Molecular Cloning of the cDNAs for the Four Subunits of Mouse DNA Polymerase α-Primase Complex and Their Gene Expression during Cell Proliferation and the Cell Cycle*

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The DNA polymerase α-primase complex purified from mouse FM3A cells is composed of four polypeptides with molecular masses of 180, 68, 54, and 46 kDa. The largest subunit has DNA polymerase activity, the two smallest subunits have DNA primase activity, and the function of the 68-kDa subunit is unknown. We have isolated the cDNAs of the four subunits by low stringency hybridization and reverse transcription polymerase chain reaction and determined their nucleotide sequences. The predicted amino acid sequence of the 180-kDa subunit shows 88, 38, 34, and 32% identity to those of the catalytic subunits of human, Drosophila melanogaster, Schizosaccharomyces pombe, and Saccharomyces cerevisiae DNA polymerase α, respectively, and contains seven regions whose orders and sequences are highly conserved among viral and other eukaryotic DNA polymerases. The deduced amino acid sequence of the 68-kDa subunit shows 25% identity to that of the 73-kDa subunit of D. melanogaster DNA polymerase α-primase, shows no significant sequence similarity to any other protein in the data bases, but contains a potential phosphorylation site(s) for cdc2 kinase. The amino acid sequence of the 54-kDa subunit shows 32% identity to that of the large subunit of S. cerevisiae DNA primase. During activation of quiescent Swiss mouse 3T3 cells to proliferate, the levels of mRNA of the four subunits of the DNA polymerase α-primase complex increased before DNA synthesis. In growing mouse FM3A cells, the transcripts of the four subunits are present throughout the cell cycle and increase slightly prior to the S phase.

Nuclear chromosomal DNA replication is a complex and tightly regulated process involving the orderly coordination of many protein-protein, protein-DNA, and protein-substrate interactions (1). Five distinct DNA polymerases have been isolated from eukaryotes and characterized (2, 3). Judging from the results with a reconstituted cell-free system of SV40 viral DNA replication (4, 5) and from genetic studies on budding yeast (6, 7), two or three of the DNA polymerases, α, δ, and/or ε, are probably involved in chromosomal DNA replication (2). Besides the DNA polymerases, many other proteins function by interacting with one another during DNA replication in vivo.

DNA polymerase α is essential for chromosomal DNA replication. Monoclonal antibodies against DNA polymerase α (8) inhibit DNA synthesis in permeabilized cells (9) or when microinjected into nuclei (10). HeLa cell extracts depleted of DNA polymerase α by immunoaffinity column chromatography do not support cell-free replication of SV40 DNA, which is a good model system of eukaryotic chromosomal DNA replication. In this system cell-free replication can be restored specifically by the addition of an appropriate (human or monkey but not murine) DNA polymerase α (11). A mutant mouse cell strain, tsF20 (12), which we isolated previously, has heat-labile DNA polymerase α activity. Characterization of the mutant strain (13–15), its revertants (16), and its purified DNA polymerase α protein (17) clearly demonstrated that DNA polymerase α activity is essential for chromosomal DNA replication.

Preparations of DNA polymerase α isolated from a wide range of species have been found to be composed of a remarkably similar set of four peptides of 165, 60, 54, and 46 kDa (for review, see Refs. 3 and 18). The largest subunit has a catalytic function (19), and DNA primase activity is associated with the two smallest subunits (for review, see Ref. 20). The function of the 68~86-kDa subunit is not known. The DNA polymerase α-primase complex might function by interacting with an initiation protein(s) of DNA replication or other replicative proteins. In SV40 viral DNA replication, the large T antigen, which binds to the origin sequences of SV40 DNA and is assumed to be an initiation protein of SV40 DNA replication, binds directly and specifically to the catalytic subunit of human DNA polymerase α (21). Recently, it was reported that single-stranded DNA-binding protein, replication protein A, forms a specific complex with purified DNA polymerase α-primase (22).

cDNAs for the catalytic subunit of DNA polymerase α-

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) D13543, Mus musculus POLA1 (180-kDa subunit); D13544, M. musculus PRIM1 (46 kDa); D13546, M. musculus PRIM2 (64 kDa); and D13547, M. musculus POLA2 (68 kDa).

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The abbreviations used are: SV40, simian virus 40; PCR, polymerase chain reaction; RT, reverse transcription; PCNA, proliferating cell nuclear antigen; kb, kilobase(s).
primase complex have been isolated from Saccharomyces cerevisiae, man, Schizosaccharomyces pombe, and Drosophila melanogaster (23–26). Analysis of the deduced primary structure of the catalytic subunit of human DNA polymerase α revealed six regions with striking similarity to regions in prokaryotic and eukaryotic replicative DNA polymerases (24). The cDNA for the third largest subunit of the DNA polymerase α-primase complex or the large subunit of DNA primase was isolated from S. cerevisiae (27), and cDNAs for the smallest subunit of the DNA polymerase α-primase complex were isolated from S. cerevisiae (28) and mouse cells (29). The genes for both subunits are essential for growth of S. cerevisiae. Most recently, the sequence of cDNA for the second largest subunit of D. melanogaster DNA polymerase α-primase complex has been reported (30). For an understanding of the molecular mechanisms of eukaryotic DNA replication it is necessary to elucidate the structure-function relationship of the DNA polymerase α-primase complex and the interactions of the complex with other replication proteins. Another important aspect of eukaryotic DNA replication is the mechanisms regulating the gene expression of replication proteins. It is important to determine the regulatory mechanisms controlling the gene expressions of the four subunits of the DNA polymerase α-primase complex are regulated since these four subunits always appear to exist as a complex. As initial steps toward these goals, we have, in the present study, isolated the cDNA clones encoding the four subunits of the mouse DNA polymerase α-primase complex, determined their nucleotide sequences, and examined their expression in mouse cells during the cell cycle.

**EXPERIMENTAL PROCEDURES**

**Materials**—[α-32P]dCTP, [γ-32P]ATP, a multiprime DNA labeling system, and a cDNA synthesis kit were purchased from Amersham International plc (Buckinghamshire, United Kingdom). A double-stranded sequencing kit for GENESIS T4 polynucleotide kinase, Klenow fragment, and nucleotide linker oligonucleotide synthesis were purchased from Applied Biosystems Inc. The plasmids carrying cDNA sequences for human EF-la (31) and mouse PCNA (32) were gifts from Dr. Y. Kajiro and Dr. A. Hoffman (39) and blunt ended with T4 DNA polymerase. After treatment with EcoRI methylase, the double-stranded cDNA was ligated into the synthetic EcoRI linker and then amplified with an Applied Biosystems model 391 DNA synthesizer. Alternatively, double-stranded cDNA was ligated to synthetic adapters because it was not necessary to digest it with any restriction enzyme. The linker- or adapter-ligated cDNA thus prepared was passed through a Sepharose CL-4B column to remove excess linker and adapter fragments and then inserted into the EcoRI site of a plasmid vector. These recombinant plasmids were packaged in vitro using Gigapack Gold (Stratagene). The Escherichia coli C600 hIΔ strain was infected with these bacteriophage particles. More than one million independent clones were obtained for both total poly(A)+ RNA and the fractionated longer sized RNA.

**Preparation of First Strand cDNA for PCR**—A sample of 2 μg of poly(A)+ RNA was converted to the first strand of cDNA by using a cDNA synthesis kit from Amersham Corp. and oligo(dT)20 as primer. The first strand cDNA synthesized was passed through a Sepharose CL-4B column to remove oligo(dT)12-18 primer, and its amount was calculated by monitoring the incorporation of [α-32P]dCTP into high molecular weight DNA.

**PCR**—PCR was performed as recommended by the supplier using 30 ng of the first strand cDNA in a total volume of 30 μl containing 50 mM KCl, 10 mM Tris-HCl, pH 8.3 (25°C), 1.5 mM MgCl2, 0.01% (w/v) gelatin, 200 μM dNTPs, 50 pmol of sense and antisense primers, 2.5 units of Taq DNA polymerase. After a denaturing step, specific amplified DNA fragments were purified from the gel by using the DESI paper method (40).

**Screening of cDNAs**—More than 8 × 106 recombinant phage plaques transferred to duplicate nitrocellulose membranes were screened by the plaque hybridization method (41). Freelyhybridized plasmids were washed out at 68°C in 1 × SSPE, 0.1% SDS, 2 × Denhardt’s solution (42), 0.5 × SSPE, and 0.1% SDS. The gradient was centrifuged at 62,300 Xg at 64°C for 7 days. Longer RNA was prepared from logarithmically growing mouse FM3A cells by homogenization with guanidinium thiocyanate followed by centrifugation through a cesium chloride cushion (36). Longer RNA was prepared for cloning the 180-kDa subunit cDNA of mouse DNA polymerase α. Approximately 6 mg of total RNA from mouse FM3A cells was heated at 69°C for 5 min and loaded onto a Sepharose 6B-DNA matrix containing 100 mM sodium acetate, 50 mM NaCl, 1 mM EDTA, and 0.1% SDS. The gradient was centrifuged at 62,300 × g for 20 h at 15°C and then collected in 35 fractions (37). The size of the RNA was examined by agarose gel electrophoresis, and total RNA (approximately 1.3 mg) of more than 2 kb was collected. Poly(A)+ RNA was purified from total RNA or longer sized fractionated RNA by a batch oligo(dT)-latex beads procedure (38). Poly(A)+ RNA was converted to double-stranded cDNA by the RNase H method of Butler and Hoffman (39) and blunt ended with T4 DNA polymerase. After treatment with EcoRI methylase, the double-stranded cDNA was ligated into the synthetic EcoRI linker and finally digested with EcoRI. Alternatively, double-stranded cDNA was ligated to synthetic adapters because it was not necessary to digest it with any restriction enzyme. The linker- or adapter-ligated cDNA thus prepared was passed through a Sepharose CL-4B column to remove excess linker and adapter fragments and then inserted into the EcoRI site of a plasmid vector. These recombinant plasmids were packaged in vitro using Gigapack Gold (Stratagene). The Escherichia coli C600 hIΔ strain was infected with these bacteriophage particles. More than one million independent clones were obtained for both total poly(A)+ RNA and the fractionated longer sized RNA.

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script as well as the human transcript under a low stringent condition as described above (data not shown). Using this probe, we screened 2.8 × 10^6 clones of the mouse FM3A cDNA library prepared with a λgt10 vector. Two positive clones (M180-16 and M180-1) were obtained. The 2.2-kb insert of clone M180-16 and the 3.5-kb insert of clone M180-1 both contained a 1.8-kb cDNA fragment and the DNA subunit of mouse DNA polymerase α. To obtain full-length cDNA, we made a new cDNA library from fractionated longer RNA by using random hexanucleotides as primers instead of oligo(dT)12-18. Two 29-mer oligonucleotides (the nucleotides of the mouse polymerase α cDNA were 2561–2579 and 2564–2593) that have sequences of 16 nucleotides complementary to each other were synthesized as high specific activity probes. Thirteen positive clones were obtained by screening 8 × 10^6 clones of the cDNA library. Among them, two clones (M180-7 and M180-9) contained a 5'-end putative initiation codon and overlapped the two previous clones.

For cloning of the DNA fragment as a probe, we used RT-PCR method (42). Two or more partial peptide sequences were obtained from the subunit; and based on these peptide sequences, two to four pairs of oligonucleotides, each in the sense and antisense orientation, were used as primers in PCR. These were designed with minimal degeneration using deoxyinosine at ambiguous codons of the amino acids. 30 positive clones were obtained. The 2.2-kb insert of clone M180-1 and the 3.5-kb insert of clone M180-9 contained a DNA region coding for the 3' end initiation codon and overlapped the two previous clones. For cloning of the DNA fragment as a probe, we used RT-PCR method (42). Two or more partial peptide sequences were obtained from each subunit; and based on these peptide sequences, two to four pairs of oligonucleotides, each in the sense and antisense orientation, were used as primers in PCR. These were designed with minimal degeneration using deoxyinosine at ambiguous codons of the amino acids. 30 positive clones were obtained. The 2.2-kb insert of clone M180-1 and the 3.5-kb insert of clone M180-9 contained a DNA region coding for the 3' end initiation codon and overlapped the two previous clones.

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FIG. 2. Nucleotide sequence and deduced amino acid sequence of cDNA for the 180-kDa catalytic subunit of the mouse DNA polymerase α-primase complex. Top numbers on the right are those of nucleotides, and lower ones are those of amino acids numbered from A of the initiation codon, and methionine 1, respectively. The asterisk indicates the termination codon, TGA. Peptide sequences derived from the immunoaffinity-purified mouse 180-kDa subunit are boxed with normal lines. Boxed and underlined amino acids 28–43 represent a potential nuclear localizing sequence. Underlined SP and TP represent loose motifs of potential phosphorylation sites for casein kinase. The shaded amino acids nucleotides represent a potential nuclear localizing sequence.
Amino Acid Sequence of the 180-kDa Subunit of the Mouse DNA Polymerase α-Primase Complex—The open reading frame predicts a polypeptide of 1,462 amino acid residues with a molecular weight of 167,037. This size is a little different from that deduced by SDS-polyacrylamide gel electrophoresis analysis. Possibly this protein is modified post-translationally. There are six copies of the sequence (Asn-X-Ser/Thr), which is a potential site of N-linked glycosylation, located at Asn461, Asn864, Asn1012, Asn1196, Asn1386, and Asn1400. These sites are perfectly conserved in the human 180-kDa subunit. No strict motif (-Ser/Thr-Pro-polar amino acid-basic amino acid-) that is a potential phosphorylation site for cdc2 kinase was found in this amino acid sequence, but there are six loose motifs (-Ser/Thr-Pro-), five of them located toward the amino terminus of the 180-kDa polypeptide and one in the carboxyl-terminal region. Information to code for two partial peptide sequences determined with the peptide sequencer was present in this reading frame (Fig. 2).

The amino acids starting from position 28 of the mouse 180-kDa subunit have two clusters of basic amino acids separated by 10 intervening spacer amino acids. A similar motif of two clusters of basic amino acids has recently been identified as a nuclear localization signal (49). These basic amino acid regions are probably a nuclear localization signal. The amino acid sequence of these regions is completely identical to that of the human counterpart. Like the human and Drosophila enzymes, the mouse 180-kDa subunit of the DNA polymerase α-primase complex has two putative zinc finger motifs in its carboxyl-terminal region (50). The first motif (amino acids 1282–1316) has the structure -Cys-X-Cys-X-Cys-, and the second (amino acids 1349–1375) the structure -Cys-X-Cys-X-Cys-. These 8 cysteine residues and the spacing between the two zinc finger motifs are conserved in the human 180-kDa subunit. These regions are predicted to be a DNA binding site.

A comparison of the deduced amino acid sequence of the mouse 180-kDa subunit with the sequences of the other known a-family polymerase α catalytic subunits is shown in Fig. 3. The amino acid sequence of the 68-kDa subunit and the mouse subunit shows 88, 38, 34, and 32% identity with the sequences of the human, Drosophila, S. pombe, and S. cerevisiae subunits, respectively. Alignment of the mouse, human, Drosophila, S. pombe, and S. cerevisiae 180-kDa amino acid sequences reveals seven highly conserved regions present in α-like DNA polymerases, namely regions I–VI indicated by Wong et al. (24) and region VII identified by Spicer et al. (51). Of these regions, sequence conservation is lowest in region IV, 5 of 42 residues being identical in the five proteins. This region includes two of the regions, ExoI and ExoII, postulated by Bernad et al. (52) to be parts of the 3′–5′ exonuclease activity site, by homology with known active site sequences in the 3′–5′ exonuclease domain of DNA polymerase I of E. coli. Structurally, the mouse 180-kDa subunit is very closely homologous to the human polymerase α catalytic subunit, the primary sequences of all of the conserved regions except region IV of the mouse and human 180-kDa catalytic subunits being identical. But the amino-terminal amino acid sequences (amino acids 1–13) of the mouse and human subunits have diverged, and the sequence on the amino-terminal side (amino acids 110–330) of the mouse subunit shows less than 70% homology with that of the human subunit. These regions may be involved in the species specificity of DNA replication observed in the SV40 cell-free DNA replication system. In addition to these regions, there are five other highly conserved regions (A–E) in the five proteins. In region A, 10 of 25 residues (40%), in region B, 20 of 43 residues (47%), in region C, 13 of 28 residues (46%), in region D, 9 of 25 residues (36%), and in region E, 8 of 19 residues (42%) are identical.

Cloning and Structure of the 68-kDa Subunit cDNA of the Mouse DNA Polymerase α-Primase Complex—The function of the 68-kDa subunit of DNA polymerase α is unknown. To obtain information on this subunit, we isolated its cDNA by the RT-PCR method as described under “Experimental Procedures.” Fig. 1B is the restriction endonuclease map of the 68-kDa subunit cDNA. The nucleotide sequence of the 68-kDa subunit cDNA of the mouse DNA polymerase α-primase complex and the deduced amino acid sequence of the protein are shown in Fig. 4. This cDNA has an open reading frame of 1,800 bases with about 200 nucleotides in the 5′-untranslated region and 324 nucleotides in the 3′-untranslated region. One cDNA is terminated by a poly(A) tail, 14 nucleotides downstream from a polyadenylation signal. In the 3′-untranslated region there is no degradation signal, ATTTA.

Amino Acid Sequence of the 68-kDa Subunit of the Mouse DNA Polymerase α-Primase Complex—The longest open reading frame of the 68-kDa subunit cDNA predicts a polypeptide of 600 amino acid residues with a molecular weight of 66,266. This size is close to that estimated for the 68-kDa subunit by SDS-polyacrylamide gel electrophoresis. The predicted polypeptide contains three oligopeptide sequences completely matching the partial amino acid sequences of the 68-kDa subunit (Fig. 4). A comparison of the predicted 68-kDa subunit amino acid sequence is compared with the D. melanogaster 73-kDa subunit of DNA polymerase α-primase (30) in Fig. 5. The sequences for the mouse and D. melanogaster subunits share 24.5% identical amino acid residues. A search of the Swiss protein data base indicated that the amino acid sequence of the 68-kDa subunit has no significant similarities to those published for any other proteins. There is no obvious consensus sequence for a nuclear localization signal. There is only one potential site for N-linked glycosylation located at Asn230 with a molecular weight of 167,037. This size is a little different from that deduced by SDS-polyacrylamide gel electrophoresis analysis. Possibly this protein is modified post-translationally. There are six copies of the sequence (Asn-X-Ser/Thr), which is a potential phosphorylation site for cdc2 kinase, located at Asn461, Asn864, Asn1012, Asn1196, Asn1386, and Asn1400. These sites are perfectly conserved in the human 180-kDa subunit. No strict motif (-Ser/Thr-Pro-polar amino acid-basic amino acid-) that is a potential phosphorylation site for cdc2 kinase was found in this amino acid sequence, but there are six loose motifs (-Ser/Thr-Pro-), five of them located toward the amino terminus of the 180-kDa polypeptide and one in the carboxyl-terminal region. Information to code for two partial peptide sequences determined with the peptide sequencer was present in this reading frame (Fig. 2).

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A comparison of the deduced amino acid sequence of the mouse 180-kDa subunit with the sequences of the other known a-family polymerase α catalytic subunits is shown in Fig. 3. The amino acid sequence of the 68-kDa subunit and the mouse subunit shows 88, 38, 34, and 32% identity with the sequences of the human, Drosophila, S. pombe, and S. cerevisiae subunits, respectively. Alignment of the mouse, human, Drosophila, S. pombe, and S. cerevisiae 180-kDa amino acid sequences reveals seven highly conserved regions present in α-like DNA polymerases, namely regions I–VI indicated by Wong et al. (24) and region VII identified by Spicer et al. (51). Of these regions, sequence conservation is lowest in region IV, 5 of 42 residues being identical in the five proteins. This region includes two of the regions, ExoI and ExoII, postulated by Bernad et al. (52) to be parts of the 3′–5′ exonuclease activity site, by homology with known active site sequences in the 3′–5′ exonuclease domain of DNA polymerase I of E. coli. Structurally, the mouse 180-kDa subunit is very closely homologous to the human polymerase α catalytic subunit, the primary sequences of all of the conserved regions except region IV of the mouse and human 180-kDa catalytic subunits being identical. But the amino-terminal amino acid sequences (amino acids 1–13) of the mouse and human subunits have diverged, and the sequence on the amino-terminal side (amino acids 110–330) of the mouse subunit shows less than 70% homology with that of the human subunit. These regions may be involved in the species specificity of DNA replication observed in the SV40 cell-free DNA replication system. In addition to these regions, there are five other highly conserved regions (A–E) in the five proteins. In region A, 10 of 25 residues (40%), in region B, 20 of 43 residues (47%), in region C, 13 of 28 residues (46%), in region D, 9 of 25 residues (36%), and in region E, 8 of 19 residues (42%) are identical.

Cloning and Structure of the 68-kDa Subunit cDNA of the Mouse DNA Polymerase α-Primase Complex—The function of the 68-kDa subunit of DNA polymerase α is unknown. To obtain information on this subunit, we isolated its cDNA by the RT-PCR method as described under “Experimental Procedures.” Fig. 1B is the restriction endonuclease map of the 68-kDa subunit cDNA. The nucleotide sequence of the 68-kDa subunit cDNA of the mouse DNA polymerase α-primase complex and the deduced amino acid sequence of the protein are shown in Fig. 4. This cDNA has an open reading frame of 1,800 bases with about 200 nucleotides in the 5′-untranslated region and 324 nucleotides in the 3′-untranslated region. In the 3′-untranslated region there is no degradation signal, ATTTA.
amino acid sequence of the protein are shown in Fig. 6. The cDNA of the mouse 54-kDa subunit, the large subunit of primase, has an open reading frame of 1,515 bases with about 150 nucleotides in the 5'-untranslated region and 305 nucleotides in the 3'-untranslated region. The cDNA is terminated with a poly(A) tail, 16 nucleotides downstream of a polyadenylation signal. In the 3'-untranslated region, there is one motif for mRNA selective degradation, ATTTA.

### Amino Acid Sequence of the 54-kDa Subunit of the Mouse DNA Polymerase α-Primase Complex

The longest open reading frame of the 54-kDa subunit cDNA predicts a polypeptide of 505 amino acid residues with a molecular weight of 58,498. This size is a little different from that estimated for the 54-kDa subunit by SDS-polyacrylamide gel electrophoresis. There is a potential site for N-linked glycosylation at Asn^26. The predicted polypeptide contains four oligopeptide sequences completely matching the partial amino acid sequences determined for the 54-kDa subunit protein (Fig. 6).

The predicted 54-kDa subunit amino acid sequence is compared with the S. cerevisiae large subunit of DNA primase (27) in Fig. 7. The sequences for the mouse and S. cerevisiae subunits share 32.0% identical amino acid residues.

### Cloning and Structure of the 46-kDa Subunit cDNA for the Mouse DNA Polymerase α-Primase Complex

The 46-kDa subunit is a component of DNA primase and has the ATP- or GTP-binding site(s). The cDNA for the mouse small subunit of DNA primase had already been cloned (29). We isolated the small subunit of DNA primase from mouse FM3A cells to confirm that the cDNA can be cloned by the RT-PCR method before isolating the cDNAs of the other subunits by the same method as described under "Experimental Procedures." The nucleotide sequence of the 46-kDa subunit cDNA and the deduced amino acid sequence of the protein are shown in Fig. 8. The mouse 46-kDa subunit cDNA had an open reading frame of 1,251 bases encoding a polypeptide of 417 amino acid residues with a molecular weight of 49,295. Its...
cDNAs for Mouse DNA Polymerase α-Primase Complex

Fig. 4. Nucleotide sequence and deduced amino acid sequence of the 68-kDa cDNA of the mouse DNA polymerase α-primase complex. Top numbers on the right are those of nucleotide residues, and lower ones are those of amino acids. The asterisk indicates the termination codon, TGA. A putative polyadenylation signal in the 3′-untranslated region is shown by a dashed line.

Mouse (FM3A)

Drosophila

Fig. 5. Amino acid sequence similarity between the 68-kDa subunit of the mouse DNA polymerase α-primase complex and the 73-kDa subunit of D. melanogaster DNA polymerase α. The alignment of amino acid sequence was optimized by computer analysis. The upper amino acid sequence is that of the 68-kDa subunit of the mouse DNA polymerase α-primase complex, and that of the 73-kDa subunit of D. melanogaster DNA polymerase α is the lower sequence. Identical residues in these two proteins are boxed. Gaps are indicated by dashes.
cDNAs for Mouse DNA Polymerase α-Primase Complex

**Fig. 6.** Nucleotide sequence and deduced amino acid sequence of 54-kDa cDNA of the mouse DNA polymerase α-primase complex. Top numbers on the right are those of nucleotides, and lower ones are those of amino acids. The asterisk indicates the termination codon, TAG. A putative polyadenylation signal in the 3'-untranslated region is shown by a bold box. The ATTTA sequence motif is underlined. Underlined and double underlined amino acids represent peptide sequences derived from the purified mouse 54-kDa subunit. Double underlined amino acids represent the positions of PCR primers. Amino acids with three circles below them are potential sites for N-linked glycosylation.

**Fig. 7.** Amino acid sequence similarity between the 54-kDa subunit of the mouse DNA polymerase α-primase complex and the large subunit of *S. cerevisiae* DNA polymerase. The alignment of amino acid sequence was optimized by computer analysis. The *upper* amino acid sequence is that of the 54-kDa subunit of the mouse DNA polymerase α-primase complex, and the *lower* sequence is that of the large subunit (PR12) of *S. cerevisiae* DNA primase. Identical residues in these two proteins are boxed. Gaps are indicated by *dashes*.

our sequence, at nucleotide position 1301, 13 nucleotides upstream from the first potential polyadenylation signal. The last difference was also in the 3’-untranslated region. Our cDNA terminated with a poly(A) tail, 59 nucleotides downstream of the second potential polyadenylation signal. There were two potential mRNA degradation signals in the 3’-untranslated region.

**Increase in mRNAs of the Four Subunits of the DNA Polymerase α-Primase Complex before DNA Synthesis upon Serum Stimulation**—After growth induction, quiescent cells progress through a series of events leading to DNA synthesis and mitosis. To examine the expressions of the genes of the mouse DNA polymerase α-primase complex subunits during this process, we arrested Swiss mouse 3T3 cells at the Go phase by serum starvation and then induced their proliferation by adding serum. The level of each mRNA was measured by Northern blot hybridization at the indicated times after serum addition. The mRNA levels all increased markedly from 8 h after serum stimulation, reaching maxima at 16–20 h, and subsequently decreasing (Fig. 9). As a control for the effect of serum stimulation on mRNA levels, the mRNA level of EF-1α was also determined. As shown in Fig. 9A, its level was not down-regulated by serum starvation or up-regulated by serum stimulation. DNA synthesis, measured as [3H]thymidine incorporation, was also shown in Fig. 9B. DNA synthesis was arrested by serum starvation and peaked after serum stimulation.
dine incorporation, began 12 h after serum stimulation and reached a maximum at 20 h (Fig. 9B), the time of the beginning of the S phase as reported previously. These results indicated that the mRNA levels of the four subunits of the mouse DNA polymerase α-prime complex increased before DNA synthesis. As a positive control, the mRNA levels of mouse PCNA, which is an auxiliary protein of DNA polymerase α, was also measured and shown to increase before the S phase. The induction of PCNA mRNA after serum stimulation seemed to be similar to those of the 46-kDa and 68-kDa subunits. The gene expression of the two larger subunits of the mouse DNA polymerase α-prime complex associated with DNA polymerase activity was maintained at the basal level in cells in the last fraction. Like the mRNA levels for the 54- and 46-kDa subunits, the mRNA level for the 180-kDa subunit mRNA that peaked a few hours later and the 180-kDa subunit mRNA that peaked at the minimum of the S phase as reported previously. These results indicate that in actively cycling cells the genes of the two larger subunits of the mouse DNA polymerase α-prime complex are expressed constitutively throughout the cell cycle.

**DISCUSSION**

We have isolated cDNA clones coding for the four subunits of the mouse DNA polymerase α-prime complex. The identification of these cDNAs as the genes encoding the 180-, 68-, 54-, and 46-kDa subunits is clearly supported by the presence of the same sequences in the deduced amino acid sequences as in the partial peptide sequences determined with a peptide sequencer. The transcripts for the four subunits of the DNA polymerase α-prime complex were detected as a single copy in the mouse genome.

**Gene Expression during the Cell Cycle**—The mRNA levels of most factors involved in DNA replication increase after serum stimulation of quiescent mammalian cells, but their levels do not fluctuate during the cell cycle in continuously growing cells. For a study of the cell cycle fluctuation of the expression of the genes for the four subunits gene in growing cells, mouse FM3A cells in the logarithmic growth phase were separated into eight fractions by centrifugal elutriation. The cell cycle stage of each fraction was determined by measuring the DNA content of propidium iodide-stained cells. As shown in Fig. 10a, most cells in fraction 1 were in the G1 phase, but cells in fractions 2-6 contained cells in progressive stages of the S phase, and fractions 7 and 8 contained cells in the G2 phase of the cell cycle. Total RNA from each fraction was isolated, and 2 μg of poly(A)+ RNA was used to detect each mRNA. The results of Northern blot analyses of transcripts for the four subunits of the DNA polymerase α-prime complex, PCNA, and EF-1α from elutriator-fractionated mouse FM3A cells are illustrated in Fig. 10B. EF-1α messages were constitutively present in all fractions throughout the cell cycle. Densitometric analyses of these results are shown in Fig. 10C. The EF-1α messages were used to normalize the intensities of the other messages. The transcripts for the four subunits of the DNA polymerase α-prime complex were found in all cell fractions. The levels of mRNAs for the 180- and 68-kDa subunits showed little change (1.5-2-fold) throughout the cell cycle, whereas the gene expression of the two larger subunits of the mouse DNA polymerase α-prime complex associated with DNA polymerase activity was constitutively expressed throughout the cell cycle, with a slight increase during the S phase, whereas the gene expression of the two smaller subunits associated with DNA primase activity shows a slight periodicity with a 2-fold increase in S phase cells.
As suggested by immunological and molecular genetic studies (54), the predicted amino acid sequence of the mouse 180-kDa subunit shares extensive homology with that of the human 180-kDa subunit. The overall lengths of the two proteins are identical, being 1,462 amino acids. The amino acid identities throughout the mouse and human 180-kDa subunit proteins are 88%. The six highly conserved regions identified by Wong et al. (24) in class B DNA polymerases (55) are almost exactly the same in the mouse and human proteins. When conservative amino acid substitutions are included, however, the similarity is 90.4%. This marginal difference between the identity and similarity of the amino acid sequences indicates the existence of significant species specificity in these two proteins. The fact is consistent with a previous observation that only the DNA polymerase α-primase complex from primate cells can efficiently replicate SV40 DNA in a cell-free system (11), probably because of the species specificity of protein-protein interactions.

In the case of D. melanogaster, a cryptic 3′→5′ exonuclease that preferentially excises 3′-terminal mismatched nucleotides is reported to be associated with the 182-kDa catalytic polypeptide of the DNA polymerase α-primase complex. Its exonuclease activity appears only upon dissociation of the second largest subunit, the 73-kDa subunit, from the enzyme complex (56). On the contrary, the association of unique 3′→5′ exonuclease activity with the 180-kDa subunit of the DNA polymerase α-primase complex of S. cerevisiae has been reported (57). Its activity was detected using substrates such as (dT)_n−[α-32P]dCTP or longer polynucleotides but not short polynucleotides such as (dT)_10 or (dT)_30 or standard 3′-terminal mismatched nucleotides as substrate. In this case, the 3′→5′ exonuclease activity associated with the catalytic subunit was not masked by the second largest subunit, the 86-kDa subunit. The physiological role of this unique exonuclease activity is not yet known. Recently, Copeland and Wang (58) overproduced the catalytic 180-kDa subunit of DNA polymerase α from recombinant baculovirus-infected insect cells as a single polypeptide of 180 kDa. This single-polypeptide polymerase α did not have any detectable proofreading 3′→5′ exonuclease activity when assayed with either mismatched or matched base-paired termini. Originally, 3′→5′ exonuclease activity in either the purified four subunits (35), two subunits (59), or the single 180-kDa subunit released from mouse FM3A DNA polymerase α devoid of primase.2 Judging from amino acid sequence homologies, the 3′→5′ exonuclease active site of E. coli DNA polymerase I has been proposed to be conserved in both prokaryotic and eukaryotic replicative DNA polymerases (52). One of the three conserved regions proposed, region III, could be realigned with that of S. cerevisiae polymerase I (3). The primary sequences of the mouse and human polymerase α 180-kDa subunits are highly conserved, and their entire protein sequences including these proposed 3′→5′ exonuclease regions can be aligned (Fig. 3A). The reason for the lack of proofreading 3′→5′ exonuclease activity, whether cryptic or not, in the human and mouse catalytic subunit of DNA polymerase α is unknown. However, it might be worthwhile to point out that in all DNA polymerases with demonstrable 3′→5′ exonuclease activity, the ExoI domain has two acidic amino acids (probably corresponding to Ala614 and Glu616 in the mouse sequence), both of which are essential for catalytic activity. This has been shown for E.

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coli polymerase I (Klenow fragment) (60) and for φ29 polymerase (52).

The function of the second largest subunit of the DNA polymerase α-primase complex has been suggested to be the masking of the 3'-5' exonuclease activity associated with 182-kDa catalytic subunit in Drosophila as described above (56). Recently, Brooke and Dumas (61) demonstrated that the 86-kDa subunit of S. cerevisiae increased the rate and extent of complex formation of a recombinant 180-kDa subunit with an immunoaffinity-purified p49-p58 DNA primase complex. In the present study, we cloned and sequenced the mouse 68-kDa subunit, hoping that we could identify some known functional domains. Unfortunately, we could not find any special amino acid sequence suggestive of a functional role of this subunit. Moreover, computer-assisted analysis did not show any significant homology of the mouse 68-kDa subunit with other known proteins. Very recently, Cotterill et al. (30) reported cloning the gene for the 73-kDa subunit of the DNA polymerase α-primase complex in D. melanogaster. At the protein level the mouse 68-kDa subunit shows significant similarity overall to the analogous subunit of D. melanogaster. However, the amino acid identity of the second largest subunit between these two species is much lower than that of the largest subunit of the DNA polymerase α-primase complex.

When sequence data for the second largest subunit of other species are available, it will be possible to obtain information about the functional domains of this subunit. We did, however, find 10 potential phosphorylation sites for p34^{{\text{cdk}}}

This finding is consistent with previous findings that the human 70-kDa subunit is phosphorylated only in the G2/M phase and that the peptides phosphorylated in vitro by p34^{{\text{cdk}}}

kinase are identical to those phosphorylated in vivo as judged by tryptic phosphopeptide mapping (62). So far, no functional role has been assigned to the second largest subunit of the mammalian DNA polymerase α-primase complex. It is tempting to speculate that phosphorylation of this subunit in the G2/M phase plays a key role in dissociation of the DNA polymerase α-primase complex from DNA and/or other replication enzymes and protein factors.

The third and the fourth largest subunits of the DNA polymerase α-primase complex have been shown to be responsible for primase activity (for review, see Ref. 63). By affinity labeling, the ATP or GTP binding site(s) of the primases from calf thymus and budding yeast were found to be localized exclusively in the fourth subunit (64, 65). Moreover, sequence comparison of the small subunit of mouse primase with that of yeast indicates high conservation of amino acid sequences in the amino-terminal halves of the polypeptides and a potential metal binding domain (zinc finger) in this region (29). On the contrary, no functional role has been assigned to the large subunit of yeast primase (27) or mouse primase (this paper). Overproduction of these subunits in E. coli or insect cells, purification of the proteins, and their reconstitution in vitro will reveal their functional roles for primase activity.

Expression of the gene for the human DNA polymerase α 180-kDa subunit is positively induced during activation of quiescent cells to proliferate, is elevated in transformed cells, and is down-regulated in terminally differentiated cells (66). In actively cycling cells, however, its expression level is almost constitutive throughout the cell cycle. Similar results have been obtained for expression of the gene for the small p49 subunit of mouse DNA primase (67). In contrast, the expression of the yeast genes for DNA polymerase I (α) and two subunits of DNA primase fluctuates during the cell cycle, the levels of their mRNAs reaching maxima at the G1/S boundary (27, 68, 69). The specific nucleotide sequence named the MluI motif or MCB (MluI cell-cycle box), commonly present in upstream regulatory regions of many yeast DNA replication-related genes, is proposed to be responsible for cell cycle-dependent expression (70–72). In case of the gene for the 180-kDa subunit of human DNA polymerase α, Pearson et al. (73) reported that a 248-base pair sequence upstream from the cap site modulates gene expression (73). This 248-nucleotide region contains sequences similar to consensus sequences for Sp1-, Ap1-, Ap2-, and E2F- binding sites. These multiple sequence elements within the 248 base pairs might be required for full induction by serum of expression of the 180-kDa catalytic subunit of DNA polymerase α.

In the present work, we examined the steady-state expression of transcripts for all four subunits of the DNA polymerase α-primase complex in mouse cells. Consistent with previous results on the human 180-kDa (66) and mouse 49-kDa (67) subunits, the steady-state levels of transcripts for all four subunits were positively and concordantly induced before the peak of DNA synthesis during induction of proliferation of quiescent cells, whereas they were constitutively expressed at all stages of the cell cycle in proliferating cells. We found, however, a slight difference between the expression of steady-state transcripts of the two larger subunits and two smaller subunits: namely the expressions of steady-state transcripts for the two smaller subunits showed slightly more periodicity. The steady-state transcript level is influenced by the rate of transcription and that of degradation of the message. We found two, zero, one, and two ATTTA sequences, which are potentially involved in selective degradation of mRNA (48),
in the 3′-untranslated regions of the cDNAs for the 180-, 68-, 54-, and 46-kDa subunits, respectively. This finding could explain why the level of the transcript for the 68-kDa subunit showed less fluctuation than those of transcripts for the two smaller subunits. However, it cannot explain why the 180-kDa transcript showed higher stability than the transcripts of the two smaller subunits. Studies of the mechanisms of transcriptional regulation of these genes are required to elucidate this problem.

Previously, we isolated a temperature-sensitive mutant cell line of mouse FMSA cells, tsFT20, and found that it has a

$k$-subunit transcript showed higher stability than the transcripts of the 3′-untranslated regions of the cDNAs for the 180-, 68-, 54-, and 46-kDa subunits, respectively. This finding could explain why the level of the transcript for the 68-kDa subunit showed less fluctuation than those of transcripts for the two smaller subunits. However, it cannot explain why the 180-kDa transcript showed higher stability than the transcripts of the two smaller subunits. Studies of the mechanisms of transcriptional regulation of these genes are required to elucidate this problem.

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