1 M NaOH at 60 °C for 10 min. The cDNA was then separated from

Table I: Peptide and Oligonucleotide Sequences Used in the Cloning of the Large Subunit of Bovine DNA Polymerase δ[a]

| | peptide sequence | | oligonucleotide sequence |
|---|---|---|---|
| V8-4 | **LSLPIDTQYYLEQ(Q)LAK(P)** | J3 (sense) | 5′ TTTGAATTC-AGC-CTG-CAA-ATC-GAC-ACC-CAG-TAC-TAC-CTG-GA(G,A)-CA(G,A)-CA 3′ |
| V8-3 | **VSHLSALEERFSRL(X)TQ(X)QR(X)QG(S)L(H)** | JR1 (antisense) | 5′ TTTGAATTC-GCA-CCG-CTG-GCA-CTG-GGT-CCA-CAG-CCG-GGA-GAA-CC(G,T)-CTC-(C,T)TC 3′ |
| V8-25 | **FVKASVR(X)GLLPQIL** | J4 (sense) | 5′ TTTGAATTC-GTG-CG(G,C)-CG(G,C)-GGC-CTG-CTG-CCC-CAG-AT(T,C)-CT 3′ |
| V8-9 | **IDH(X)VAPARPLPGAPP(P)(S)QD(S)(V)(P)(I) (L) (L) (A) (F)** | J5 (sense) | 5′ TTTGAATTC-GTG-GCC-CCC-GCC-CG(G,C)-CCC-CTG-CCC-GG(G,C)-GC(T,C)-CC 3′ |
| | | EDT (antisense) | 5′ TTTGAATTC-TTTTTTTTTTTTTTTT 3′ |
| | | BAG (sense) | 5′ TTTGGATCC-GGGGGGGGGGGGGG 3′ |
| | | J2 (sense) | 5′ TTTGAATTC-AGAGCTGTACCAGAAGGAGGT 3′ |
| | | PF1 (sense) | 5′ TTTGAATTC-TCAGTTCCCATCCTCCGCGCC 3′ |
| | | DF1 (sense) | 5′ TTTGAATTC-AGTCAGGGGTCACGGCGGCGTGG 3′ |
| | | PR1 (antisense) | 5′ TTTGAATTC-GGGAAGTTCGTTGAGGTCAGAGGT 3′ |
| | | PR2 (antisense) | 5′ TTTGAATTC-GGGGTCTGTCTCCTTGGCCAGCTC 3′ |
| | | PR3 (antisense) | 5′ TTTGAATTC-TGCAGCTCGCTCAGGTGCTCAGGT 3′ |
| | | HR1 (antisense) | 5′ TTTGAATTC-ATGACCGAGTCTGTGTCACCATACACCAC 3′ |
| | | PR4 (antisense) | 5′ TTTGAATTC-AAGGCGCGGAGGATGGGAACTGAG 3′ |

[a] Peptide sequences used in the design of oligonucleotides are boldfaced. Tentative amino acid assignments in the amino acid sequence analysis are shown in parentheses. Restriction sites in oligonucleotides to facilitate cloning are underlined. Positions in the synthetic oligonucleotides containing more than one base are also shown in parentheses.

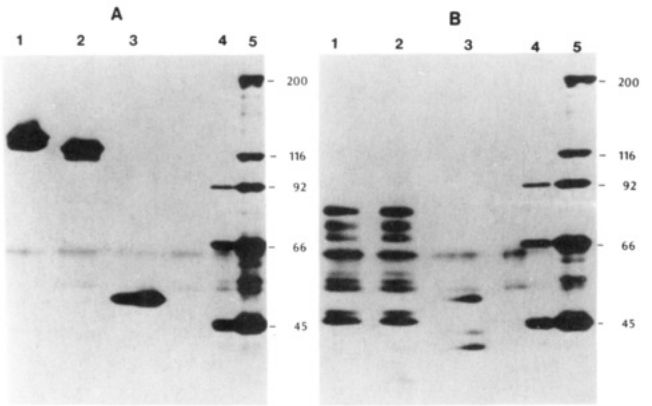


FIGURE 1: Partial peptide maps of the 125-, 116-, and 48-kDa polypeptides from calf thymus DNA polymerase δ. *N*-Chlorosuccinimide partial peptide mapping was carried out as described under Materials and Methods. Approximately 4 μg/slot of pol δ core enzyme was run on a 6.5% SDS–polyacrylamide gel, stained with Coomassie Blue, and the separated 125-, 116-, and 48-kDa polypeptides were excised. Individual gel slices were treated with urea/$H_2O$/acetic acid (1 g/1 mL/1 mL) in the absence (panel A) or presence (panel B) of 0.015 M *N*-chlorosuccinimide for 30 min at room temperature and then rerun on an 8–18% gradient SDS–polyacrylamide gel and silver stained. Panels A and B: lane 1, 125-kDa polypeptide; lane 2, 116-kDa polypeptide; lane 3, 48-kDa polypeptide; lane 4, low molecular weight markers; lane 5, high molecular weight markers.

the oligonucleotide primer and hydrolyzed RNA by chromatography on Sepharose CL-6B equilibrated with 50 mM KOH and 0.1 mM EDTA. Fractions that contained the oligonucletoide primer were predetermined in a separate experiment in which $^{32}$P-labeled primer was chromatographed under identical conditions. Fractions containing the cDNA without primers were neutralized with 1 M Tris-HCl, pH 7.5. Carrier glycogen (10 μg) (Boehringer Mannheim) was added and the cDNA precipitated by ethanol. Oligo(dC) tails were added to the 3′ end of the cDNA by terminal deoxynucleotidyltransferase (BRL) in the presence of 1 mM dCTP (Roychoudhury et al., 1976). The 5′ end of the pol δ cDNA was then amplified in a PCR reaction with the specific primer PR3 and an oligo(dG)-containing primer, BAG (Table I). The amplified DNA fragment was purified as described above.

*Cloning and DNA Sequencing.* Overlapping fragments of the pol δ cDNA obtained by PCR and subsequently recovered from low melting point agarose gel slices were cloned into M13mp18 or -19 (Pharmacia) digested with appropriate restriction enzymes. Recombinant M13 plaques containing the 5′- and 3′-end fragments of bovine pol δ cDNA obtained by the modified RACE protocol were identified by hybridization to the specific $^{32}$P-labeled oligonucleotides PR4 and JR1, respectively (Table I). Recombinant phage adsorbed on nitrocellulose filters were hybridized to oligonucleotides phosphorylated by T4 polynucleotide kinase in the presence of [γ-$^{32}$P]ATP under the conditions described (Benton & Davis, 1977). Single-stranded templates were sequenced by the dideoxy chain terminator method (Sanger et al., 1977) using a universal sequencing primer (Pharmacia) and specific synthetic oligonucleotide primers. All sequencing reactions were performed with T7 DNA polymerase (Sequenase, U.S. Biochemicals) and 7-deaza-dGTP (Mizusawa et al., 1986).

## RESULTS

*Subunit Structure of Pol δ and Partial Peptide Mapping.* Calf thymus pol δ purified to apparent homogeneity consists of 125- and 48-kDa subunits present in a 1:1 molar ratio (Lee et al., 1984; Ng et al., 1991). A variable amount of 116-kDa polypeptide is usually observed in pol δ preparations. Partial

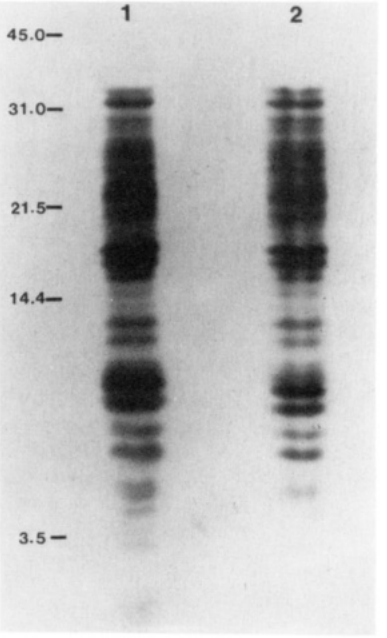


FIGURE 2: Coomassie Blue stained blot of peptides generated by *S. aureus* V8 protease digestion of the 125- and 116-kDa polypeptides: lane 1, 125-kDa polypeptide (80 μg); lane 2, 116-kDa polypeptide (60 μg). The positions of the molecular weight standards (×$10^3$) are given on the left. Procedures are given under Materials and Methods.

peptide mapping of the isolated 125-, 116-, and 48-kDa polypeptides by *N*-chlorosuccinimide cleavage (Figure 1) revealed that the 125- and 116-kDa polypeptides generated essentially identical partial peptide maps, suggesting that the 116-kDa polypeptide is a degraded form of the 125-kDa subunit. This was further substantiated by the generation of identical partial peptide maps following digestion of the 125- and 116-kDa polypeptides with either *S. aureus* V8 protease (Figure 2) or cyanogen bromide (not shown). *N*-Chlorosuccinimide cleavage of the 48-kDa polypeptide, however, resulted in an entirely different peptide pattern (Figure 1), indicating that it is not derived from the higher molecular weight subunit. These data suggested that pol δ is a heterodimer composed of 125- and 48-kDa subunits.

*Primary Sequence of the Large Subunit of Bovine Pol δ.* Partial amino acid sequence data from peptides derived from either *S. aureus* V8 protease, CNBr, or trypsin digestion of the combined 125- and 116-kDa polypeptides of pol δ showed that peptides V8-4 and V8-3 (Table I) are homologous to two regions of the yeast pol δ sequence that are 110 amino acid residues apart (Boulet et al., 1989). Accordingly, these peptide sequences were used to design two PCR primers (J3 and JR1, Table I) that were employed to amplify the corresponding region of the cDNA for bovine pol δ. To minimize degeneracy in the primer sequences, preferred bovine codons were chosen which, by coincidence, are rich in G and C nucleotides. The PCR using J3 and JR1 produced a fragment of approximately 340 base pairs with a sequence consisting of a single open reading frame (nucleotides 2895–3233, Figure 4). The predicted amino acid sequence (amino acid residues 948–1060, Figure 4) is 36% identical to the yeast pol δ sequence and contains cysteine residues in conserved positions that are characteristic of zinc finger motifs (Berg, 1986, 1990). In addition, the predicted amino acid sequence is identical to a peptide sequence (amino acid residues 980–1007, Figure 4) obtained by *S. aureus* V8 protease digestion of bovine pol δ. These data indicate that the PCR-amplified fragment was derived from a cDNA coding for the 125-kDa subunit of bovine pol δ.

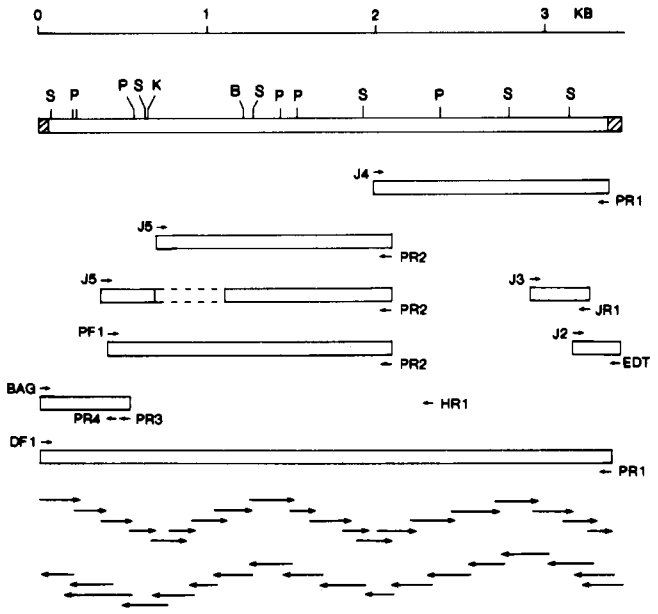


FIGURE 3: Partial restriction map and overlapping cDNAs for the large subunit of bovine DNA polymerase δ. Coding sequences are represented by open bars; 5′ and 3′ untranslated sequences are represented by hatched bars. KB represents kilobase. Restriction sites are abbreviated as follows: B, *Bam*HI; P, *Pst*I; S, *Sma*I; K, *Kpn*I. Oligonucleotides used in PCR and cDNA synthesis are represented by short arrows. The longer arrows at the bottom show the direction and extent of the sequences that were determined.

The 3′ end of the pol δ cDNA was then amplified with the primers J2 and EDT (Table I). Since EDT is essentially a poly(dT)-containing primer, a large number of unrelated background products were generated. The clones containing the authentic 3′-end fragment were identified by hybridization to oligonucleotide JR1. Analysis of the combined nucleotide sequence revealed that the partial cDNA was GC-rich (66%), and by analogy with other GC-rich cDNAs, it was anticipated that the entire cDNA would also be GC-rich. Accordingly, unique peptide sequences V8-25 and V8-9 (Table I) were used for the design of additional PCR primers that contained bovine preferred codons, and the redundancy was further reduced by the choice of GC-rich codons.

Primers J4 and PR1 produced a partial clone that overlapped the sequences in the primer regions J3 and JR1 (Figure 3). Primer J5 was designed after peptide V8-9. This oligonucleotide, however, apparently hybridized to two regions of the cDNA. The PCR using J5 and PR2 produced predominantly a fragment that resulted from J5 priming at an unexpected proximal location (Figure 3). A rare clone that resulted from J5 priming at the expected distal site was also identified but contained a deletion of the proximal site. Primers PF1 and PR2 were then used to establish the sequences between the two J5 priming sites.

The 5′ end of the cDNA (nucleotides 1–544, Figure 4) was obtained by specifically priming the cDNA synthesis using HR1 instead of oligo(dT). The cDNA was then tailed with dC residues and the 5′-end fragment amplified by primers PR3 and BAG (Figure 3). Since BAG is nonspecific, the resultant clones were further screened by hybridization with oligonucleotide PR4, which identified a collection of clones containing heteogeneous 5′-end fragments, likely the result of either partial degradation or incomplete reverse transcription of the mRNA. The longest clone from the 5′ end was sequenced to establish the entire cDNA sequence. Finally, primers DF1 and PR1 were used to amplify the entire coding region of the cDNA in one continuous segment, and the nucleotide sequence was again verified on both strands using 7-deaza-dGTP to minimize compressions due to the high GC content. The sequencing strategy is outlined in Figure 3.

The entire cDNA sequence and its predicted amino acid sequence are shown in Figure 4. Peptide sequences that are identical to the predicted amino acid sequence are overlined. The tryptic peptide GGLXDEDEAYRPSQFEEE is located prior to Met41, indicating that translation initiates prior to this site. An in-frame stop codon at nucleotides 45–47 also precludes initiation prior to nucleotide 45. These results suggest that the ATG codon at nucleotides 54–56 is the initiator methionine and that the 5′ untranslated region consists of 53 nucleotides. The 3′ noncoding region was found to consist of 77 nucleotides with a polyadenylation signal (AATAAA) 14 nucleotides upstream from the poly(A) tail.

The base composition of the cDNA coding for pol δ was particularly rich in G and C. The total nucleotide composition for the full-length cDNA was calculated to be 17.6% A, 32.7% G, 32.9% C, and 16.8% T. This gives a total GC content of 65.5%, which is substantially higher than most DNAs.

The predicted amino acid sequence of the large subunit of bovine pol δ (1106 amino acids) has a calculated molecular weight of 123 707. This value is in good agreement with the molecular weight of 125 000, estimated from SDS–polyacrylamide gel electrophoresis. These data suggest that substantial posttranslational modification of the polypeptide is unlikely. In contrast, the large subunit of human pol α has been shown to undergo both glycosylation and phosphorylation, resulting in an approximately 15 000-Da increase in the mass of the mature polypeptide (Hsi et al., 1990).

Two nucleotide differences were observed from the analysis of different PCR clones which suggested that amino acid residue 131 is either Glu or Asn and residue 263 is either Asn or Ser. These differences are not due to sequencing ambiguities but are attributed to either PCR errors or true polymorphism.

The amino acid composition for the mature protein was calculated as $Ala_{89}$, $Cys_{25}$, $Asp_{55}$, $Glu_{81}$, $Phe_{39}$, $Gly_{80}$, $His_{19}$, $Ile_{49}$, $Lys_{45}$, $Leu_{132}$, $Met_{25}$, $Asn_{18}$, $Pro_{76}$, $Gln_{54}$, $Arg_{93}$, $Ser_{66}$, $Thr_{44}$, $Val_{73}$, $Trp_{10}$, $Tyr_{33}$. The high levels of Leu and Arg represented 11.9% and 8.4% of the total protein, respectively. These data as well as the molecular weight were calculated on the basis of residues 131 and 263 as Asn.

*N-Terminal Sequence of the 116-kDa Polypeptide.* Attempts to determine the N-terminal sequence of the isolated 125-kDa polypeptide by amino acid sequence analysis were unsuccessful, suggesting that the N-terminal amino acid is blocked with an acyl group such as acetyl. Sequencing of the isolated 116-kDa polypeptide resulted in the sequence GGLXDEDEAYXP, corresponding to amino acids 20–31 of the deduced sequence of the 125-kDa subunit. These results are consistent with the partial peptide mapping data which indicated that the 116-kDa polypeptide is a degradation product of the 125-kDa polypeptide. Furthermore, this degradation may have been catalyzed by an endopeptidase with a specificity for an Arg or Lys at P1 and P4. A number of these endopeptidases involved in the processing of plasma proteins, prohormones, and viral proteins have been reported in recent years (Barr, 1991). A loss of 19 amino acids from the N-terminus of pol δ, however, does not correspond to the reduction in apparent molecular mass of 9000 Da observed by SDS–polyacrylamide gel electrophoresis. Whether this is due to anomalous behavior of the 116-kDa polypeptide on SDS gels or to the additional loss of a C-terminal peptide is not clear.

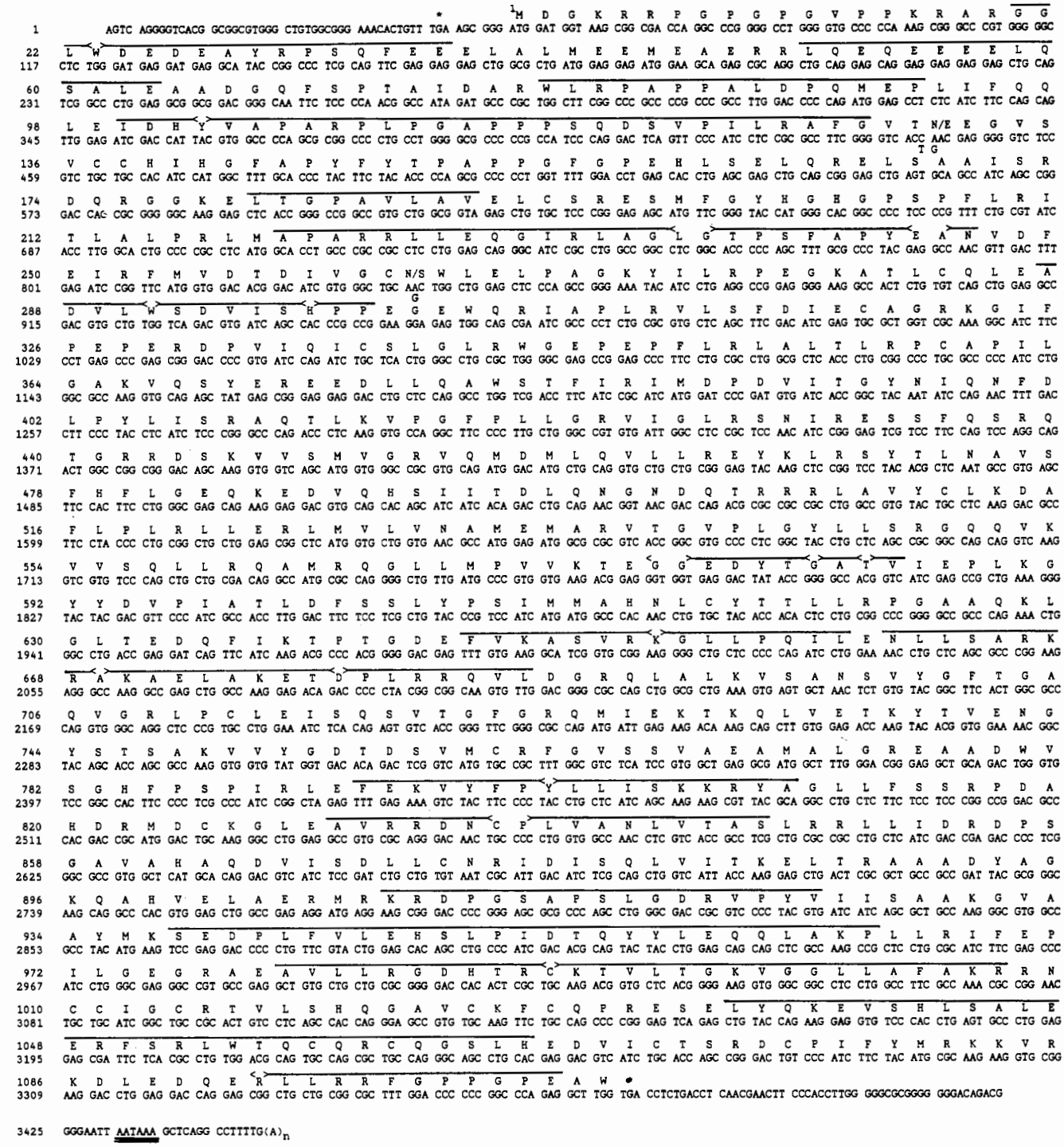

FIGURE 4: Nucleotide sequence and predicted amino acid sequence for the cDNA coding for the large subunit of bovine DNA polymerase δ. Amino acid sequences obtained by automated Edman degradation of peptides are shown with overlines. ⟨ ⟩ represents amino acids not determined. The polyadenylation site is double underlined.

*Sequence Similarities with Other DNA Polymerases.* Alignment of the deduced amino acid sequence of the catalytic subunit of bovine pol δ with that of yeast pol δ (Boulet et al., 1989) revealed that these two polymerases are highly homologous over the entire length of the polypeptide (Figure 5). The overall sequence similarity between bovine and yeast pol δ is 44%, as compared to human and yeast pol α which are 31% homologous (Wang et al., 1989).

Seven highly conserved regions present in the YGDTDS class of DNA polymerases have been identified, including regions I–VI by Wong et al. (1988) and region VII by Spicer et al. (1988), in a number of bacterial, phage, viral, and eukaryotic DNA polymerases (Figure 5). The present data identified five additional regions that are very highly conserved between yeast and bovine pol δ designated as regions A–E (Figure 5). The degree of sequence conservation between yeast and bovine pol δ in these five regions is striking. In region A, 17 of 28 residues are identical (61%), in region B, 30 of 40 residues are identical (75%), in region C, 79 of 97 residues are identical (81%), in region D, 14 of 16 residues are identical (88%), and in region E, 28 of 43 residues are identical (65%). The high degree of sequence conservation between such phylogenetically distant species may indicate some functional significance of these regions.

Regions A and B were previously identified by Boulet et al. (1989) as highly conserved between yeast pol δ and the

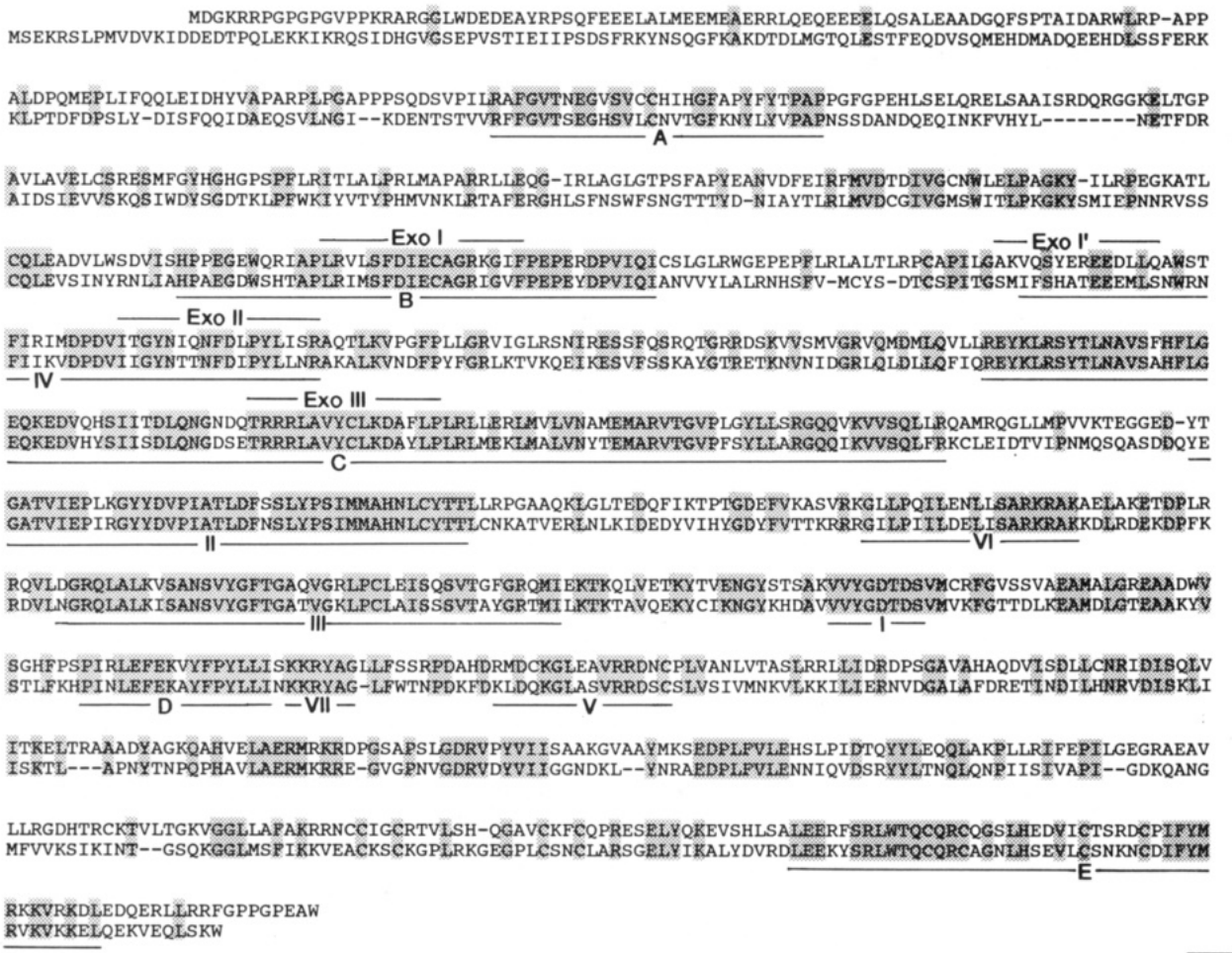


FIGURE 5: Amino acid homology between the large subunits of bovine (upper sequence) and yeast (lower sequence) pol δ. Polypeptide sequences were aligned using the GENEPRO program (Riverside Scientific, Seattle, WA). Identical amino acids are shaded. Regions I–VII and A–E are defined in the text.

herpes virus family of DNA polymerases. Region C is also quite homologous to the herpes virus family of DNA polymerases (Figure 6); 26–36% of the residues are identical, and the homology increases to 42–46% with conservative amino acid substitutions. There is also significant homology to the REV3 protein of *S. cerevisiae* (Morrison et al., 1989) and to DNA polymerase II of *Escherichia coli* (Iwasaki et al., 1991) but little or no homology to yeast pol ε (Morrison et al., 1990) or to pol α from human cells (Wong et al., 1988), budding yeast (Pizzagalli et al., 1988), or fission yeast (Damagnez et al., 1991). Region C also includes one of the regions (EXO III) postulated by Bernad et al. (1989) to be part of the 3′–5′ exonuclease active site, by homology with known active-site residues in the 3′–5′ exonuclease domain of DNA polymerase I of *E. coli* (Ollis et al., 1985; Derbyshire et al., 1988). Region D is homologous to the REV3 protein of *S. cerevisiae* (63% identical) and to the herpes virus family of DNA polymerases (50–56% identical) (Figure 6). Region E, although highly homologous to yeast pol δ, is not homologous to any other DNA polymerase that has been sequenced thus far (Figure 6).

As is the case with other eukaryotic cellular DNA polymerases, e.g., human pol α, yeast pol α, yeast pol δ, yeast pol ε, and the REV3 protein of *S. cerevisiae*, bovine pol δ contains cysteine-rich sequences near the C-terminus of the protein (residues 1011–1075). These sequences are predicted to be DNA binding sites or zinc fingers (Berg, 1986, 1990). Similar cysteine-rich sequences are present in the same position in yeast pol δ (residues 1005–1070). The pattern, $[CX_2C]X_{10-11}[CX_2C]X_{29}[CX_2C]X_9[CX_4C]$, is conserved between the bovine and yeast polypeptides, and 30 of 66 residues are identical.

## Discussion

The eukaryotic replicative DNA polymerases appear to be very highly conseved in both primary and quaternary structure. For example, mammalian and yeast pol α have identical subunit structures. Both are heterotetramers with subunits of nearly identical size. Both enzymes have DNA primase activity, catalyzed by the two smallest subunits, and both are moderately processive. Similarly, mammalian and yeast pol δ are both heterodimers with catalytic subunits of 125 kDa. Both enzymes have endogenous 3′–5′ exonuclease activity, and both have very low intrinsic processivities but become highly processive in the presence of the proliferating cell nuclear antigen (PCNA), an accessory protein for pol δ (Tan et al., 1986; Prelich et al., 1988). Remarkably, bovine PCNA can increase the processivity of yeast pol δ and vice versa, suggesting that the protein–protein interactions between pol δ and PCNA have been highly conserved during evolution (Burgers, 1988; Bauer & Burgers, 1988). Herpes simplex virus DNA polymerase is also structurally similar to bovine and yeast pol δ. It is composed of two subunits of 137 and 55 kDa, with the larger polypeptide being the catalytic subunit (Vaughn et al., 1984; Crute & Lehman, 1989; Gottlieb et al., 1990). It has intrinsic 3′–5′ exonuclease activity (O'Donnell et al., 1987; Knopf, 1979; Dorsky & Crumpacker, 1988) and is capable of highly processive DNA synthesis (Hernandez & Lehman, 1990; Gottlieb et al., 1990).

**REGION C**

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boδ      464  REYKLRSYTLNAVSFHFLGEQK-EDVQHSIITDLQNGNDQTR--RRL
yeδ      468  REYKLRSYTLNAVSAHFLGEQK-EDVHYSIISDLQNGDSETR--RRL
EBV      447  DKLSLSDYKLDTVARHLLGAKK-EDVHYKEIPRLFAAGPEGR--RRL
CMV      492  AKTNSPNYKLNTMAELYLRQRK-DDLSYKDIPRCFVANAEGR--AQV
HSV      531  DKIKLSSYKLNAVAEAVLKDKK-KDLSYRDIPAYYATGPAQR--GVI
VZV      512  ELKKLSSYKLDSVAREALNESK-RDLPYKDIPGYYASGPNTR--GII
REV3     832  SDVNLTQYTIESAAFNILHKRL-PHFSFESLTNMWNAKKSTTELKTV
PolII    284  AFWNFSSFSLETVAQELLGEGKSIDNPWDRMDEIDRRFAEDK--PAL

boδ      AVYCLKDAFLPLRLLERLMVLVNAMEMARVTGVPLGYLLSRGQQVKVVSQLLR
yeδ      AVYCLKDAYLPLRLMEKLMALVNYTEMARVTGVPFSYLLARGQQIKVVSQLFR
EBV      GMYCVQDSALVMDLLNHFVIHVEVAEIAKIAHIPCRRVLDDGQQIRVFSCLLA
CMV      GRYCLQDAVLVRDLFNTINFHYEAGAIARLAKIPLRRVIFDGQQIRIYTSLLD
HSV      GEYCIQDSLLVGQLFFKFLPHLELSAVARLAGINITRTIYDGQQIRVFTCLLR
VZV      GEYCIQDSALVGKLFFKYLPHLELSAVARLARITLTKAIYDGQQVRIYTCLLG
REV3     LNYWLSRAQINIQLLRKQDYIARNIEQARLIGIDFHSVYYRGSQFKVESFLIR
PolII    ATYNLKDCELVTQIFHKTEIMPFLLERATVNGLPVDR---HGGSVAAFGHLYF
```

**REGION D**

```
boδ      788  PIRLEFEKVYFPYLLI
yeδ      793  PINLEFEKAYFPYLLI
EBV      790  PISLEAEKTFSCLMLI
CMV      945  PVKLEFEKVFVSLMMI
HSV      921  PIKLECEKTFTKLLLI
VZV      886  PIKLECEKTFIKLLLI
REV3    1176  PIFLKFEKVYHPSILI
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**REGION E**

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boδ     1046  LEERFSRLWTQCQRCQGSLHEDVICTSRDCPIFYMRKKVRKDL
yeδ     1041  LEEKYSRLWTQCQRCAGNLHSEVLCSNKNCDIFYMRVKVKKEL
```

FIGURE 6: Highly conserved regions between bovine and yeast δ polymerases (regions C–E in Figure 5). Numbers indicate the amino acid position relative to the N-terminus of each polymerase. Similar or identical amino acids are shaded. The following conservative amino acid substitutions have been used: A = G, D = E, F = Y, L = I = V, K = R, N = Q, and S = T. Abbreviations are defined in Table II.

Bovine pol δ is a member of the YGDTDS class of DNA polymerases which have six highly conserved regions (regions I–VI in Figure 5) in corresponding positions in a number of DNA polymerases from bacteria, bacteriophage, animal viruses, and eukaryotic cells (Wong et al., 1988). These consensus regions are likely to be important functional domains. For example, sequence analysis of herpes simplex virus mutants with altered sensitivity to drugs which are dNTP or pyrophosphate analogues has suggested that consensus regions II and III may be involved in dNTP binding (Larder et al., 1987; Coen et al., 1983; Tsurumi et al., 1987; Gibbs et al., 1988). Site-directed mutagenesis of individual amino acids in the YGDTDS motif (region I), the most highly conserved region, has been carried out in bacteriophage $\phi$29. Measurements of the activity of the mutant DNA polymerases have shown that several of these amino acids are critical for enzymatic activity (Bernad et al., 1990a,b). Similar studies with herpes simplex virus DNA polymerase demonstrated that site-directed mutagenesis of region I resulted in the loss of polymerase activity as well as the failure of the enzyme to be stimulated by cotranslation of the accessory protein UL42 in an in vitro transcription–translation system (Dorsky & Crumpacker, 1990). It was further demonstrated by Marcy et al. (1990) that mutation of region I led to altered sensitivity to nucleotide and pyrophosphate analogues, suggesting that region I, similar to regions II and III, is involved directly or indirectly in substrate recognition.

Pairwise comparisons of members of the YGDTDS class of DNA polymerases (Table II) showed that bovine and yeast pol δ are more similar to each other than are human and yeast pol α or the members of the herpes virus family of DNA polymerases, i.e., the herpes simplex, Epstein–Barr, and cytomegalovirus enzymes. These data suggest that the δ polymerases have diverged less rapidly than the other members of this class. The δ polymerases are more closely related to the herpes virus polymerases than to the α polymerases. Regions A–D in Figure 5 have been identified as having significant homology to corresponding regions in the herpes virus family of DNA polymerases, but not to the α polymerases, possibly reflecting a function or functions for these regions which is (are) absent in the α polymerases, e.g., 3′–5′ exonuclease activity. Similarly, region E, which is highly conserved between the δ polymerases but not present in any other DNA polymerase sequenced thus far, may be involved in a function unique to the δ polymerases, e.g., interaction with an accessory protein. The δ polymerases also share significant homology with two repair polymerases, DNA polymerase II of *E. coli*, which is involved in SOS repair (Bonner et al., 1990: Iwasaki et al., 1991), and the REV3 protein of *S. cerevisiae*, a nonessential DNA polymerase thought to be required for translesional repair processes (Morrison et al., 1989).

Both yeast and bovine pol δ have intrinsic 3′–5′ exonuclease activity, and the exonuclease active site is thought to be located on the 125-kDa subunit (Lee et al., 1991; Simon et al., 1991). Bernad et al. (1989) have suggested that three regions in the N-terminal portion of a number of DNA polymerases (EXO I, EXO II, and EXO III) contain highly conserved residues which have been identified in the Klenow fragment of DNA polymerase I of *E. coli* as being involved in either metal binding or catalysis of 3′–5′ exonucleolytic hydrolysis (Ollis

Table II: Amino Acid Sequence Similarity between Various DNA Polymerases and Bovine Pol δ

| protein | PolII | T4 | Ad2 | Vacc | HSV | CMV | EBV | REV3 | yeε | yeα | huα | yeδ | boδ |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| boδ | 44 | 13 | 7 | 25 | 94 | 108 | 130 | 83 | 12 | 62 | 66 | 372 | 1106 |
| yeδ | 38 | 21 | 8 | 30 | 71 | 91 | 100 | 67 | 12 | 52 | 66 | 1093 | |
| huα | 42 | 14 | 9 | 25 | 39 | 30 | 32 | 28 | 12 | 239 | 1462 | | |
| yeα | 34 | 15 | 8 | 33 | 39 | 43 | 36 | 28 | 13 | 1468 | | | |
| yeε | 13 | 9 | 9 | 12 | 9 | 8 | 10 | 9 | 2222 | | | | |
| REV3 | 27 | 11 | 11 | 23 | 39 | 43 | 64 | 1504 | | | | | |
| EBV | 38 | 12 | 11 | 25 | 218 | 227 | 1015 | | | | | | |
| CMV | 21 | 12 | 14 | 22 | 182 | 1242 | | | | | | | |
| HSV | 13 | 11 | 9 | 25 | 1235 | | | | | | | | |
| Vacc | 8 | 13 | 8 | 937 | | | | | | | | | |
| Ad2 | 9 | 6 | 1056 | | | | | | | | | | |
| T4 | 23 | 898 | | | | | | | | | | | |
| PolII | 783 | | | | | | | | | | | | |

[a] Homologies were scored on the Intelligenetics IFIND program, which used the Wilber and Lipman algorithm. Window size was set at 20, word length at 1, density at less, and gap penalty at 2. Abbreviations: boδ, bovine DNA polymerase δ; yeδ, yeast DNA polymerase δ; huα, human DNA polymerase α; yeα, yeast DNA polymerase α; yeε, yeast DNA polymerase ε; REV3, the REV3 protein of *S. cerevisiae*; EBV, Epstein–Barr virus DNA polymerase; CMV, cytomegalovirus DNA polymerase; HSV, herpes simplex virus DNA polymerase; Vacc, vaccinia virus DNA polymerase; Ad2, adenovirus type 2 DNA polymerase; T4, bacteriophage T4 DNA polymerase; PolII, DNA polymerase II of *E. coli*.

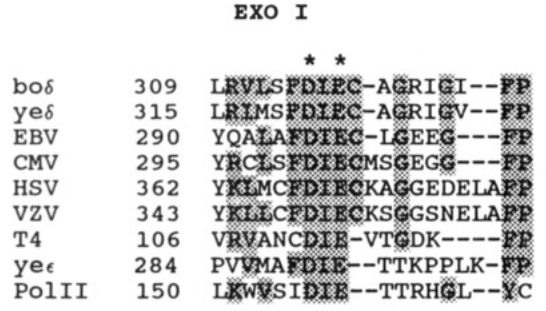


FIGURE 7: Putative EXO I domain in exonuclease-containing DNA polymerases. The aspartic acid and glutamic acid residues indicated by asterisks (*) have been shown to be required for exonucleolytic proofreading in yeast pol δ (Simon et al., 1991). Abbreviations are defined in Table II.

et al., 1985); Derbyshire et al., 1988). In spite of the overall lack of homology between *E. coli* pol I and the YDGTDS class of DNA polymerases, the EXO domains can be aligned in a variety of DNA polymerases (Bernad et al., 1989; Blanco et al., 1991). Site-directed mutagenesis of the critical residues in *E. coli* pol I (Derbyshire et al., 1988, 1991) or φ29 DNA polymerase (Bernad et al., 1989) was found to result in the loss of 3′–5′ exonuclease activity and/or a mutator phenotype, consistent with a function in exonucleolytic proofreading. A significant loss of exonuclease activity was also found to result from the mutation of a critical residue in the EXO III region of T4 DNA polymerase (Reha-Krantz et al., 1991); however, site-directed mutagenesis of two of the metal binding residues in the proposed EXO I region of T4 DNA polymerase did not result in either a significant loss of exonuclease activity or a significant increase in mutation frequency, suggesting either that some of the proposed metal binding residues are not essential for the 3′–5′ exonuclease activity of T4 DNA polymerase or that the EXO I site has been misaligned.

Recently, an alternative EXO I domain has been proposed for yeast pol δ (Simon et al., 1991). This domain is located in region B and is amino terminal to the original EXO I region (Bernad et al., 1989) which is now designated EXO I′ (Figure 5). Site-directed mutagenesis of putative metal binding residues in the new EXO I domain, but not in the EXO I′ domain, was found to produce a mutator phenotype (Simon et al., 1991). The EXO I domain, shown in Figure 7, is also highly conserved among the herpes virus family of DNA polymerases, as well as yeast pol ε, T4 DNA polymerase, and PolII of *E. coli*, all of which have intrinsic 3′–5′ exonuclease activity. Consistent with the proposal that this conserved region might contain metal binding ligands essential for 3′–5′ exonuclease activity is the observation that the EXO I domain is absent from the α polymerases, which lack proofreading exonuclease activity. However, whether this conserved region is involved in 3′–5′ exonuclease activity in any or all of these DNA polymerases awaits further mutagenesis and structural studies.

## Added in Proof

Recently, the cDNA coding for the catalytic subunit of human DNA polymerase δ has been cloned. The amino acid sequence of the human protein is 94% identical to the bovine enzyme (Chung et al., 1991).

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**Registry No.** DNA polymerase, 9012-90-2.

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