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Molecular cloning, structure and expression of the yeast proliferating cell nuclear antigen gene

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ABSTRACT

The budding yeast *Saccharomyces cerevisiae* is proving to be an useful and accurate model for eukaryotic DNA replication. It contains both DNA polymerase α (I) and δ (III). Recently, proliferating cell nuclear antigen (PCNA), which in mammalian cells is an auxiliary subunit of DNA polymerase δ and is essential for *in vitro* leading strand SV40 DNA replication, was purified from yeast. We have now cloned the gene for yeast PCNA (*POL30*). The gene codes for an essential protein of 29 kDa, which shows 35% homology with human PCNA. Cell cycle expression studies, using synchronized cells, show that expression of both the PCNA (*POL30*) and the DNA polymerase δ (*POL3*, or *CDC2*) genes of yeast are regulated in an identical fashion to that of the DNA polymerase α (*POL1*) gene. Thus, steady state mRNA levels increase 10 – 100-fold in late G1 phase, peak in early S-phase, and decrease to low levels in late S-phase. In addition, in meiosis mRNA levels increase prior to initiation of premeiotic DNA synthesis.

INTRODUCTION

Evidence for the model that, in mammalian cells, DNA polymerase δ is responsible for leading strand DNA replication (1) derives from studies of the proliferating cell nuclear antigen (PCNA, or cyclin), which is a known cofactor of DNA polymerase δ (2−4) in *in vitro* replication of SV40 DNA (5,6). The high degree of structural and functional conservation of components of the DNA replication apparatus between mammalian cells and yeast (7) gives relevance to genetic information obtained in yeast for understanding eukaryotic DNA replication in general. Recently, we (8), and others (9), have shown that the polymerase subunit of DNA polymerase III (10), the yeast analog of mammalian Polδ, is encoded by the *CDC2* gene (11), indicating an essential function for this enzyme during the S phase of the yeast cell cycle. Here, we describe the cloning, primary structure and cell cycle regulated expression of the yeast PCNA (yPCNA) gene, and show that it is an essential gene as well.

MATERIALS AND METHODS

Strains and Media. The *E. coli* strain was DH1. Yeast strains were prototrophic diploids NCYC239 and SK1 (12), as well as PY2/YM599 (*a/α ura3-52/ura3-52 trp1Δ/trp1Δ leu2-3,112/+ +/ade2-101 +/lys2-801 can1/+*). All rich and minimal media were as described (13).

Isolation of the gene for yPCNA. Fifty μg of yPCNA, Fraction VIII (14) was purified by electrophoresis on a preparative 12% SDS-polyacrylamide gel. The protein was transferred electrophoretically to a polyvinylidene difluoride membrane (15). Half of the appropriate membrane was directly subjected to sequencing on an automated Applied Biosystems sequenator. The other half was treated with trypsin, peptides released from the membrane were separated by reverse phase HPLC and some of these sequenced as well. A 41 aminoterminal sequence was obtained from the intact protein. And an octapeptide sequence, which was later shown to map near the carboxy terminus, was obtained from a tryptic fragment (Figure 2). Using yeast codon usage data, a sense strand 64-fold degenerate 44-mer oligonucleotide was made to amino acid residues 1−14 2/3 and an antisense strand 32-fold degenerate 42-mer oligonucleotide to residues 40−27 (16). These oligonucleotides, at 1 μM nucleotide each, were used as primers in a 25 cycle polymerase chain reaction (PCR) with total yeast DNA at 20 μg/ml as template (17). A 120 bp fragment was detected and purified by 10% PAGE and used as a probe for the isolation of the full length gene from a library of 10−20 kb genomic DNA *Hind*III fragments ligated into the centromere vector yCp50. Subcloning using the 120 bp PCR fragment as a probe located the gene to a 1.1 kb *Mlu*I-*Xba*I fragment, which was sequenced using sequenase (United States Biochemicals) and the dideoxy methodology. Both strands were completely sequenced from deletions generated with restriction enzyme cutting or by the use of appropriate synthetic oligonucleotide primers.

Plasmids. The 2.1 kb *Mlu*I fragment containing the yPCNA gene was ligated into the *Sal*I site of pUC19 after both the *Mlu*I and *Sal*I sites were filled in with DNA polymerase I, Klenow fragment, and dNTP's (pBL203). The orientation of the insert in pBL203 was such that a 1.0 kb *Xba*I fragment could be deleted by cutting and religating using the *Xba*I site in the polylinker

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of pUC19 and a single *Xba*I site in the insert, resulting in plasmid pBL205 containing the full length yPCNA gene on the remaining 1.1 kb *Mlu*I-*Xba*I fragment (Figure 1A). A 1.2 kb *Hind*III fragment from plasmid yEp24 which contained the *URA3* gene (18) was made blunt-end with DNA polymerase I, Klenow fragment, and dNTP's and ligated into the *Eco*RV sites of plasmid pBL203. This replaced the 160 bp *Eco*RV fragment internal to the yPCNA gene by the *URA3* gene (pBL206, Figure 1B). Plasmid pBL205 was cut with *Eco*RV and *Sty*I and religated after filling in the *Sty*I site, thus deleting a 360 bp fragment internal to the yPCNA gene (pBL207). The *URA3* gene was inserted in this plasmid into the *Hind*III site present in the polylinker region (pBL208, Figure 1C). Plasmid pBL203 was cut with *BamH*I and *Hind*III, which cut at sites in the pUC19 polylinker region such that the fragment contained the 2.1 kb *Mlu*I insert. The resulting fragment was ligated into the *BamH*I-*Hind*III site of centromere vector yCp50 (pBL211).

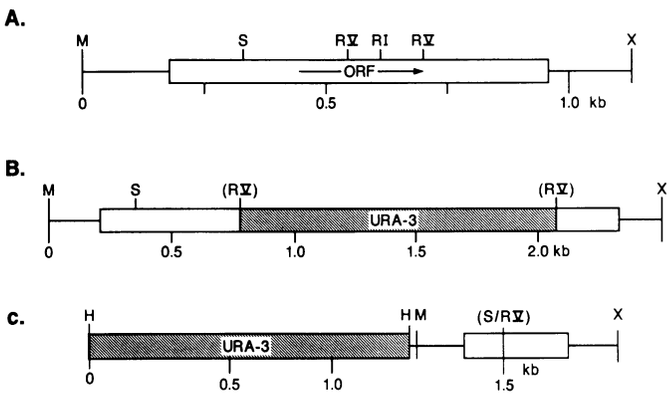

Figure 1. Restriction maps of the yeast PCNA gene region and deletion derivatives. All fragments are inserted into pUC19 in a counter clockwise direction as described in **Materials and Methods**. A, The *Mlu*I-*Xba*I wild-type fragment in pBL205; B, The disruption construction in pBL206 used for insertional mutagenesis; C, The deletion construction in pBL208 used for making a chromosomal deletion. Abbreviations are: M, *Mlu*I; S, *Sty*I; RV, *Eco*RV; RI, *Eco*RI; X, *Xba*I; H, *Hind*III.

Expression studies of the POL30 (yPCNA) gene. Strain NCYC239 was synchronized by the feed-starve method (12,19). Synchronous entry into meiosis for strain SK1 was affected through a shift to potassium acetate containing media (12,20). At appropriate times cells were collected by centriguation and frozen in dry ice. Total RNA was isolated by the glass-bead method (21), separated by agarose gel electrophoresis and transferred to Gene-screen (DuPont) according to

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   1                                  ACGCGTAACTTTTTTTTTTTTGGATTTCAACTGATAGTTTTCGTAC

  46 TTTGCTTCCTCTGGTACATAAAATTATATATAAGAAACACTTTTGCTTTAGCCTTCCTTTCTTTCCACTTGCACC
                                        ▼  ▼
 121 TTTCACTTTCGCCGTCCTTTTTCACTCACAGCAACAAGCAGCAAGCACTAAGTACGCAGTCAAAAGAGAGAAAAA

 196 ATGTTAGAAGCAAAATTTGAAGAAGCATCCCTTTTCAAGAGAATAATTGATGGTTTCAAAGATTGTGTCCAGTTG
   1 MetLeuGluAlaLysPheGluGluAlaSerLeuPheLysArgIleIleAspGlyPheLysAspCysValGlnLeu

 271 GTCAATTTCCAATGTAAAGAAGATGGTATCATTGCACAAGCTGTCGATGACTCAAGAGTTCTATTGGTCTCCTTG
  26 ValAsnPheGlnCysLysGluAspGlyIleIleAlaGlnAlaValAspAspSerArgValLeuLeuValSerLeu

 346 GAAATAGGTGTCGAAGCCTTCCAAGAATATAGATGTGACCATCCTGTTACGTTAGGTATGGATCTAACCTCACTA
  51 GluIleGlyValGluAlaPheGlnGluTyrArgCysAspHisProValThrLeuGlyMetAspLeuThrSerLeu

 421 AGTAAAATCCTACGTTGTGGTAACAACACCGATACATTAACACTAATTGCTGACAACACACCGGATTCCATCATC
  76 SerLysIleLeuArgCysGlyAsnAsnThrAspThrLeuThrLeuIleAlaAspAsnThrProAspSerIleIle

 496 TTATTATTTGAGGATACCAAGAAAGACCGTATAGCCGAATACTCTCTGAAATTGATGGATATCGATGCTGATTTC
 101 LeuLeuPheGluAspThrLysLysAspArgIleAlaGluTyrSerLeuLysLeuMetAspIleAspAlaAspPhe

 571 TTAAAGATTGAAGAATTACAGTACGACTCCACCCTGTCATTGCCATCTTCCGAATTCTCTAAAATTGTTCGTGAC
 126 LeuLysIleGluGluLeuGlnTyrAspSerThrLeuSerLeuProSerSerGluPheSerLysIleValArgAsp

 646 TTGTCCCAATTGAGTGATTCTATTAATATCATGATCACCAAAGAAACAATAAAGTTTGTAGCTGACGGTGATATC
 151 LeuSerGlnLeuSerAspSerIleAsnIleMetIleThrLysGluThrIleLysPheValAlaAspGlyAspIle

 721 GGATCAGGTTCAGTCATAATAAAACCATTCGTGGATATGGAACATCCTGAAACAAGCATCAAACTTGAAATGGAT
 176 GlySerGlySerValIleIleLysProPheValAspMetGluHisProGluThrSerIleLysLeuGluMetAsp

 796 CAACCTGTCGACTTGACGTTCGGAGCTAAATATTTATTGGACATCATTAAGGGCTCCTCCCTTTCTGATAGAGTT
 201 GlnProValAspLeuThrPheGlyAlaLysTyrLeuLeuAspIleIleLysGlySerSerLeuSerAspArgVal

 871 GGTATCAGGCTCTCCAGCGAAGCTCCTGCTTTATTCCAATTTGATTTGAAGAGTGGGTTCCTACAGTTTTTCTTG
 226 GlyIleArgLeuSerSerGluAlaProAlaLeuPheGlnPheAspLeuLysSerGlyPheLeuGlnPhePheLeu

 946 GCTCCTAAATTTAATGACGAAGAATAAATGTAAATTATCTATATAGTTGTATACTAAAAATAATAAACAAAAAAA
 251 AlaProLysPheAsnAspGluGlu

1021 AAACAGTAAAGTTTGTTTTAAATGAAAATAAATAACAAAGAAAATAAAGACTAAGTAGTCAGTTAATATCAGCAT

1096 TTTTGTGTGACTTATACAGTATTTATGACATATCTTACATTAATCTAGA
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Figure 2. DNA sequence of the *Mlu*I-*Xba*I fragment and deduced protein sequence of yPCNA. The *POL30* sequence data will appear in the EMBL/GenBank/DDBJ Nucleotide Sequence Databases under the accession number X16676. The underlined amino acid residues correspond to those obtained from protein sequencing of the intact protein (residues 1−41), or a tryptic fragment thereof (residues 231−240). Arrows indicate RNA start sites as mapped by primer extension analysis (34). Both start sites are used with approximately equal efficiency. The underlined DNA sequence is identical with a sequence upstream of the *POL3* gene.

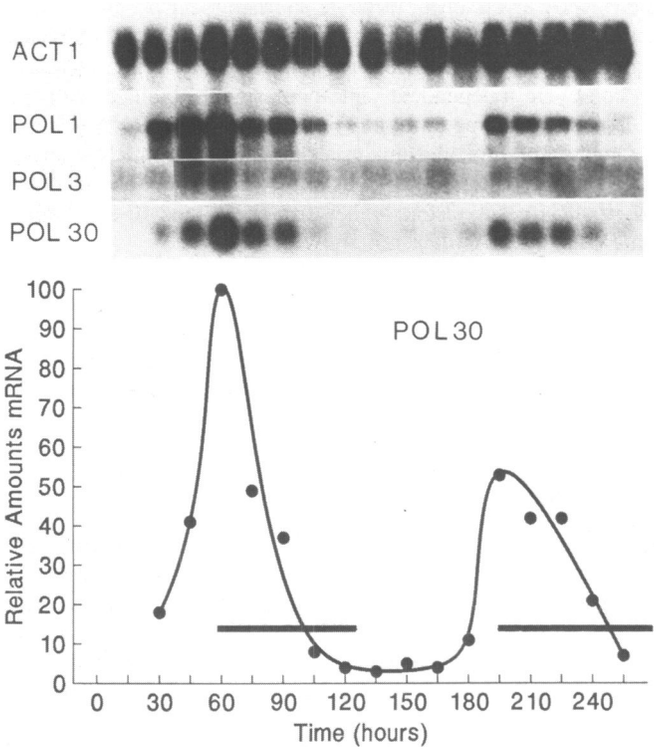

Figure 3. Cell cycle regulated expression in mitotic cells. NCYC239 cells, synchronized by the feed-starve method were shifted to rich media. Samples were removed at intervals and total RNA extracted for Northern blot analysis. A, The blot was probed with radioactively labelled fragments of the *ACT1*, *POL1*, *POL3* and *POL30* genes. B, Densitometric scanning of the autoradiogram obtained from probing of the blot with the *POL30* gene. The bar in the graph identifies the period of DNA synthesis (from ref. 12,20).

the manufacturer's directions. The blots were probed with ^{32}P-labelled probes to the coding sequences of the *ACT1*, *POL1*, *POL3* and *POL30* genes (8,22,23). Probes were prepared using the random primer method (24).

RESULTS

The sequence of the amino-terminal 41 residues of yPCNA was determined on an automated sequenator from the protein bound to a polyvinylidene difluoride membrane (15). Using this information, we made two oppositely-oriented degenerate oligonucleotides (see **Materials and Methods**) and subjected them to the polymerase chain reaction (PCR) with genomic yeast DNA (17). The resulting amplified 120 bp DNA fragment was used as a probe to *Hind*III-digested yeast genomic DNA in a Southern blot analysis. Hybridization was to a single 17 kb band. Accordingly, a yeast genomic library of 10−20 kb *Hind*III fragments was made in the centromere vector YCp50. Three identical clones were obtained by screening this library with the 120 bp fragment.

The yPCNA gene is designated *POL30* because PCNA is a subunit of DNA polymerase δ (III) and the gene designation for the catalytic polypeptide (formerly designated *CDC2*) should be considered as *POL3* (8,9). The *POL30* gene was mapped to the left arm of chromosome II (25) (results not shown). After restriction mapping, a 2.1 kb *Mlu*I fragment was subcloned into pUC19 in such a way that the *Mlu*I sites were regenerated and all further manipulations were done with this plasmid (pBL203) or a smaller derivative (pBL205) (see **Materials and Methods**). Further mapping and sequencing defined the gene to a *Mlu*I-*Xba*I fragment (Figure 1A). The DNA sequence of the 1.1 kb *Mlu*I-*Xba*I fragment and the deduced amino acid sequence of yPCNA are given in Fig. 2. An open reading frame for a 28,916 dalton protein was found. This is larger than the M_r of purified yPCNA as observed by SDS-PAGE (26−27 kDa), suggesting that the purified yPCNA may be a proteolytic fragment. However, amino acid sequence data from a tryptic fragment obtained from pure yPCNA yielded sequence data near the carboxy terminus (residues 231−240), indicating that our purified yPCNA could at most be missing the 18 carboxy terminal residues. In addition, we have recently expressed yPCNA in *E. coli* using an appropriate expression system, and the resulting purified protein has exactly the same SDS-PAGE size and enzymatic properties as the protein purified from yeast (Burgers *et al.*, unpublished results).

A null mutant of yPCNA was created *in vitro* by deleting the internal *Eco*RV fragment (160 bp) and inserting a 1.1 kb *Hind*III fragment from YEp24 containing the *URA3* gene (Fig. 1B). The resulting modified *POL30* gene was removed from the plasmid by digestion with *Mlu*I and transformed into diploid strain PY2/YM599 (see **Materials and Methods**) to disrupt one of the two copies of the gene by a double crossover event (26). Tetrad analysis of the sporulated diploid showed that the *POL30* gene is essential for spore viability. Of 18 tetrads analyzed, 10 produced two viable spores and 8 produced one viable spore. All viable spores were Ura$^-$. Sporulation of the control diploid strain gave (of 10 tetrads dissected) 6 tetrads with 4 viable spores, 3 tetrads with 3 viable spores, and 1 tetrad with 2 viable spores, indicating that the low spore viability of the parental strain was the cause of the relatively high number of tetrads with 1 viable spore in the disruption strain. These results show that the *POL30* gene is essential for growth. A large chromosomal deletion of the yPCNA gene was generated by transforming the PY2/YM599 diploid to Ura$^+$ with the integrating plasmid pBL208 (Figure 1C). Cells which had then excised the plasmid by intramolecular recombination leaving the deleted copy of the gene in the chromosome were selected by plating on 5-fluoroorotic acid containing plates and subsequently screened by Southern blot analysis (27,28). Sporulation and tetrad analysis showed that this deletion was also lethal. The deletion mutant, however, was complemented by a plasmid containing the *POL30* gene on a 2.1 kb *Mlu*I fragment inserted into centromere vector yCp50 (pBL211, see Materials and Methods) when the heterozygous diploid was transformed with this plasmid prior to sporulation.

190 bp upstream from the initiation codon is a *Mlu*I (ACGCGT) site which is also present in one or more copies upstream from a large number of genes involved in DNA metabolism that are periodically expressed during the cell cycle (23). This sequence is also present upstream from the start site of the *POL3* (*CDC2*) gene (8). Even more strikingly, a 14 bp stretch at the *Mlu*I site (ACGCGTAACTTTTT) is identical for the *POL3* (*CDC2*) and *POL30* (yPCNA) genes (Ref. 8, Fig. 2), suggesting that both genes may be similarly regulated. Cell cycle dependent expression of the *POL3* and *POL30* genes was measured after synchronization of NCYC239 cells by the feed-starve method (12,19). This type of synchronization gave results identical to elutriation or α-Factor synchronization when cell-cycle dependent expression of the *POL1* and *CDC9* genes was studied (12,20). The timing of *POL3* and *POL30* expression was indistinguishable from each other and from that of the *POL1* gene, with steady-

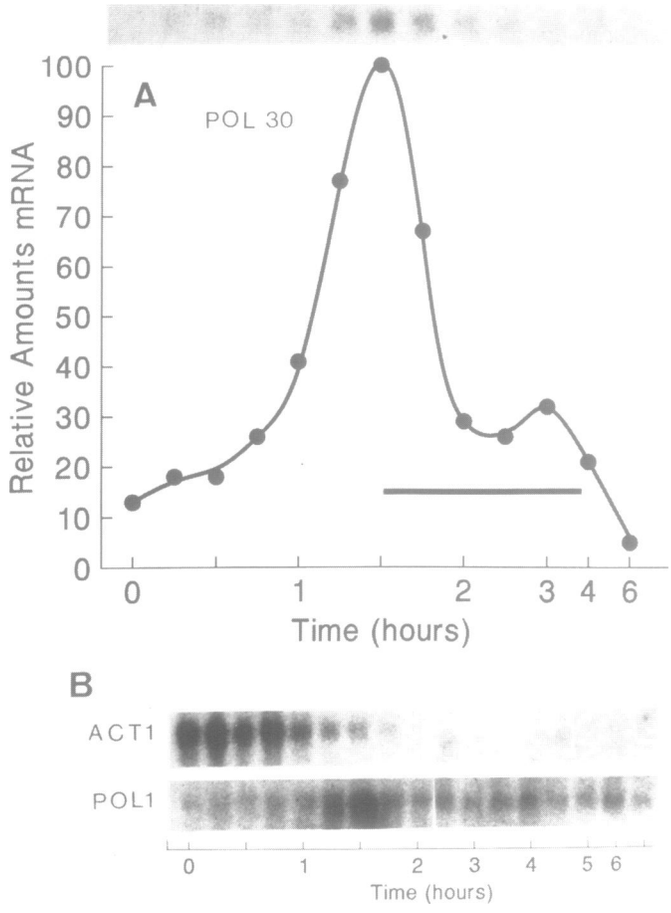

Figure 4. Regulation of mRNA levels during meiosis. Late-log phase SK-1 cells were transferred to sporulation medium and samples taken and processed as described in **Materials and Methods** and in the Legend to Figure 3. The same RNA samples were used for different blots in *A* and *B*. *A*. The blot was probed with the *POL30* gene and the autoradiogram scanned with a densitometer. The bar indicates the region of premeiotic DNA synthesis (from ref. 12,20). *B*. The blot was probed with the *ACT1* and *POL1* genes.

state mRNA levels of all three genes rising sharply in late G1, continuing through S, and decreasing again in the late S phase (Figure 3). As a control, expression of the actin gene (*ACT1*) was cell-cycle independent. The poor signal obtained with the *POL3* probe was due to the low levels of *POL3* mRNA (Figure 3). Quantitative dot blot analysis of steady state mRNA levels in asynchronously growing cells showed that the abundance of *POL30* mRNA is 2−3 fold higher than *POL1*, and 10-fold higher than *POL3* mRNA (data not shown).

The *POL30* and *POL1* mRNA levels also rose coordinately when the diploid strain SK-1 was induced to sporulate in a synchronous fashion (Figure 4). In these blots, however, the *POL3* (*CDC2*) mRNA could not be detected above background noise. Again, increase in the mRNA levels precedes meiotic DNA replication.

DISCUSSION

The use of amino acid sequence derived oppositely oriented degenerate oligonucleotides as primers for a polymerase chain reaction with total nuclear DNA proved to be a facile way to generate a probe for isolation of the full-length gene (29). Only one fragment of the expected size (120 bp) was produced. The coding region of the *POL30* gene is preceded by a 190 bp regulatory region which contains a consensus TATA box, the RNA start site(s) and a ACGCGT sequence (a *MluI* site) presumed to be required for cell cycle regulated gene expression (23). This 190 bp upstream region was sufficient to allow complementation of a *POL30* deletion mutant by the wild-type gene on a centromere plasmid. Whether the entire region is required for proper expression has not yet been determined.

The putative amino acid sequence of yPCNA shows ca. 35% homology to that of the human PCNA (30) (Fig. 2). While yPCNA can fully stimulate calf thymus DNA polymerase δ by increasing its processivity of action, it cannot substitute for human PCNA in the *in vitro* SV40 DNA replication system (14). This low homology at the amino acid level can also explain why human polyclonal antisera from lupus patients, which react with

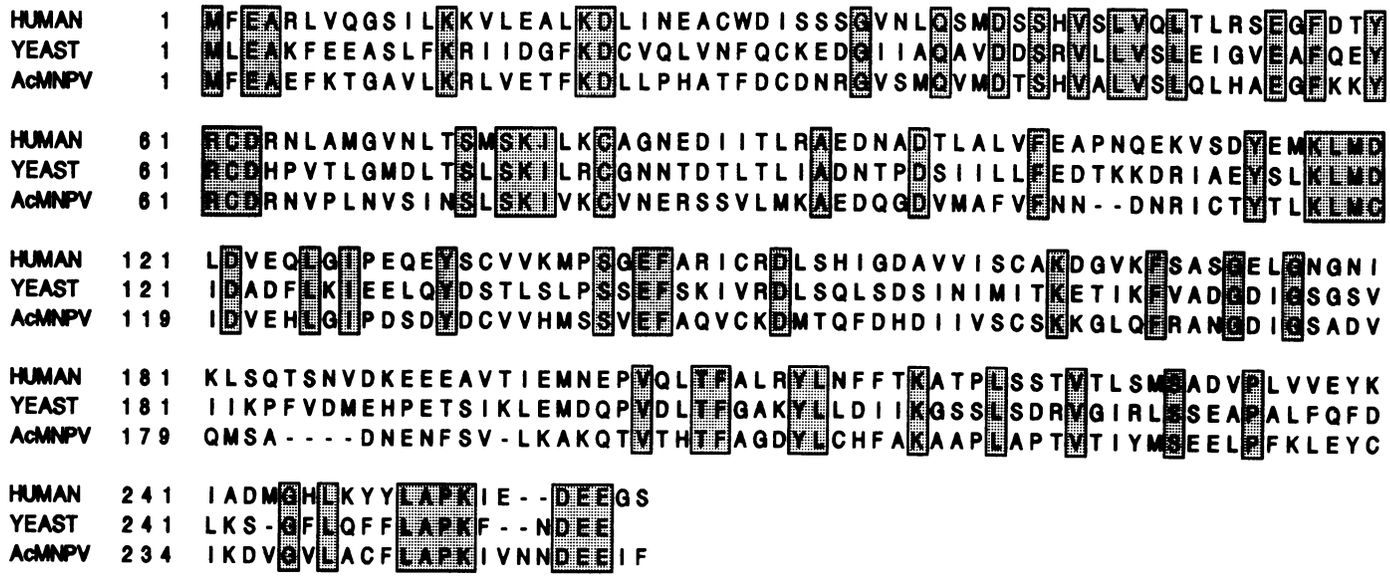

Figure 5. Protein comparison of human, yeast, and AcMNPV PCNA sequences. Protein sequence homology was determined using the Wilbur/Lipman PRTALN alignment program (35). Boxed residues indicate identity between all three PCNA sequences.

mammalian PCNA, do not react with yPCNA (14). Recently it has been reported that the baculovirus *Autographa californica* nuclear polyhedrosis virus (AcMNPV) has a gene that encodes a protein, ETL, that has significant homology to rat PCNA (31), which in turn is virtually identical to the human PCNA. The possibility that ETL functions similarly to yeast and calf thymus PCNA is further supported by the finding that the AcMNPV also encodes a DNA polymerase with δ-like properties, i.e. aphidicolin sensitivity, and a 3′→5′ exonuclease activity (32). This AcMNPV ETL protein is 34% homologous to yeast PCNA (Fig. 5). When all three PCNA sequences are analyzed together (human, yeast, and AcMNPV), there are definite domains of homology which might be important for protein-protein interaction with the δ polymerase (33).

These results indicate that yPCNA plays a necessary role in yeast, presumably in its interaction with DNA polymerase δ during DNA replication. This interaction appears to be conserved through evolution, since yeast PCNA can sustitute for calf thymus PCNA *in vitro*. Also, domains of homology between human, yeast and AcMNPV PCNA suggest a functional conservation between these three very diverse organisms.

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