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The small subunits of human and mouse DNA polymerase ε are homologous to the second largest subunit of the yeast *Saccharomyces cerevisiae* DNA polymerase ε

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ABSTRACT

Human DNA polymerase ε is composed of a 261 kDa catalytic polypeptide and a 55 kDa small subunit of unknown function. cDNAs encoding the small subunit of human and mouse DNA polymerase ε were cloned. The predicted polypeptides have molecular masses of 59.469 and 59.319 kDa respectively and they are 90% identical. The human and mouse polypeptides show 22% identity with the 80 kDa subunit of the five subunit DNA polymerase ε from the yeast *Saccharomyces cerevisiae.* The high degree of conservation suggests that the 55 kDa subunit shares an essential function with the yeast 80 kDa subunit, which was earlier suggested to be involved in S phase cell cycle control in a pathway that is able to sense and signal incomplete replication. The small subunits of human and mouse DNA polymerase ε also show homology to the C-terminal domain of the second largest subunit of DNA polymerase α. The gene for the small subunit of human DNA polymerase ε (*POLE2*) was localized to chromosome 14q21–q22 by fluorescence *in situ* hybridization.

INTRODUCTION

Five distinct DNA polymerases, designated α, β, γ, δ and ε, have been identified in mammalian cell extracts. DNA polymerases α, β, δ and ε are located in the nucleus, whereas DNA polymerase γ is predominantly a mitochondrial enzyme. Three of the nuclear DNA polymerases, α, δ and ε, belong to a group of α-like DNA polymerases that share many structural and catalytic properties. The homologous yeast enzymes are all essential for cell viability and nuclear DNA replication (1–4). The other nuclear enzyme, DNA polymerase β, functions in base excision repair (5,6). In contrast, DNA polymerase γ is required for replication of mitochondrial DNA (7).

Replication of SV40 DNA has been used as a model system for mammalian DNA replication. DNA polymerases α and δ are sufficient for replication of the circular genome of this virus *in vitro* (8,9) and apparently also *in vivo* (10). Human DNA polymerase ε does not seem to be involved in replication of SV40 DNA (11). However, a recent study showed cross-linking of DNA polymerases α, δ and ε to nascent cellular DNA in replicating chromosomes, suggesting that DNA polymerase ε plays a role in replication of cellular DNA or in a process closely associated with replication (10). This observation is consistent with proliferation-dependent expression of the catalytic subunit of human DNA polymerase ε (12). DNA polymerase ε has also been implicated in repair of UV-damaged DNA in human fibroblasts (13). It also seems to have a specific role in meiotic recombination and repair in spermatogenesis (14) and was found to be a component of a mammalian protein complex that is able to repair double-strand breaks and deletions by recombination (15).

DNA polymerase ε purified from HeLa cells consists of two subunits with molecular weights of >200 and 55 kDa (16,17), while the yeast *Saccharomyces cerevisiae* DNA polymerase ε is a five subunit protein consisting of the >200 kDa catalytic subunit and smaller subunits with molecular weights of 80, 34, 31 and 29 kDa (18). We have previously cloned the cDNA encoding the catalytic subunit of the human enzyme (19), but the identity and function of the small subunit of mammalian DNA polymerase ε has so far remained unknown. We report here the cloning of the cDNAs encoding the small subunits of human and mouse DNA polymerase ε and its putative role revealed by primary structure comparisons. The human gene was also localized.

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MATERIALS AND METHODS

Amino acid sequencing

HeLa S3 cells (American Type Culture Collection) were cultivated in suspension as 'spinner cells' in Joklik's modification of minimum essential medium supplemented with glutamine, penicillin, streptomycin and 5% newborn calf serum. Human DNA polymerase ε was purified from a 100 l culture of HeLa cells in logarithmic phase as previously described (16), except that ammonium sulfate fractionation was omited. DNA polymerase activity was assayed as described by Syväoja and Linn (16) and Syväoja *et al.* (20). Glycerol gradient fractions were concentrated by the methanol/chloroform/H_2O method (21) and loaded onto an 8% SDS–polyacrylamide gel. The gel was stained with Coomassie brilliant blue and the small subunit (~500 pmol) was excised from the gel for subsequent in-gel digestion as described by Hellman (22). SDS and the Coomassie dye were removed by washing with Tris–HCl/acetonitrile, the gel dried and the small subunit digested with 0.5 µg modified trypsin (Promega). Rehydration with 0.2 M ammonium bicarbonate was allowed to proceed overnight at 30°C and the incubation mixtures were acidified by adding trifluoroacetic acid. The generated internal peptides were extracted from the gel and isolated by narrow bore reversed phase liquid chromatography on a µRPC/C18 2.1/10 column operated in a SMART system (Pharmacia Biotech). The individual peptides were sequenced by automated Edman degradation on an Applied Biosystems 470A or 494A sequencer.

Cloning and sequencing

Escherichia coli strains XL1-Blue, DH5α, Y1090 and c600Hfl were used for propagation of plasmids and phages respectively. Human T cell (HUT-78), human thymus and mouse erythroleukemia cDNA libraries (Clontech) were screened using [^{32}P]dCTP-labeled DNA probes (23). The 5′-ends of the human and mouse cDNAs were further extended by 5′-RACE from placenta mRNA using a Marathon™ cDNA Amplification kit (Clontech) and from mouse embryo 7-day Marathon-Ready™ cDNA (Clontech) respectively. Positive λ clones were subcloned into pBluescript KS+ vector and 5′-RACE products into pUC18 vector with blunt end ligation (SureClone ligation kit; Pharmacia). DNA sequencing was performed by the dideoxy method (24) using 7-deaza-dGTP analogs and T7 DNA polymerase (Pharmacia Biotech) or by automated sequencing using ABI PRISM™ AmpliTaq FS dye and dRhodamine terminator cycle sequencing kits (Perkin-Elmer). Sequences were tested against non-redundant GenBank, PDB, SwissProt, PIR, EMBL, DDBJ and PDB databases.

Production of antibodies and Western analysis

For production of polyclonal antibodies against the small subunit of human DNA polymerase ε a peptide with sequence LEDPTGTVQLDLS (amino acids 197–209) was synthesized with an Applied Biosystems 433A synthesizer and conjugated to keyhole limpet hemocyanin (Sigma) according to Ausubel *et al.* (23). Rabbits were immunized with 1 mg coupled peptide by conventional methods (25). Antibodies were purified from rabbit serum on protein A–Sepharose (Pharmacia) according to Harlow and Lane (25). For Western analysis protein samples were separated by SDS–PAGE and transferred to Immobilon P membrane (Millipore) by a wet method. The membrane was blocked with non-fat milk powder in TBS buffer (50 mM Tris–HCl, 150 mM NaCl, pH 7.5), followed by incubation with the primary antibodies. Rabbit polyclonal K28 antibody was used for detection of the small subunit and mouse monoclonal antibodies 93H3B, 93G1A and 93E24C (26) for detection of the catalytic subunit. The membrane was incubated with alkaline phosphatase-conjugated anti-rabbit IgG (Sigma) or anti-mouse IgG (BioRad) and developed using BioRad colorimetric reagents.

Chromosomal mapping of human *POLE2*

Fluorescence *in situ* hybridization for localization of the human *POLE2* gene was performed as described elsewhere (27,28).

RESULTS

The nucleotide sequence data reported in this article has been deposited in the DDBJ/EMBL/GenBank databases under accession nos. AF036899 (human cDNA) and AF036898 (mouse cDNA).

Cloning of the cDNAs for the small subunits of human and mouse DNA polymerase ε

Partial amino acid sequence data from peptides derived from trypsin digestion of the small subunit of human DNA polymerase ε (Table 1) was used to search the non-redundant database of the GenBank EST Division. A mouse embryo cDNA clone (AA008627, 441 bp) similar to *S.cerevisiae* DNA polymerase ε subunit B was found and amplified by PCR from a mouse erythroleukemia cDNA library. The 5′- and 3′-ends of this 441 bp fragment were sequenced, confirming its identity with the EST sequence, and thereafter used as a probe for screening both mouse and human cDNA libraries. Out of 2.35×10^6 colonies screened from human T cell and thymus cDNA libraries four incomplete overlapping positives were found, isolated and analyzed. The very 5′-end of the cDNA was obtained by RACE using placenta mRNA as the starting template. For cloning of the corresponding mouse cDNA 9.0×10^6 colonies were screened from a mouse erytroleukemia cDNA library and 13 positive clones were obtained. The 5′-end of the mouse cDNA was further extended by 5′-RACE from mouse embryo Marathon-Ready cDNA. The mouse cDNA is incomplete, lacking only the initial A of the ATG start codon, as compared with the human cDNA.

The putative translation initiation codon of the human cDNA is located at nt 15–17. It is likely to encode the initiator methionine, since amino acid sequence alignment with *S.cerevisiae* DNA polymerase ε subunit B reveals conservation of the N-terminal halves of these enzymes (Fig. 1). Furthermore, the nucleotide content surrounding the translation start site fulfils the Kozak rules for translation initiation well (29).

Primary structures of the small subunits of human and mouse DNA polymerase ε

The human cDNA encodes a protein of 527 amino acid residues with a calculated molecular mass of 59.469 kDa. This is in reasonably good agreement with the size of 55 kDa estimated from SDS–PAGE (16). The identity of the cDNA was confirmed by sequencing tryptic fragments obtained from the small subunit of this enzyme. Reliable sequence data was obtained from eight fragments and five of them corresponded to the amino acid sequence deduced from the cDNA (Table 1). Three of the eight peptides were found to be contaminants of the DNA polymerase

Table 1. Amino acid sequence of tryptic fragments from human DNA polymerase ε small subunit[a]

Cycle	Tryptic fragment	Residues 10-15	Tryptic fragment	Residues 106-122	Tryptic fragment	Residues 28-43	Tryptic fragment	Residues 344-360	Tryptic fragment	Residues 195-210
1	A	A	F	F	Y	Y	T	T	F	F
2	L	L	L	L	L	L	L	L	F	F
3	S	S	P	P	T	T	A	A	L	L
4	A	A	L	L	E	E	D	D	E	E
5	F	F	I?	**L**	A	A	I	I	D	D
6	K	K	M	M	L	L	I	I	P	P
7			T	T	Q	Q	E	**C**	T	T
8			N	N	X	S	E	E	G	G
9			H	H	I	I	Y	Y	T	T
10			P	P	A	**S**	P	P	V	V
11			A	A	E	E	D	D	Q	Q
12			Q	**P**	L	L	I	I	L	L
13			N	N	E	E	H	H	D	D
14			K?	**L**	L	L	Q	Q	L	L
15			F	F	E	E	S	S	S	S
16			F	**G**	D	D	S?	S	K	K
17			T	T			S?	**R**		
18							R			

[a]The sequenced oligonucleotides were obtained upon cleavage of the human small subunit with trypsin and subsequent purification of derived oligopeptides as described in Materials and Methods. The numbering of the residues is from the amino acid sequence deduced from the nucleotide sequence of the human cDNA. Unclear residues are labeled with a question mark and unassigned with X. The differences between the tryptic fragments and the amino acid sequence of the small subunit of DNA polymerase ε are shown in bold.

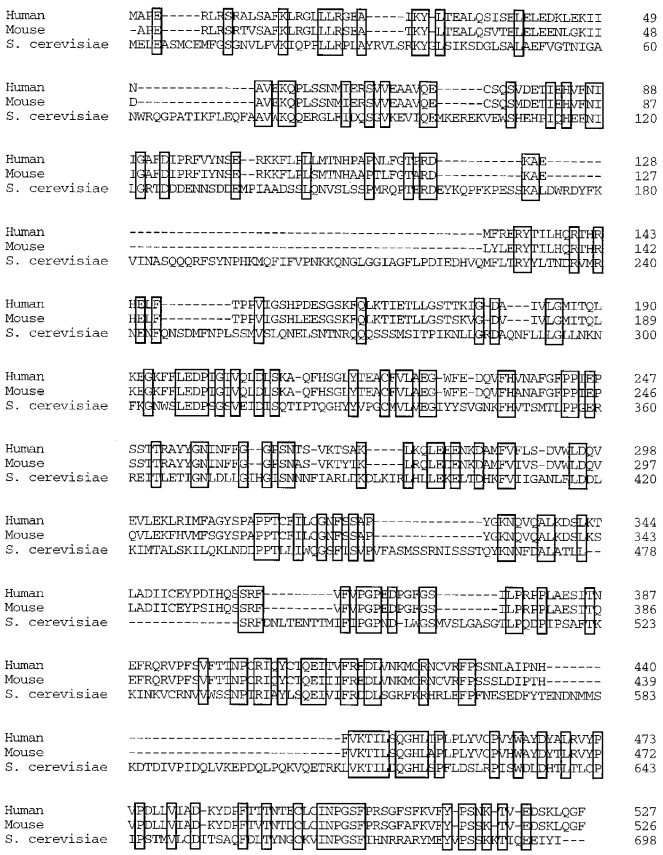

Figure 1. Comparison of amino acid sequences of the small subunit of human and mouse DNA polymerase ε and the 80 kDa subunit of *S.cerevisiae* DNA polymerase ε. Identical amino acid residues are in boxes.

ε preparation. Antiserum was raised against a synthetic peptide from the human small subunit. In Western analysis it specifically recognized a 55 kDa polypeptide which was not recognized by the preimmune serum. As seen in Figure 2, both the catalytic and the 55 kDa subunits co-eluted from a gel filtration column in a complex with an apparent molecular mass of ~400 kDa, providing further evidence on the identity of the human cDNA. The mouse counterpart for the small subunit encodes a protein of 527 amino acid residues with a calculated molecular mass of 59.319 kDa. The human and mouse amino acid sequences are 90% identical (data not shown), indicating high evolutionary conservation. The primary structures of the small subunits of human and mouse DNA polymerase ε show 22% identity to the 80 kDa subunit B of *S.cerevisiae* DNA polymerase ε (Fig. 1), indicating that subunit B is the yeast counterpart for the mammalian small subunit. The conserved areas are distributed throughout the polypeptides, although higher conservation is observed in the C-terminal halves. Searches of the protein databases with human and mouse polypeptides detected homology to mouse DNA polymerase α 70 kDa subunit, mouse DNA polymerase ε small subunit being 23% identical in 348 amino acid overlap, and the human subunit 25% in 211 amino acid overlap. Amino acid alignment of the human small and human DNA polymerase α 70 kDa subunits reveals 26% identity in a 191 amino acid overlap, as seen in Figure 3. These homologous areas also seem to be conserved in the yeast *S.cerevisiae* DNA polymerase ε 80 kDa subunit. Examples of the conserved motifs in the C-terminus are P---I--G-F, F-P---D, DL, K-IL-Q and C---NPG (Figs 1 and 3).

Localization of the gene for the small subunit of human DNA polymerase ε (*POLE2*)

The *POLE2* gene was first assigned to chromosome 14 by Southern blot analysis of 13 human × rodent hybrid clones (30) with a fragment of the cloned human cDNA (723 bp). In *Xba*I-digested human DNA the probe detected 11 fragments. The most intense signals were due to fragments of 5.0 and 2.9 kb (seven fragments <2.5 kb gave weak signals). The 5.0 and 2.9 kb fragments could be followed unambiguously in the hybrids. They segregated together and with human chromosome 14 (at least two discordant clones; data not shown). To further localize the gene along chromosome 14 the regional assignment of the *POLE2* gene was determined by fluorescence *in situ* hybridization using a mixture of two human cDNA clones (559 and 915 bp inserts) in plasmids. Double spots (two labeled sister chromatids) formed by the probes were found only on chromosome 14. The fluorescent signals were located at 42–53% of the chromosome length (starting from the p telomere), corresponding to bands 14q21–q22. The average position was the lower limit of 14q21 (illustrated in Fig. 4).

DISCUSSION

The catalytic subunit of the yeast *S.cerevisiae* DNA polymerase ε is structurally homologous to human DNA polymerase ε (1,19).

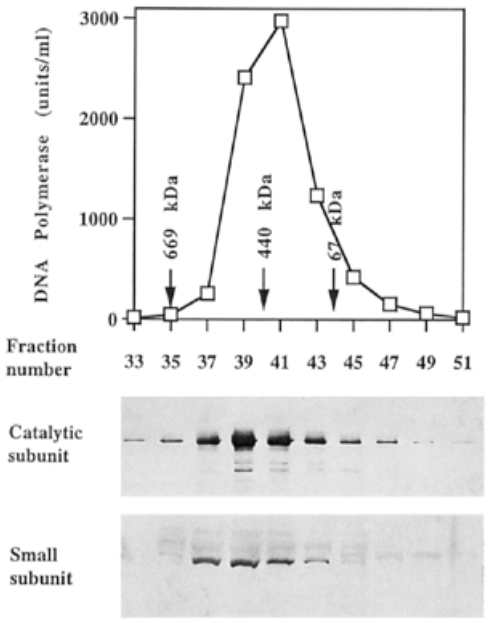

Figure 2. Recognition of the small subunit of human DNA polymerase ε by a polyclonal antibody against a synthetic peptide representing a tryptic fragment derived from the putative small subunit. The enzyme was purified from HeLa cells to step V (hydroxyapatite) as described in Materials and Methods. A hydroxyapatite fraction containing 1 mg protein was concentrated by Centriplus 30 concentrator (Amicon), applied to a Superose 6 PC 3.2/30 gel filtration column operated by a SMART HPLC system (Pharmacia) and eluted at the flow rate of 40 µl/min with a buffer containing 150 mM potassium phosphate, pH 7.5, 10% glycerol, 5 mM DTT and 0.02% Triton X-100. The polymerase activity was assayed from gel filtration fractions and found to elute at 400 kDa. Purified DNA polymerase ε was detected by Western analysis with antibodies against the small and the catalytic subunits. Molecular weight markers of 67 (bovine serum albumin), 440 (ferritin) and 669 kDa (thyroglobulin) were used in gel filtration.

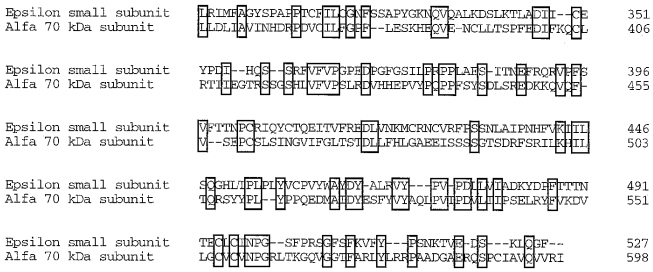

Figure 3. Comparison of amino acid sequences of the small subunit of human DNA polymerase ε (upper) and the 70 kDa subunit of DNA polymerase α (lower). Identical amino acid residues are in boxes.

However, the polypeptide structures of purified enzymes are different: while the human enzyme is composed of two subunits, the >200 kDa catalytic subunit and the 55 kDa small subunit (16,20), the yeast enzyme is composed of a >200 kDa catalytic subunit and at least four smaller subunits with molecular weights of 80, 34, 31 and 29 kDa (18). The human 55 kDa subunit was here shown to be a structural homolog of the yeast 80 kDa subunit. This yeast subunit is essential for viability of cells and a temperature-sensitive mutant is defective in DNA synthesis at the

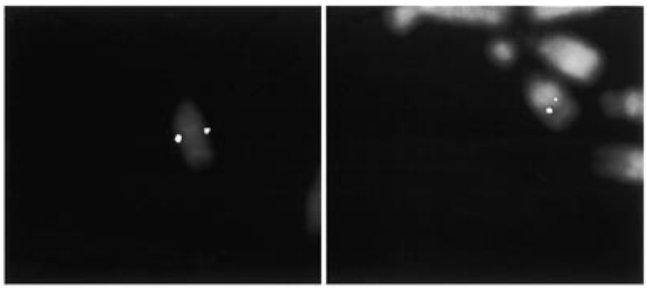

Figure 4. Regional mapping of the *POLE2* gene. Two metaphase regions are shown with one copy of chromosome 14 labeled by two fluorescent signals at 14q21–q22. The chromosomes were DAPI counterstained and the banding and probe signal images were captured and treated with the ISIS imaging system (MetaSystems).

restrictive temperature (31). The transcript also fluctuates periodically during the cell cycle in a manner that is typical for replication genes. The C-terminal half of the catalytic subunit of the yeast enzyme acts as a sensor of DNA replication progression, coordinating transcriptional and cell cycle responses with blockage of DNA replication (32,33). Another study strongly suggests that this cell cycle checkpoint control in yeast is mediated by the 80 kDa subunit (34). The DNA polymerase ε complex seems to be unstable both *in vivo* and *in vitro* when the 80 kDa subunit is mutated. The polymerase activity of the defective complex is also low (34). Conservation of the primary structure of the 80 kDa subunit from yeast to human suggests that this subunit also has a conserved function. The requirement of the small subunit for a stable polymerase complex may also explain our failure to purify the catalytic subunit of human DNA polymerase ε in an active form when expressed in conventional expression systems (unpublished results). Cloning of the cDNA for the small subunit provides an opportunity to co-express it with the catalytic subunit, hopefully resulting in an active complex.

The human and mouse 55 kDa subunits of DNA polymerase ε were here shown also to have homology to the 70 kDa subunit of DNA polymerase α. The function of this subunit is not currently known. It may interact with cellular replication initiator proteins, as suggested by its interaction with T antigen in SV40 DNA replication (35). However, this interaction is mediated by the N-terminal 240 amino acids of the 70 kDa subunit, while the region showing high homology to the small subunit of DNA polymerase ε is the C-terminal half of the polypeptide. The homologous region could be needed for other protein–protein interactions and/or could be a target for phosphorylation, which then modulates activity of the catalytic subunit in a cell cycle-dependent manner, as has been suggested for the 70 kDa subunit of *S.cerevisiae* DNA polymerase α (36).

The gene for the small (55 kDa) subunit of human DNA polymerase ε was assigned to chromosome 14q21–q22. To our knowledge no disease genes have been mapped to this locus so far. The gene for the catalytic subunit was earlier assigned to chromosome 12q24.3 (37).

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