The Fission Yeast Protein, Ker1p, is an Ortholog of RNA Polymerase I Subunit A14 in Saccharomyces cerevisiae, and is Required for Stable Association of Rrn3p and RPA21 in Pol I*

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Running Title: Ker1p heterodimerizes with RPA21
A heterodimer formed by the A14 and A43 subunits (A14/A43) of RNA polymerase I\(^1\) (pol I) in *Saccharomyces cerevisiae* is proposed to correspond to the Rpb4/Rpb7 and C17/C25 heterodimers in RNA polymerases II and III, respectively, and to play a role(s) in the recruitment of pol I to the promoter. However, the question of whether the A14/A43 heterodimer is conserved in eukaryotes other than *S. cerevisiae* remains unanswered, although both Rpb4/Rpb7 and C17/C25 are conserved from yeasts to human. To address this question, we have isolated a *Schizosaccharomyces pombe* gene named *ker1\(^+\)*, using a yeast two-hybrid system including *rpa21\(^+\)*, which encodes an ortholog of A43 as bait. Although no homolog of A14 has previously been found in the *S. pombe* genome, functional characterization of Ker1p and an alignment between Ker1p and A14 show that Ker1p is an ortholog of A14. Disruption of *ker1\(^+\)* results in temperature-sensitive (ts) growth and the ts-deficit of *ker1\(^-\)* is suppressed by either overexpression of *rpa21\(^+\)* or *rrn3\(^+\)*, which encodes the rDNA transcription factor Rrn3p, suggesting that Ker1p is involved in stabilizing the association of RPA21 and Rrn3p in pol I. We also found that Ker1p dissociates from pol I in post-log-phase cells, suggesting that
Ker1p is involved in growth-dependent regulation of rDNA transcription.

**INTRODUCTION**

There are three distinct types of eukaryotic nuclear RNA polymerases (pols): RNA polymerase I (pol I), RNA polymerase II (pol II), and RNA polymerase III (pol III). Among eukaryotic organisms, the structure and function of pols in *Saccharomyces cerevisiae* have been studied fairly extensively (1–4). *S. cerevisiae* pol I consists of 14 subunits. The core structure contains 10 subunits (A190, A135, AC40, AC19, Rpb5, Rpb6, Rpb8, Rpb10, Rpb12 and A12.2), and is believed to be sufficient for non-specific transcription, but not for accurate initiation of transcription (5). In fact, pol I requires 4 specific subunits, designated as A49, A43, A34.5 and A14, for specific transcription of rDNA. A43 is also essential for cell growth (6), whereas A49 (7), A34.5 (8) and A14 (9) are dispensable.

Much attention has recently been focused on the A14 and A43 subunits, in view of the structural and functional conservation of these two subunits in eukaryotes. A43 is conserved in a variety of eukaryotes (10), and shows amino acid sequence similarity to Rpb7 (a specific
subunit of pol II, C25 (a specific subunit of pol III), and RpoE (a subunit of archaeal pols) across multiple pols (11). Furthermore, A43 forms a heterodimer with A14 (A14/A43) that is similar to the Rpb4/Rpb7 (11, 12), C17/C25 (13) and RpoF/RpoE (14) heterodimers in pol II, pol III and archaeal pols, respectively. It should be noted that Rpb4, C17 and RpoF have mutual sequence similarity and are grouped into a gene family, but no obvious homolog of A14 is found in available databases. A14 and Rpb4 are required for the stable assembly of A43 and Rpb7, respectively, in their respective pols, suggesting a functional similarity of A14 with Rpb4 (5, 11, 15, 16). The position of A14/A43 in the three-dimensional structure of pol I is deduced to be similar to that of Rpb4/Rpb7, forming an upstream interface with CTD to interact with TFIIB for pol II recruitment to the pol II promoter (4), and, furthermore, playing a role in the processing of the nascent RNA transcript (17). Consistent with the proposed position in pol I, A14/A43 also interacts with a rDNA specific transcription factor, Rrn3p, for pol I recruitment to the rDNA promoter (10), and is able to bind to single-stranded RNA (18). Interestingly, C17/C25 in pol III is also reported to interact with TFIIB, a factor that recruits pol III to the pol III promoter (19).
The mechanism of the down regulation of rDNA transcription (20–24) is now believed to be as follows: only a small fraction of pol I associated with Rrn3p is able to recognize the components of the pre-initiation complex, resulting in pol I recruitment to the rDNA promoter (25–28); A43 in pol I is responsible for associating with Rrn3p (10), and the association of A43 with Rrn3p is inhibited in post-log-phase cells (including nutrient-starved or growth-arrested cells) (24, 26), resulting in a drastic decrease of pol I recruitment to the promoter (29).

Thus, the molecular function of A43 and Rrn3p deserves further study to resolve long-standing questions regarding growth-dependent transcription of rDNA (30).

It is firmly established that all 12 and all 17 subunits of *S. cerevisiae* pol II and pol III, respectively, are conserved in human pol II and pol III (31, 32). However, it is not clear whether all the 14 subunits identified in *S. cerevisiae* pol I are conserved in other eukaryotes (33, 34). To gain further insight into the structure and function of pol I, we have been studying pol I of *Schizosaccharomyces pombe*, which is only distantly related to *S. cerevisiae*, but is amenable to genetic analysis (35). To date, it is known that *S. pombe* pol I consists of at least 12 subunits: the two largest, RPA190 and RPA140, are homologous to A190 and A135,
respectively (36, 37); the two smaller subunits, RPA42/RPA17, correspond to AC40/AC19, respectively (38, 39); five common subunits (Rpb5, Rpb6, Rpb8, Rpb10, and Rpb12) are shared by pol II and pol III (12); and SpRPA12 is a functional homolog of A12.2 (40). Thus, the 10-subunit core structure of pol I has been well conserved between the two yeasts through evolution. Moreover, the two specific subunits in S. pombe, RPA21 and RPA51, have been identified to be related to A43 and as a functional homolog of A49, respectively, suggesting that the pol I architecture in S. pombe is likely to be analogous to that of S. cerevisiae (41, 42).

In this paper, we demonstrate that a newly isolated protein, Ker1p, is an ortholog of A14, and that the Ker1p/RPA21 heterodimer in S. pombe is the functional counterpart of A14/A43 in S. cerevisiae. We also show novel aspects of Ker1p that have not been previously observed in A14, and suggest that Ker1p is involved in growth-dependent transcription of rDNA.

**EXPERIMENTAL PROCEDURES**

**Media, Strains and Genetic Techniques:** Yeast plasmids and strains are listed in Table 1. Minimal medium (MM) with or without thiamine and supplemented with appropriate amino
acids, and YE medium were prepared to grow *S. pombe* cells, as previously described (35).

YEPD and synthetic dextrose (SD) medium were prepared as previously described (43). SD-Trp-Leu medium lacks tryptophan and leucine, and SD+3-AT medium contains 25mM 3-aminotriazole (3-AT). MM +AbA medium contains 0.1-0.4μg/ml of Aureobasidin A (AbA) in MM. Disruption of chromosomal ker1+ was carried out as follows: diploid cells (a cross between JY742 and JY745 cells) were transformed with the 4.5-kb XhoI-Saci linear fragment having ker1Δ::ura4+ from pYI186. To replace Ker1p with Ker1p-(HA)3, a 5.0-kb XhoI-Saci fragment from pYI176 (see below) was transformed into IZ2, resulting in YI28.

*Plasmids:* For two-hybrid screening, GAL4DB–rpa21+ (pYI77) was constructed as follows: 0.52-kb rpa21+ cDNA was amplified by PCR using pKI45 containing a full-length 0.52-kb cDNA of rpa21+ (41) as a template, and cloned between the Smal and XhoI sites of pAS2-1 (44) to fuse GAL4DB to RPA21 in-frame. To construct deletion derivatives of rpa21+, pKI45 was also used as template DNA for PCR amplification. pYI106 expresses RPA21 (with the N-terminal 56 amino acids truncated) fused to GAL4DB, while pYI105 expresses RPA21 (with the C-terminal 60 amino acids truncated) fused to GAL4DB. To replace chromosomal
ker1\textsuperscript{+} with ker1\textsuperscript{+}-(HA)\textsubscript{3}, a PCR-amplified 1,317-bp XhoI-Smal fragment of the 5\textquotesingle-untranslated region and ORF of ker1\textsuperscript{+} and a 1,017-bp NotI-SacI fragment of the 3\textquotesingle-flanking region of ker1\textsuperscript{+} were cloned successively between the XhoI and Smal sites and the NotI and SacI sites of pYN1237, generating pYI176. To express Ker1p under the control of a CMV promoter in S. pombe, a full-length (441 bp) of ker1\textsuperscript{+} was amplified from JY742 DNA by PCR and cloned between the XhoI and BamHI sites of pAUR222 (TaKaRa), resulting in pYI195. To study the cellular localization of Ker1p, a full-length ker1\textsuperscript{+} was amplified from JY742 DNA and the 441-bp fragment was cloned between the NotI and BamHI sites of pKS406 to express a GFP-Ker1p fusion protein, resulting in pYI193. A GFP-fibrillarin fusion construct was made using a 912-bp fragment of the fib gene encoding fibrillarin, which was amplified by PCR from JY742 DNA and cloned between the NotI and BamHI sites of pKS406, resulting in pYI200. To construct a disrupted allele ker1\textDelta::ura4\textsuperscript{+}, we amplified the 880-bp 5\textquotesingle-untranslated sequence of ker1\textsuperscript{+} flanked by the XhoI and BamHI sites and the 1.0-kb 3\textquotesingle-untranslated sequence of ker1\textsuperscript{+} flanked by the NotI and SacI sites, respectively, from the JY742 genome. Each PCR product was cloned successively between the XhoI and BamHI sites and between
the NotI and SacI sites of KS (+), resulting in pYI185. Then, the 2.5-kb BamHI-BamHI DNA fragment of *ura4* obtained from pYN1235 was cloned into the BamHI site of the resulting plasmid, generating pYI186. To construct pKI27, a full-length *rrn3*+ was amplified by PCR from JY742 DNA and cloned between the SacI and SmaI sites of pREP81. To express *ker1*+ under the control of the nmt1 promoter, a full-length *ker1*+ was amplified by PCR from JY742 DNA and cloned between the SacI and BamHI sites of pYI40, generating pYI210. *Pfu* DNA polymerase was used for PCR, and DNA sequencing analysis was used to confirm the PCR product.

**Two-Hybrid Screening:** pYI77 expressing a bait GAL4DB-RPA21 was transformed into the reporter strain Y190. Y190 carrying pYI77 was transformed with a *S. pombe* cDNA library fused to GAL4AD in pGAD GH (Clonetech). The 3-AT resistant and His*+* transformants were screened on SD-Trp-Leu plates containing 25mM 3-AT. *LacZ* activation was examined by a filter-lifting assay (38).

**Fluorescent Microscopy of GFP-fusion:** To visualize the nuclear chromatin region, cells were stained with 4',6-diamidino-2-phenylindole (DAPI) at 1 mg/ml. Fluorescent images were
obtained with a Fujix HC-2500 CCD camera using a Zeiss Axioskop fluorescent microscope.

**Immunoprecipitation:** *S. pombe* cells were grown in YE medium and harvested in mid-log phase. Preparation of cell extracts and immunoprecipitation with 12CA5 (monoclonal antibody against the HA epitope) and anti-RPA190 antibodies were carried out as described in Mitsuzawa *et al.* (45). 12CA5 was purchased from Roche Molecular Biochemicals. Immunoblot was performed essentially as previously described (39), using polyclonal antibodies against RPA190, RPA140, RPA21 and Rpb1 (pol II) (41, 46).

**Biochemical Fractionation of Pol I:** Pol I was partially purified as previously described (46). Whole cell extract from the YI28 strain was loaded onto a N^{2+}-nitrilotriacetic acid agarose column. The proteins eluted with 200mM imidazole were loaded onto a DEAE-Sephadex A25 column and eluted by a 50-620mM gradient of ammonium sulfate. Fractions were examined by SDS-PAGE followed by Western blotting, using antibodies against RPA190, RPA21 and HA [Ker1p was tagged by (HA)_3 in the YI28 strain].

**Phosphatase Treatment:** Whole cell extract was prepared form strain YI28, and 1.6 mg of protein was immunoprecipitated by antibodies against RPA190 (10μl of antiserum), as
described above. The precipitates were washed three times with A buffer [20mM HEPES-KOH (pH7.6), 150mM potassium acetate, 20% glycerol, 0.1% Nonidet P-40, and 1mM dithiothreitol] and once with HM buffer [50mM HEPES-KOH (pH7.6) and 1mM MgCl₂]. The pellet was resuspended in 1 ml of HM buffer and divided into 4 aliquots, centrifuged again, resuspended with 100μl of HM buffer and incubated for 10 min at 30°C. Thirty units (1.5μl) of calf intestine alkaline phosphatase (Roche Molecular Biochemicals) were added to one tube and incubated for 20 min at 30°C. The reaction was stopped by addition of SDS sample buffer and heating at 95°C for 5 min. In controls, sodium pyrophosphate (final concentration of 5.4mM) was added with or without alkaline phosphatase, and the sample was then treated as above. No treatment was performed for the fourth sample. All the samples were subjected to 8% SDS-PAGE, followed by immunoblot analysis with the antibody against HA.

RESULTS

Identification of a Novel Protein, Designated Ker1p, that Interacts with the RPA21
Subunit: To identify protein(s) interacting with RPA21, we generated a GAL4DB-RPA21 fusion construct in pAS2-1 (pYI77) and introduced it into a two-hybrid reporter *S. cerevisiae* strain Y190. Subsequently, we introduced a *S. pombe* cDNA library fused to GAL4AD into the Y190 strain carrying pYI77. We selected about $10^7$ Leu$^+$ transformants and screened colonies showing 3-AT resistance and a *LacZ* positive phenotype. In total, 27 transformants showing 3-AT resistance and the *LacZ* positive phenotype were obtained and the responsible plasmids carrying cDNA fused to GAL4AD were retrieved (data not shown). Nucleotide sequencing of the retrieved plasmids indicated that all the cDNAs encoding the protein shown to interact with RPA21 were derived from the same gene; one group lacked the C-terminal 30 amino acids and another retained the full-length gene, indicating that the C-terminal 30 amino acids are not required for interaction with RPA21 in the yeast two-hybrid method. The gene isolated by the two-hybrid system encodes a protein of 147 amino acids with a calculated molecular weight of 16,976 and a calculated pI of 6.25. The predicted protein is very hydrophilic and contains many charged amino acids: 21 lysine residues, 9 arginine residues, 24 glutamic acid residues and 7 aspartic acid residues (see Fig. 7). Therefore, we have named
this protein Ker1p [lysine (K) and glutamic acid (E) rich protein 1] and the gene encoding it 

ker1". No proteins homologous to Ker1p were observed in an initial database search.

**Apparent Molecular Mass of Ker1p-(HA)₃**: To determine the apparent molecular mass of 

Ker1p, a YI28 strain expressing Ker1p tagged with (HA)₃ [Ker1p-(HA)₃] was constructed. 

Whole cell extracts prepared from YI28 and the parental strain IZ2, in which Ker1p had not 

been tagged, were subjected to SDS-PAGE followed by immunoblotting with a monoclonal 

antibody against HA (12CA5). Fig. 1A shows that Ker1p-(HA)₃ is detected as a doublet of 

bands of 30 kDa and 32 kDa, which include a triple HA sequence (4.3 kDa). Since the 

calculated molecular mass of Ker1p is about 17 kDa, it appears that Ker1p-(HA)₃ migrates 

abnormally in SDS-PAGE, for unknown reasons.

**Ker1p is Phosphorylated**: The predicted amino acid sequence of Ker1p suggested that it 

contains many consensus phosphorylation sites for PKA (S14), PKC (S14, S22 and S94), CKI 

(S45), CKII (S41 and T89) and GSKI (S41, S45, S94 and T89) (see Fig. 7). We considered 

the possibility that Ker1p is phosphorylated, and that both phosphorylated and non- 

phosphorylated forms were detected as doublet bands by immunoblotting in Fig. 1A.
Therefore, Ker1p-(HA)₃ was first immunoprecipitated with the HA antibody, and the immunoprecipitants were then treated with alkaline phosphatase in the absence or presence of a phosphatase inhibitor. As shown in Fig. 1B, phosphatase treatment results in appearance of only the faster-moving 30 kDa band (lane 2). No treatment (lane 1), treatment with phosphatase and an inhibitor (lane 3), or treatment with the inhibitor only (lane 4) generates two bands, 30 kDa and 32 kDa, similarly to those observed in lane 2 of Fig. 1A. Therefore, we conclude that the 30 kDa band represents non-phosphorylated Ker1p-(HA)₃ and the 32 kDa band represents phosphorylated Ker1p-(HA)₃.

*Ker1p is Predominantly Localized in the Nucleolus:* Because Ker1p interacts with RPA21 of pol I, which localizes specifically in the nucleolus, we examined whether Ker1p also localizes in the nucleolus, using a GFP-Ker1p fusion protein. Figs. 2A, 2B and 2C show that GFP-Ker1p forms a dense, crescent-shaped structure that occupies one side of the nucleus, and that the crescent-shaped region is not stained well by DAPI. The observed crescent-shaped structure with much-reduced DNA staining is the most obvious characteristic of the yeast nucleolus (37, 47, 48). However, GFP-Ker1p is also observed in the DAPI-stained region (Fig.
2B), and it might be possible that GFP-Ker1p also localizes outside the nucleolus, due to overproduction under the control of the strong \textit{nmt1} promoter. For controls, we examined localization of GFP itself and observed a clear cytoplasmic distribution (Figs. 2D, 2E and 2F).

We also examined the localization of the nucleolar protein fibrillarin (GFP-fibrillarin) expressed in the same GFP-fusion vector, and found that it localizes specifically in the crescent-shaped nucleolus (Figs. 2G, 2H and 2I). As expected, the crescent-shaped region in the nucleus shows much lower DAPI staining in Fig. 2G. Taken together, we conclude from these results that Ker1p is predominantly localized in the nucleolus, although it is also possible that a certain fraction of Ker1p localizes in the nucleoplasm.

\textit{Co-immunoprecipitation of Ker1p and pol I:} The apparent nucleolar localization of Ker1p prompted us to study the physical interaction of RPA21 (pol I) and Ker1p \textit{in vivo}. Therefore, extracts prepared from cells expressing Ker1p-(HA)\textsubscript{3} were immunoprecipitated with HA antibody beads (12CA5 beads), and co-precipitated proteins were detected by immunoblotting. Fig. 3A shows that pol I subunits RPA190, RPA140 and RPA21 were co-immunoprecipitated with Ker1p, suggesting that Ker1p associates with pol I \textit{in vivo}. The association is specific for
pol I, because pol II subunit Rpb1 was not co-immunoprecipitated (Fig. 3A, lowest panel).

Conversely, Ker1p was co-immunoprecipitated with RPA140 and RPA21 when anti-RPA190 antibodies were used for immunoprecipitation (Fig. 3B). These results confirm that Ker1p associates with pol I in vivo. No subunit was precipitated without the specific antibodies (Fig. 3B, lane 3). It should be noted that the bands of Ker1p again appear to be doublets (Fig. 3B, lane 2), suggesting that the upper and lower bands correspond to the 32 kDa and 30 kDa forms of the protein, respectively, as seen in Fig. 1B. The results suggest that pol I associates with both phosphorylated and non-phosphorylated Ker1p.

*Ker1p Is Co-fractionated Biochemically with pol I:* The above results suggest that Ker1p is a pol I subunit. To confirm biochemically that Ker1p is a novel pol I subunit, we have purified pol I from an *S. pombe* strain expressing both Ker1p-(HA)$_3$ and RPA140 tagged with a (His)$_6$-FLAG epitope (YI28). The whole cell extract was first affinity-purified using a nickel-agarose column, and then fractionated using DEAE-Sephadex A25 column chromatography. We observed that Ker1p was co-eluted with the peak fractions (fractions 13 and 14) of pol I detected through the RPA190 and RPA21 subunits (Fig. 4). Although more rigorous
biochemical purification is needed, the elution pattern through the DEAE column confirms that Ker1p is a subunit of pol I. We note that fractions 11 and 12 might also contain pol I without Ker1p and RPA21, but we have not examined these fractions further in this study.

**Genetic Interaction between Ker1p and RPA21:** We have previously shown that overproduced Rrn3p is able to suppress the temperature-sensitive (ts) growth defect of *rpa21* mutants, indicating a genetic interaction between RPA21 and Rrn3p (41). To examine the genetic interaction between Ker1p and RPA21, we introduced a multi-copy vector expressing Ker1p under the control of a CMV promoter into the three ts *rpa21* mutants [*ts152*, *ts296* and *ts2817* (41)] and examined whether the growth defects of the mutants were suppressed at the restrictive temperature. As can be seen in Fig. 5, overproduction of Ker1p clearly suppresses the growth deficiency of the three mutants, indicating a genetic interaction between Ker1p and RPA21. Since A14 was previously shown to be required for stable association of A43 with pol I in *S. cerevisiae* (11), it appears that Ker1p is also required for stable association of RPA21 with pol I.

*Ker1p Has a Limited Homology with S. cerevisiae A14:* No apparent homologous protein
was initially found when the Ker1p sequence was used in a database search. However, the
results obtained above clearly indicate that Ker1p is a pol I subunit and that it interacts with
RPA21. Since RPA21 is an ortholog of A43 of *S. cerevisiae*, we re-examined the homology of
Ker1p with *S. cerevisiae* A14, which heterodimerizes with A43. Previously, A14 was
suggested to have homology to a putative open reading frame of IPF1568 from *Candida
albicans* (11), suggesting that the A14 gene family is conserved in *C. albicans*, although no
genetic or biochemical evidence was presented. This suggestion prompted us to directly
investigate the homologies among Ker1p, A14 and IPF1568 (hereafter referred to as CaA14),
and Fig. 6 shows an alignment of these proteins, constructed using ClustalW. We found that
Ker1p shows 21% identity and 27% similarity to the 126-amino acid sequence of ScA14, and
that the N-terminal 60 amino acids of Ker1p shows especially high identity (37%) and
similarity (43%) to ScA14. The N-terminal region also showed a significant identity between
ScA14 and CaA14 (11), and Ker1p shows 26% identity and 36% similarity to the 132-amino
acid sequence of CaA14. The local identity of Ker1p with ScA14 and CaA14 is especially
high (38% and 42%, respectively) between the 42nd and 65th amino acids of Ker1p, and this
region contains a motif that may be conserved between ScA14 and CaA14 (SQLKRIQR), as already suggested by Peyroche et al. (11). Taken together, we conclude from these results that Ker1p is an ortholog of both ScA14 and CaA14.

*ker1+ is Required for Growth Only at High Temperatures:* To examine whether *ker1* is essential for cell growth, we replaced one of the chromosomal copies of *ker1* with a disrupted *ker1Δ::ura4* in the *S. pombe* diploid. A Ura+ transformant (YI29) was chosen and subjected to tetrad analysis upon sporulation (Fig. 7A). Of the 20 asci dissected, one yielded four viable spores, seven yielded three viable spores and the remaining twelve yielded two viable spores on YEPD plates at 30°C. Large colonies were invariably Ura-, while all the small colonies, including the extremely small ones, were Ura+. Correct disruption of the *ker1* locus in the Ura+ segregant YI30 was verified by PCR (data not shown). Growth of the colonies was tested at 25, 30 and 36°C; none of the Ura+ colonies grew at 36°C, but all of the Ura- colonies did so, indicating that *ker1* is not essential for cell growth at 30°C or 25°C, but is required for cell growth at 36°C (Fig. 7B).

*ker1Δ is Suppressed by Overproduction of RPA21 or Rrn3p:* We subsequently examined
whether complete deletion of ker1+ (ker1Δ) is also suppressed by overproduction of RPA21.

Fig. 8 shows that overexpression of RPA21 suppressed the growth defect of ker1Δ, again suggesting (see Fig. 5) that Ker1p is required for stable association of RPA21 with pol I. This result also suggests that RPA21 can associate with pol I independently of Ker1p. Since the rDNA-specific transcription factor Rrn3p interacts with RPA21 (41), we also examined whether overexpression of Rrn3p is able to suppress ts growth of ker1Δ. As also shown in Fig. 8, overproduction of Rrn3p can suppress the ts phenotype of ker1Δ, suggesting that Ker1p interacts with Rrn3p, and directly stabilizes the association of Rrn3p with pol I. However, the alternative possibility remains that overproduced Rrn3p can interact with RPA21 and perhaps stabilize the association of RPA21 with pol I without the participation of Ker1p. Fig. 8 also shows that multiple copies of rpa190+ were unable to suppress the ker1Δ phenotype.

*Dissociation of Ker1p from pol I in Post-Log-Phase Cells:* The data in Fig. 8 suggest that Ker1p is involved in stabilizing the association of Rrn3p with pol I, either directly or indirectly. Since Rrn3p is released from pol I in post-log-phase or growth-arrested cells (23, 24, 26), we examined if Ker1p is also released from pol I, resulting in destabilization of
Rrn3p in pol I in post-log-phase cells. As shown in Fig. 9, pol I from cells expressing Ker1p-(HA)3 in mid-log phase and post-log phase, respectively, was immunoprecipitated with antibodies against RPA190, and the relative amounts of RPA190, RPA140 and Ker1p were compared. We found that a drastic decrease in the ratio of Ker1p (both phosphorylated and non-phosphorylated Ker1p) to RPA190 was observed (Fig. 9; lanes 2 and 3, compared to lane 1 in IP) in pol I prepared from post-log-phase cells, although the ratio of RPA140 to RPA190 did not change significantly, suggesting that Ker1p is dissociated from pol I in the post-log phase. The dissociation of Ker1p from pol I may cause instability of Rrn3p in pol I, either directly or indirectly, resulting in dissociation of Rrn3p from pol I, which inactivates rDNA transcription.

**DISCUSSION**

In the present paper, we have shown that Ker1p isolated by a yeast two-hybrid system using RPA21 as bait is the counterpart of the *S. cerevisiae* pol I subunit A14. This is the first demonstration that a pol I-specific A14 ortholog is conserved in eukaryotes other than *S. cerevisiae*, despite no apparent homolog of A14 being identified in the *S. pombe* genome. We
have successfully aligned the amino acid sequence of almost the entire length of Ker1p with those of A14 and IPF1568 (Fig. 6), indicating that these subunits are indeed grouped into a gene family. Our investigation of Ker1p has, however, also revealed features of Ker1p that are distinct from those of A14: first, Ker1p is phosphorylated (Fig. 1) whereas phosphorylation of A14 has never been observed; second, Ker1p is suggested to interact, either directly or indirectly, with Rrn3p and stabilize the association of Rrn3p with pol I \textit{in vivo} (Fig. 8), while it is unclear whether A14 affects the stability of Rrn3p with pol I \textit{in vivo}; and third, Ker1p is released from pol I in post-log-phase cells (Fig. 9), whereas such instability of A14 in post-log-phase or growth-arrested cells is unknown in \textit{S. cerevisiae}. The significance of these differences must await future studies to determine if A14 can be phosphorylated, or does dissociate from pol I in post-log-phase cells.

A comparison of the pol I subunits in \textit{S. pombe} and \textit{S. cerevisiae} is shown in Table 2. Ten subunits constituting the core structure (RPA190, RPA140, RPA42, RPA17, Rpb5, Rpb6, Rpb8, Rpb10, Rpb12 and SpRPA12) are conserved from \textit{S. cerevisiae} to \textit{S. pombe} pol I. RPA190 and RPA140 were not examined, but seven of the remaining eight subunits (all
except Rpb8) could substitute for the corresponding subunits in *S. cerevisiae*, suggesting functional conservation of most of the subunits (38-40, 49). In three specific subunits, including Ker1p, RPA51 was tested and found to rescue an *rpa49* mutation in *S. cerevisiae* (42). While RPA21 encodes only 174 amino acids and appears to be much diversified from *S. cerevisiae* A43 (*S. cerevisiae* A43 contains 326 amino acids), its role in pol I recruitment to the rDNA promoter is conserved (41), and it is plausible that the N-terminal region conserved in the A43 gene family plays a role in the interaction with Rrn3p. The question of whether *S. pombe* pol I conserves a counterpart of *S. cerevisiae* A34.5 is unresolved. We believe that the primary sequence of the A34.5 homolog, if any such protein exists, may be poorly conserved in *S. pombe*. It is noted that mouse pol I has been found to contain distinct subunits from lower eukaryotes, such as PAF67 and PAF49, (33, 34, 50). These results tempt us to speculate that lower eukaryotes like yeasts might conserve the 14 subunits found in *S. cerevisiae* and that higher eukaryotes might have more specific subunits such as PAF67 and PAF49, in addition to the 14 conserved subunits.

It is known that A14 is not phosphorylated in *S. cerevisiae* pol I (1, 51, 52), whereas here
Ker1p was found to be phosphorylated, suggesting that specific subunit phosphorylation has also evolved independently among pol I subunits. Indeed, *S. cerevisiae* A43 is multiphosphorylated (53), whereas mammalian A43 is barely phosphorylated (24). It has been argued that A43 must be phosphorylated to associate with Rrn3p in *S. cerevisiae*, whereas Rrn3p phosphorylation is a prerequisite to the association of A43 with Rrn3p in mammalian cells (24, 53, 54). In this context, the functional dissection of Ker1p phosphorylation/dephosphorylation may provide a novel insight into the pol I recruitment mechanism.

It appears that Ker1p is required for the stability of RPA21, based on multi-copy suppression experiments: (i) ker1Δ exhibits a ts-growth deficit and the ts deficit is suppressed by overproduction of RPA21 (Fig. 8); and (ii) the ts-growth deficit of three rpa21 mutants, *ts152, ts296* and *ts2817*, was suppressed by overexpression of ker1+ (Fig. 5). Since it is known that A14 is also required for the stability of A43 in *S. cerevisiae* (9, 11), the role of Ker1p may be, as expected, similar to that of A14. To verify the Ker1p function, purification of pol I from extracts of ker1Δ mutants deserves future study. Unexpectedly, as can be seen in
Fig. 8, overproduction of Rrn3p also suppresses the ts phenotype of the ker1Δ mutant, suggesting that Ker1p is also required for the stable association of Rrn3p with pol I. Alternatively, these suppression data could also suggest that overproduction of Rrn3p suppresses the instability of RPA21 in pol I without participation of Ker1p, leading to indirect suppression of the ts phenotype of ker1Δ. Clearly, future biochemical experiments are required to reveal whether Rrn3p directly interacts with Ker1p.

Accumulating evidence has shown that a dissociation of Rrn3p from pol I in post-log phase or growth-arrested cells causes a decrease of pol I recruitment to the promoter, resulting in a decrease or halting of rDNA transcription. As described above, it has been argued that post-translational modification (phosphorylation/dephosphorylation) of A43 and Rrn3p regulates the stability of the pol I-Rrn3p complex, causing dissociation of Rrn3p from pol I in the post-log-phase or growth-arrested cells. The immunoprecipitation experiments shown in Fig. 9, using antibodies against RPA190, showed that the amounts of both forms of Ker1p (phosphorylated and non-phosphorylated forms) relative to RPA140 and RPA190 are reduced drastically after cells enter the post-log phase. The results suggest that dissociation of Ker1p
from pol I in post-log-phase cells is one of the regulatory mechanisms of Rrn3p dissociation from pol I, since the dissociation of Ker1p may lead to instability of RPA21 and Rrn3p in pol I. It is also possible that release of Ker1p induces certain modification(s) of RPA21 and Rrn3p, resulting in release of Rrn3p from pol I. Therefore, it is tempting to speculate that the association/dissociation of Ker1p might primarily regulate growth-dependent transcription of rDNA in *S. pombe*.

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**FOOTNOTES**

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1The abbreviations used are: pol I, RNA polymerase I; pols, RNA polymerases; pol II, RNA
polymerase II; pol III, RNA polymerase III; MM, minimal medium; YE, yeast-dextrose;
YEPD, yeast-peptone-dextrose; SD, synthetic dextrose; 3-AT, 3-amino-(1,2,4)-triazole; AbA,
Aureobasidin A; PCR, polymerase chain reaction; DAPI, 4',6-diamidino-2-phenylindole; HA,
hemagglutinin antigen; PAGE, polyacrylamide gel electrophoresis.

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**FIGURE LEGENDS**

**FIG. 1. Ker1p can be phosphorylated.** *A*, Apparent molecular mass of Ker1p. Extracts prepared from *S. pombe* cells of IZ2 (WT) and Y128 expressing Ker1p-(HA)₃ (HA-KER1) were subjected to SDS-PAGE followed by immunoblot analysis with the monoclonal antibody 12CA5. In both lanes, 40 µg of crude extract was loaded. *B*, Ker1p is a phosphorylated protein. Ker1p-(HA)₃ was immunoprecipitated from a whole cell extract of Y128 with monoclonal antibody 12CA5. The immunoprecipitates were treated as follows: lane 1, no treatment; lane 2, alkaline phosphatase; lane 3, alkaline phosphatase and a phosphatase inhibitor (sodium pyrophosphate); lane 4, the inhibitor alone (sodium pyrophosphate). After treatment, samples were subjected to SDS-PAGE followed by
immunoblot analysis with monoclonal antibody 12CA5. Molecular mass standards (kDa) are indicated on the right (A) and left (B).

**FIG. 2. Ker1p predominantly localizes in the nucleolus.** The indicated GFP-fusion proteins (GFP-KER1 or GFP-FIB) or GFP alone, as shown on the left of the figure, were expressed in *S. pombe* JY742. top row, GFP-Ker1p; second row, GFP; third row, GFP-FIB (fibrillarin). DNA was visualized by DAPI staining in the left column (A, D, and G). The localization of GFP-fusion proteins or GFP is shown in the middle column (B, E, and H). Merged images are shown in the right column (C, F, and I).

**FIG. 3. Ker1p associates with pol I.** A, Extracts from strains IZ2 (KER1) and YI28 (HA-KER1) expressing Ker1p (lane 3) or Ker1p-(HA)₃ (lane 4), respectively, were immunoprecipitated with 12CA5 beads (anti-HA). The extracts (lanes 1 and 2) and immunoprecipitates (lanes 3 and 4) were subjected to SDS-PAGE, followed by immunoblot analysis with antibodies against RPA190, RPA140, RPA21 and Rpb1 (from top to bottom, respectively). Twenty-five μg of crude extract was used for each control. B, Extracts from strains expressing Ker1p-(HA)₃ were immunoprecipitated with antibodies against RPA190.
The extract (lane 1), the immunoprecipitates with RPA190 antibodies (lane 2), and a sample without anti-RPA190 treatment (lane 3) were subjected to SDS-PAGE, followed by immunoblot analysis with antibodies against HA, RPA140 and RPA21 (from top to bottom, respectively). The position of each band is indicated by the bar on the right hand side of the panels. Ker1p appears as a doublet of bands. The dense bands seen at the top of the bottom panel (lanes 2 and 3) are derived from the light chain of immunoglobulin G.

**FIG. 4. Ker1p co-fractionates with pol I.** Extracts from strains expressing Ker1p-(HA)₃ were loaded onto a nickel agarose column. The eluted fractions were then loaded onto a DEAE-Sephadex A25 column and eluted with a linear gradient of 50 mM to 500 mM ammonium sulfate. Peaks of RPA190, RPA21 and Ker1p were detected by Western blotting using antibodies against RPA190, RPA21 and HA, respectively. The numbers on the upper panel show the column numbers, and arrows on the right indicate each subunit [RPA190, Ker1p-(HA)₃ and RPA21].

**FIG. 5. Overproduction of Ker1p suppresses ts-growth of three rpa21 mutants.** Each of the ts mutants (ts152, ts296 and ts2817) transformed with pYI195 (ker1⁻) or pAUR222
(vector) was restreaked on MM medium with thiamine (+thi) containing AbA and incubated for 5 days at 36°C. *ker1* + was expressed under the control of the CMV promoter.

**FIG. 6. Amino acid sequence alignment of *S. pombe* Ker1p (Sp) with *S. cerevisiae* A14 (Sc) and *C. albicans* IPF1568 (Ca).** Identical and similar residues are highlighted in black and gray, respectively. The alignment was generated with the ClustalW program using the ID matrix (the default BLOSUM matrix yielded a different alignment, in which Ker1p appeared to be less homologous to *S. cerevisiae* A14). The DDBJ/GenBank™/EMBL accession numbers for the *ker1*+ sequence are AB079137 and AL157874.

**FIG. 7. Gene disruption of *ker1*+.** A, Left: The 4.5-kb XhoI-SacI DNA fragment used for *ker1*+ disruption. Right: Tetrad of diploids (*ker1Δ::ura4+/ker1*+) grown at 30°C. B, Growth of four haploid segregants derived from the ascospore produced by tetrad dissection of the *ker1Δ::ura4+/ker1*+ diploid.

**FIG. 8. Ts-growth of a *ker1Δ* strain is suppressed by overexpression of *rpa21*+ or *rrn3*+ but not by *rpa190*+.** Vector, *ker1*+, *rpa190*+, *rrn3*+ and *rpa21*+ indicate YI28 (*ker1Δ*) carrying pREP41 (an empty vector), pYI210 (*ker1*+), pGK100 (*rpa190*+), pK127 (*rrn3*+) and
pYI82 (rpa21<sup>+</sup>), respectively. Each transformant was restreaked on MM plates without leucine and thiamine, and incubated at 36°C or 30°C for 5 days.

**FIG. 9. Dissociation of Ker1p from pol I in post-log-phase cells.** 

_A_ Extracts were prepared from YI28 in mid-log-phase cells (lane 1) and post-log-phase cells (lanes 2 and 3), respectively, at the indicated times shown in panel B. Crude extract: for detection of each subunit in the crude extract, 50 μg of each extract was loaded onto an SDS-PAGE gel. Ker1p tagged with (HA)₃, RPA190, RPA140 and Rpb1 (pol II for a control) were detected by immunoblot analysis with antibodies against HA, RPA190, RPA140 and Rpb1 (from top to bottom, respectively). _IP:_ Pol I in the crude extract was immunoprecipitated by antibodies against RPA190. The immunoprecipitates were subjected to SDS-PAGE, followed by immunoblot analysis with antibodies against HA, RPA190, RPA140 and Rpb1 from top to bottom, respectively. We note that bands detected by anti-Rpb1 were non-specific. 

_B_ YI28 was grown in YE medium at 30°C. The culture was harvested at the indicated times (1, 2 and 3). The A₆₀₀ values of the culture at harvesting times 1, 2 and 3 were ~1.0 (mid-log phase), ~5.3 and ~6.8 (post-log phase), respectively. The doubling times at each point were ~2 h.
(time point 1), ~6 h (time point 2) and ~20 h (time point 3). In this paper, we defined a temporary slow-growth phase, such as those at time points 2 and 3, as a post-log phase.
| Strains or Plasmids | Description |
|---------------------|-------------|
| **Strains**         |             |
| *S. pombe*          |             |
| JY742               | $h^+ \text{ ade}6\text{-}M216 \text{ ura}4\text{-}D18 \text{ leu}1$ |
| JY745               | $h^- \text{ ade}6\text{-}M210 \text{ ura}4\text{-}D18 \text{ leu}1$ |
| IZ2                 | Derivative of JY742 expressing RPA140 tagged with (His)$_6$-FLAG (42) |
| YI28                | Derivative of IZ2 expressing ker$1^+$-(HA)$_3$ |
| YI29                | Diploid (crossing JY742 with JY745) carrying ker$1\Delta:\text{ura}4^+/\text{ker}1^+$ |
| YI30                | $h^- \text{ ker}1\Delta:\text{ura}4^+ \text{ ade}6 \text{ ura}4\text{-}D18 \text{ leu}1$ |
| **S. cerevisiae**   |             |
| Y190                | MAT$\alpha\ \text{ ade}2\text{-}101\ \text{ ura}3\text{-}52\ \text{ his}3\Delta200\ \text{ lys}2\text{-}801\ \text{ trp}1\text{-}901\ \text{ leu}2\text{-}3,112$ |
gal4Δ gal80Δ LYS2::GAL1-HIS3 URA3::GAL1-lacZ

Plasmids

pKI45 Derivative of KS (+) cloned with a full-length cDNA of *rpa21* between the *XhoI* and *EcoRI* sites

pYI77 Derivative of pAS2-1 expressing GAL4DB-RPA21, *TRP1*, 2μm

pKS406 Derivative of pSGA (47) expressing green fluorescence protein (GFP) fusion proteins under the control of the *nmt1* promoter, *ars1*, *LEU2*

pYI193 Derivative of pKS406 expressing GFP fused to Ker1p

pYI200 Derivative of pKS406 expressing GFP fused to fibrillarin

pYN1235 Derivative of pGEM3ZpBR322ura4+pBR322, in which the *BamHI* site was introduced into the *HindIII* site (55)

pYN1237 Derivative of KS(+). The (HA)$_3$-TAG sequence (triple HA epitope tagged with TAG) is inserted between the *SmaI* and *SpeI* sites. A 2.5-kb *BamHI*-*BamHI* *ura4* fragment excised from pYN1235 was inserted at
a BglII site created downstream of the (HA)$_3$-TAG sequence.

pAUR224 Expression vector under the control of the CMV promoter, *aur1*, *ars1*

pYI195 Derivative of pAUR224 expressing a full-length *Ker1p* under the control of the CMV promoter

pYI176 Derivative of pYN1237 carrying a 1.0-kb fragment of the 5′-flanking and coding sequence of *Ker1p* fused to (HA)$_3$, *ura4* and a 1.0-kb fragment of the 3′-flanking sequence of *ker1*

pGK100 Derivative of pDB248 carrying *nuc1*/rpa190 (37)

pYI185 Derivative of KS (+) carrying a 0.88-kb fragment of the 5′-flanking region of *ker1* between the *XhoI* and *BamHI* sites and a 1.0-kb fragment of the 3′-flanking region of *ker1* between the *NotI* and *SacI* sites

pYI186 Derivative of pYI185 with *ura4* inserted at the *BamHI* site

pREP41 Expression vector under the control of a modified *nmt1* promoter,
LEU2, ars1 (56)

pREP81 Expression vector under the control of a modified nmt1 promoter, LEU2, ars1 (56)

pYI40 Derivative of pREP81 in which the NdeI site was converted to a BglII site (41)

pYI82 Derivative of pYI40 expressing rpa21+ cDNA under the control of a weak nmt1 promoter (41)

pKI27 Derivative of pREP81 expressing a full-length rrn3+ under the control of the nmt1 promoter

pYI210 Derivative of pYI40 expressing a full-length ker1+ under the control of the nmt1 promoter
# TABLE II

*Comparison of the RNA polymerase I subunits of S. pombe and S. cerevisiae*

| Gene   | No. of amino acids | Gene   | No. of amino acids | Identity (%) |
|--------|--------------------|--------|--------------------|--------------|
| rpa190 | 1,689              | RPA190 | 1,664              | 50           |
| rpa140 | 1,228              | RPA135 | 1,203              | 63           |
| rpa42  | 348                | RPC40  | 335                | 58           |
| rpa17  | 125                | RPC19  | 142                | 63           |
| Sprpa12| 119                | RPA12  | 125                | 55           |
| rpb5   | 210                | RPB5   | 215                | 56           |
| rpb6   | 142                | RPB6   | 155                | 54           |
| rpb8   | 125                | RPB8   | 146                | 39           |
| rpb10  | 71                 | RPB10  | 70                 | 72           |
| Gene   | Position | Accession | Start | End |
|--------|----------|-----------|-------|-----|
| rpb12+ | 63       | RPB12     | 70    | 39  |
| rpa51+ | 425      | RPA49     | 415   | 30  |
| rpa21+ | 173      | RPA43     | 326   | 36  |
| ker1+  | 147      | RPA14     | 137   | 21  |
| ?      |          | RPA34     | 233   |     |

The sequence data and identity were obtained as follows: rpa190+ (36, 37), rpa140+ (GenBank<sup>TM</sup>/EMBL accession number for the rpa140+ is AL136535), rpa42+ (38), rpa17+ (39), Sprpa12+ (40), rpb5+, rpb6+, rpb8+, rpb10+, and rhp12+ (12), rpa51+ (42), rpa21+ (41), ker1+ (this paper).
**FIG. 1 (Imazawa et al.)**

**A**

- HA-WT KER1
- KER1

**B**

- Phosphatase inhibitor
  - Phosphatase
  - Non-P

| 1 | 2 | 3 | 4 |
|---|---|---|---|
| - | + | + | - |
| - | - | + | + |
FIG. 4 (Imazawa et al.)

- RPA190
- KER1-(HA)$_3$
- RPA21

[Image of a gel electrophoresis pattern with markers 5, 10, 15, 20, 25]
FIG. 5 (Imazawa et al.)
A

\[ \text{XhoI} \quad \text{ker}^+ \quad \text{SacI} \]

\[ \text{ura}^+ \]

B

\begin{align*}
\text{ker}^+ & \quad \text{ker}^- \\
\text{ker}^+ & \quad \text{ker}^- \\
30^\circ C & \quad 36^\circ C
\end{align*}

FIG. 7 (Imazawa et al.)
FIG. 8 (Imazawa et al.)

The diagram shows the distribution of different bacterial strains and vectors at two temperatures: 30°C and 36°C. The strains include:

- rpa21+
- rrn3+
- ker1+
- rpa190+
- Vector

The images on the left and right represent the growth patterns of these bacterial strains at 30°C and 36°C, respectively.
FIG. 9 (Imazawa et al.)

A

|       | crude extract | IP |
|-------|---------------|----|
| 1     |               |    |
| 2     |               |    |
| 3     |               |    |

- α-HA
- α-RPA190
- α-RPA140
- α-Rpb1

B

- KER1-P
- KER1
- RPA190
- RPA140
- Rpb1

Time (H)

A600

0 10 20 30 40

0.001 0.01 0.1 1 10

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The fission yeast protein, Ker1p, is an ortholog of RNA polymerase I subunit A14 in *Saccharomyces cerevisiae*, and is required for stable association of Rrn3p and RPA21 in Pol I

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