Mouse RNA Polymerase I 16-kDa Subunit Able to Associate with 40-kDa Subunit Is a Homolog of Yeast AC19 Subunit of RNA Polymerases I and III

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We have previously isolated a mouse RPA40 (mRPA40) cDNA encoding the 40-kDa subunit of mouse RNA polymerase I and demonstrated that mRPA40 is a mouse homolog of the yeast subunit AC40, which is a subunit of RNA polymerases I and III, having a limited homology to bacterial RNA polymerase subunit α (Song, C. Z., Hanaida, K., Yano, K., Maeda, Y., Yamamoto, K., and Muramatsu, M. (1994) J. Biol. Chem. 269, 26976–26981). In an extension of the study we have now cloned mouse RPA16 (mRPA16) cDNA encoding the 16-kDa subunit of mouse RNA polymerase I by a yeast two-hybrid system using mRPA40 as a bait. The deduced amino acid sequence shows 45% identity to the yeast subunit AC19 of RNA polymerases I and III, known to associate with AC40, and a local similarity to bacterial α subunit. We have shown that mRPA40 mutants failed to interact with mRPA16 and that neither mRPA16 nor mRPA40 can interact by itself in the yeast two-hybrid system. These results suggest that higher eukaryotic RNA polymerase I conserves two distinct α-related subunits that function to associate with each other in an early stage of RNA polymerase I assembly.

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In eukaryotes RNA polymerases I, II, and III (pol I, pol II and pol III) transcribe specifically nuclear encoded genes. Each of pol I, pol II, and pol III forms a complex, multisubunit structure (1), whereas prokaryotic RNA polymerase forms a relatively simple structure consisting of only three core subunits (β′, β, and α) and an initiation factor (σ) conferring promoter specificity. The structure of Saccharomyces cerevisiae RNA polymerases has been studied extensively among eukaryotic RNA polymerases (2, 3). For example, S. cerevisiae pol I is composed of 14 distinct polypeptides. Five of them (ABC27, ABC23, ABC14.5, ABC10α, and ABC10β) are shared by pol I, pol II, and pol III; two of them (AC40 and AC19) are shared by pol I and pol III; and the remaining seven subunits (A190, A135, A49, A43, A34.5, A14, and A12.2) are unique to pol I. The largest (A190) and the second largest (A135) are homologous to the β′ and β subunits of Escherichia coli RNA polymerase, respectively. AC40 and AC19 have a limited homology to E. coli α subunit, indicating that they are functional homologs to α (3). Thus analogous to E. coli core RNA polymerase αββ′, these four subunits A190, A135, AC40, and AC19 may form a core in pol I. Indeed, AC40 and AC19 were shown to associate in vivo by a yeast two-hybrid system (4). In contrast to the well-characterized yeast pol I, pol I from mammalian cells has not been sufficiently purified and analyzed biochemically. To study the structure and subunit composition of mammalian pol I, we have purified mouse pol I to apparent homogeneity and have shown that mouse pol I is composed of at least 11 subunits (180, 114, 44, 40, 27, 20, 18, 16, 14, and 12 kDa) and three associated proteins (PAF53, 51, and 49) and appears to have an organization similar to S. cerevisiae pol I (5, 6). Indeed, molecular cloning of the mouse 40-kDa subunit of mouse pol I (hereafter referred to as mRPA40) has revealed that mRPA40 shares a high similarity with yeast AC40 (7) and a limited homology to E. coli α subunit (5). Furthermore, pol I-associated factor PAF53 has been shown to have local similarities to yeast A49 subunit (6). However, the correspondence for the remaining subunits between yeast and mouse is not yet clear. Recently, conservation of five common subunits between yeast and mammalian pol II has been shown with identification of cDNAs encoding human homologs of yeast ABC27, ABC23, ABC14.5, ABC10α, and ABC10β (8–10). Remarkably, all but one, ABC27, can complement the corresponding null mutations in yeast (9, 10). Considering the high conservation of components of RNA polymerases through evolution, we could expect that a smaller subunit related to yeast AC19 (and E. coli α) exists in mouse pol I and that the assumed homolog, if any, is able to interact with mRPA40. In this paper, using a yeast two-hybrid system, we have cloned mRPA16 cDNA encoding the 16-kDa subunit able to associate with mRPA40 and found that mRPA16 is indeed a homolog of yeast AC19. We have also shown that mRPA40 mutants failed to interact with mRPA16 and that neither mRPA16 nor mRPA40 could associate by itself. These results suggest that higher eukaryotic pol I conserves two distinct α-related subunits and give supportive evidence that they will form a heterodimer in the assembly pathway of pol I.

EXPERIMENTAL PROCEDURES

Strains, Plasmids, and Media—SD synthetic medium is 2% glucose, 0.67% Bacto yeast nitrogen base (Difco) supplemented with the required bases and amino acids as described by Sherman et al. (11). SD–Leu lacks leucine. SD–Leu–Trp lacks leucine and tryptophan. To select His+ transformants, 25 mM 3-amino-(1,2,4)-triazole (3AT), a
chemical inhibitor of the HIS3 gene product, is supplemented to 
SD–Leu–Trp (SD–Leu–Trp+3AT). For making a solid medium, 2% 
agar was added.

The yeast and E. coli strains and plasmids used in this study 
are listed in Table I. pY4 carrying GAL4DB-mRPA40 fusion 
gene driven by PADC1 was constructed in the following way. A 1.4-kb SauI-SmaI fragment of PADC1-GAL4DB cut out from pAS1 (12) was ligated between the SauI and SmaI sites of YEps51 (15). The resultant plasmid was digested with SmaI and BamHI and ligated with a 0.5-kb SmaI–BamHI fragment containing TAD1 derived from pPC97 (14), resulting in pYN521. The cDNA encoding the entire coding region of mRPA40 (5) modified for subcloning in-frame to GAL4DB was prepared and inserted between the NcoI and SmaI sites of pYN521, creating pY4. 
pY11068 was constructed as follows. A 0.7-kb SfiI-NorI fragment was cut out from pPC67-B22 (Table I) by digestion with SfiI and NorI and inserted between the SfiI and NorI sites of pPC97 to express in-frame GAL4DB-B22 (mRPA16) fusion protein. pS1 was identified in the pPC67 library as a clone expressing the GAL4 transcriptional activation domain (GAL4<sub>AD</sub>)-mRPA40 (amino acid position 4–355) fusion protein able to interact with GAL4<sub>DB</sub>-B22 (mRPA16) fusion protein produced from pYN1068 in the yeast two-hybrid system.2

**Table I**

| Strains and plasmids used in this work |
|--------------------------------------|
| **Strains** | **Description** |
| **Yeast** | **MH1066** |
| | ΔlacI△lacZ, hsr-ripl, pyrF/Tn5, leuB600, trpC9830, galE, galK (31). |
| | ΔlacI△lacZl69, his3–Δ200, ade2–101, galΔ gal80ΔURA3::GAL-LacZ, LYS2::GAL-HIS3 (12). |
| **E. coli** | **DH5α** |
| | supE44, ΔlacI169 (50 lacIΔ152), hsdR36 proxB lacIΔ152 lacZΔM15]. |
| | supE44, ΔlacI169 (50 lacIΔ152), hsdR36 proxB lacIΔ152 lacZΔM15]. |
| **E. coli** | **TG1** |
| | supE44, ΔlacI169 (50 lacIΔ152), hsdR36 proxB lacIΔ152 lacZΔM15]. |

**Plasmids**

| pPC86 | Expresses GAL4<sub>AD</sub>, fusion gene under P<sub>DGC</sub>, TRP1, CEN6, ARSH4, and amp (14). |
| pFC97 | Derivative of pPC62 (14) to express GAL4<sub>AD</sub>, fusion gene under P<sub>DGC</sub>, carrying the same multicloning site with pPC86 |
| pYN901 | Derivative of YEp351 (13) carrying P<sub>DGC</sub>-GAL4DB<sub>T</sub>AD1 derived from pPC97, LEU2, 2μ and amp. |
| pY4 | Carries GAL4<sub>DB</sub> derived from pAS1 (12) fused to BcaBEST DNA polymerase (TaKaRa) on both ends of the coding region of B22. The NorI site was rendered blunt with the Klenow enzyme and inserted at the SmaI site of pGE1 (17) in-frame to the coding region of glutathione S-transferase (GST), resulting in pGEX1-B22. The plasmid pGEX1-B22 was transformed into E. coli TG1, and the synthesis of GST-B22 fusion protein was induced with the addition of 1 μM isopropyl-1-thio-β-D-galactoside. The fusion protein was affinity-purified as described (6) using glutathione-Sepharose 4B beads (Pharmacia) followed by a preparative sodium dodecyl sulfate (SDS)-polyacrylamide gel. The fusion proteins were excised from the gel, and polyclonal antibodies were prepared by MBL Co. (Nagano, Japan).

**Immunoblot experiments** were done as described (6). Pol I purified from mouse ascites cell MH134 as described (5) was subjected to SDS-polyacrylamide gel electrophoresis and transferred to an Immobilon membrane (Millipore). The membrane was treated with anti-B22 antibodies, and horseradish peroxidase-conjugated antibody to rabbit immunoglobulin was used as a second antibody. The bound antibodies were visualized by ECL reagents (Amersham Corp.).

**RESULTS AND DISCUSSION**

**Isolation of Clones Encoding Proteins Able to Interact with mRPA40 Subunit**—To identify mouse cDNAs encoding proteins able to associate with mRPA40, the reporter yeast Y153 carrying pY4 expressing GAL4<sub>DB</sub>-mRPA40 fusion protein was transformed with mouse cDNA library pPC67. In total, about 1.5 x 10<sup>6</sup> transformants were screened on SD–Leu–Trp+3AT plates. 250 His<sup>−</sup> transformants were obtained in the initial HIS<sup>−</sup> screening, and 30 of 250 His<sup>−</sup> clones were found to be LacZ<sup>−</sup> by the filter-lifting assay. We recovered library-derived plasmids from the 30 positive candidates individually, and the recovered plasmids were introduced into Y153 carrying either pY4 (the bait) or pYN901 (the control) to eliminate false positives. 21 clones showed His<sup>−</sup> and LacZ<sup>−</sup> in a GAL4<sub>DB</sub>-mRPA40-dependent manner. Subsequently the 21 clones were assigned into five groups by restriction enzyme mapping, and 5 representative clones were subjected to DNA sequence determination. Of the five clones sequenced, we found that cDNA designated B22 encodes a sequence that is highly homologous to the yeast pol I and pol III subunit, AC19 encoded by RPC19 gene (yRPC19) (18). Fig. 1 shows that Y153 cotransformed with pY4 (GAL4<sub>DB</sub>-mRPA40) and B22 cDNA can activate HIS3 as well as LacZ<sup>−</sup>, resulting in growth on plates containing 3AT (Fig. 1a) and blue color on a filter containing 5-bromo-4-chloro-3-indolyl-β-D-galactoside (Fig. 1b). B22 encodes a protein of 153 amino acids with a calculated relative molecular mass of 15.1 kDa and a calculated isoelectric point of 6.5. Fig. 2 shows the nucleotide and deduced amino acid sequences of the protein encoded by...
B22 cDNA. The coding region starts at nucleotide 128 and ends at 526, and the polyadenylation signal (AATAAA) lies from 679 to 684. The putative first methionine shows a good context in translation initiation; 5 out of 6 nucleotides are in accordance with Kozak’s rule (18). The identity and similarity of the deduced amino acid sequence between the ORF of B22 and yeast AC19 are 44 and 83%, respectively (Fig. 3), suggesting that B22 cDNA encodes a mouse homolog of AC19. Almost the entire region of the ORF of B22 is well conserved between yeast and mouse, whereas the amino-terminal part of yeast AC19 (amino acid position 1–21) and the carboxy-terminal part of the ORF of B22 (amino acid position 121–133) appear to be species-specific. Yeast AC19 is a phosphoprotein; the potential phosphorylation sites are observed at amino acid positions 54–56, 91–97, and 136–138 (19); however, the corresponding region of mRPA16 is not conserved well. Hence it remains to be determined whether mRPA16 is phosphorylated in vivo.

As anticipated, there are no translation stop codons in-frame preceding the ATG at nucleotide 128 in the original B22 clone. Therefore, to confirm the predicted ORF (133 amino acids), several overlapping cDNA clones were isolated independently by the screening of mouse MH134 ascites cell cDNA libraries with B22 as a probe. We found the longest cDNA contained the same ORF but extended 17 nucleotides (1–17 in Fig. 2) at the 5’ end of B22. The results indicate that the cloned B22 contains an entire ORF, and ATG at nucleotide 128 is an authentic translation-initiating ATG. The region between GAL4TA and the first ATG at nucleotide 128 did not appear to be involved in the interaction with mRPA40 since a derivative of B22 consisting of the ORF of 133 amino acids fused directly to GAL4TA on pCP86 showed the interaction with mRPA40 in the yeast two-hybrid system. Northern blot analysis was performed using B22 as a probe and detected an endogenous B22 mRNA in mouse ascites MH134 cells as a single transcript of 0.8–0.9-kb size, which is sufficient for encoding B22.

Mouse Pol I 16-kDa Subunit Is a Homolog of Yeast AC19—Mouse pol I consists of at least 11 subunits, ranging from 180 to 10 kDa (5). To examine which subunit of mouse pol I is encoded by B22 cDNA, we prepared specific polyclonal antibodies against the polypeptide encoded by B22. Purified mouse pol I was subjected to SDS-polyacrylamide gel electrophoresis followed by Western blot analysis using anti-B22 antibodies. We found that anti-B22 antibodies detect a subunit with an apparent molecular mass of 16 kDa (Fig. 4), which is very close to the calculated molecular mass of B22 (15.1 kDa). We have designated the protein encoded by B22 cDNA mouse RPA16 (mRPA16) according to the apparent molecular mass of the subunit on SDS-polyacrylamide gel. The results show that the 16-kDa subunit, mRPA16, in mouse pol I is a homolog of yeast AC19 shared by yeast pol I and pol III. The demonstration that mammalian pol I contains a homolog to yeast AC19 and the homolog, mRPA16, can interact with mRPA40, which is a homolog to the AC40 subunit, raises the question of whether both mRPA40 and mRPA16 are also contained in mammalian pol III since AC19 and AC40 are shared by yeast pol I and pol III (7, 19). Western blot analysis using the antibodies against mRPA40 shows that human pol III contains a subunit corresponding to mRPA40, suggesting that mRPA40 is shared by mouse pol I and pol III. It remains to be determined whether the mRPA16 subunit is present in mammalian pol III.

Two Distinct α-Related Subunits in Both Pol I and Pol II—In addition to mRPA40 identified previously, our identification of the mRPA16 underscores the existence of two α-related subunits in eukaryotic nuclear RNA polymerases. Two distinct α-related subunits have also been identified in both yeast and human pol II: B44.5 and B12.5 in yeast (20, 21) and hRPB33 and hRPB14 in human (22, 23). Furthermore, we have recently identified mouse RPB14, a homolog to yeast B12.5,4 and mouse RPB31, a homolog to yeast B44.5, in mouse pol II.2 Taken together, it is now clear that two distinct α-related subunits are

3 Y. Yao, K. Yamamoto, Y. Nogi, and M. Muramatsu, unpublished results.
4 Y. Nishi, K. Yamamoto, and M. Muramatsu, unpublished results.

Fig. 1. Demonstration of the interaction between mRPA40 and a positive clone B22 by His3 assay (panel A) and LacZ assay (panel B). The reporter strain Y153 was transformed with a pair of plasmids: a, pPC86 (GAL4p) and pY4 (GAL4mpRPA40); b, pYN901 (GAL4mp) and pPC67-B22 (GAL4p-B22); and c, pY4 and pPC67-B22.

Fig. 2. Nucleotide and deduced amino acid sequences of B22 clone (mRPA16). The coding sequence starts at nucleotide 128 and ends at nucleotide 526.
The especially marked in the region covering 19-amino acid named subunits including mRPA16 confirms that the similarity is perfectly conserved among the small α-related subunits (mRPA16, hRBP14, AC19, and B12.5), suggesting that mRPA16, AC19, B12.5, and hRBP14 are highly related to one another (21, 23). The other α-related subunits (mRPA40, hRBP33, AC40, B44.5, and SpRPB3) have also been suggested to be related to one another (5, 26).

Neither mRPA16 nor mRPA40 Can Associate by Itself in the Yeast Two-hybrid System—In E. coli it has been established that the assembly pathway of core polymerase is: α-β-αβ-ββ* (27). Hence, dimerization of α is an important step for the initiation of enzyme assembly in E. coli. In yeast, earlier work suggested that α-related subunits like B44.5 or AC40 play roles in the assembly pathway of cognate enzymes. For example, using temperature-sensitive mutants of B220 (β* homolog), B150 (β homolog), and B44.5 (α-related subunits), Kolodziej and Young (28) observed similar steps for the assembly of core polymerase, i.e., B220, AC19, and B12.5 or AC40, B44.5, and B150. However, the exact functions of these subunits remain unclear.

Consistent with previous observations (for review, see Ref. 2), amino acid sequence comparison among these α-related subunits including mRPA16 confirms that the similarity is especially marked in the region covering 19-amino acid named the α-motif (4, 19) located from amino acid 48 to 66 of mRPA16 (Fig. 5). Close inspection of the α-motif reveals that the haptapeptide EDHTLGN located from amino acid 47 to 53 is perfectly conserved among the small α-related subunits (mRPA16, hRBP14, AC19, and B12.5), suggesting that mRPA16, AC19, B12.5, and hRBP14 are highly related to one another (21, 23). The other α-related subunits (mRPA40, hRBP33, AC40, B44.5, and SpRPB3) have also been suggested to be related to one another (5, 26).
higher eukaryotes are also involved in the early step of enzyme assembly by forming either heterodimers or homodimers. Therefore, we examined whether mRPA16 or mRPA40 can self-associate in the yeast two-hybrid system. All pairwise combinations of plasmids expressing GAL4DB-mRPA40, GAL4TA-
mRPA40, GAL4DB-mRPA16, or GAL4TA-mRPA16 were co-
transformed into reporter yeast Y153 individually and the growth of the transformants were examined together with a positive control (wild-type) on SD-Leu-Trp plates at 25 or 34 °C. Wild-type, pY4 and pPC67-B22, A82D, pY4-A82D and pPC67-B22, A82R, pY4-A82R and pPC67-B22. Western blotting analysis with anti-mRPA40 antibody (Santa Cruz Biotechnology) revealed that expression of the wild-type and mutant mRPA40 was almost equal in these yeast cells (see Footnote 3).

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