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An essential *Saccharomyces cerevisiae* single-stranded DNA binding protein is homologous to the large subunit of human RP-A

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Single-stranded DNA binding proteins (SSBs) are known to play a role in DNA replication and recombination in prokaryotes. An SSB was previously purified from the yeast *Saccharomyces cerevisiae*. This SSB stimulated the activity of a cognate strand exchange protein (SEP1) *in vitro* suggesting a role in recombination. We have cloned and functionally analyzed the gene encoding this protein. DNA sequencing of the cloned DNA revealed a 621 amino acid open reading frame with a coding potential for a M_r 70 269 polypeptide. Highly significant amino acid homology was detected between this *S.cerevisiae* gene and the M_r 70 000 subunit polypeptide of human RP-A, a cellular protein essential for SV40 DNA replication *in vitro*. Therefore, we named the *S.cerevisiae* gene *RPA1*. *RPA1* encodes an essential function in this organism as shown by tetrad analysis of heterozygous insertion mutants and is continuously required for mitotic growth. Cells lacking *RPA1* accumulate as multiply budded cells with a single nucleus suggesting a defect in DNA replication.

Key words: DNA recombination/DNA replication/ *S.cerevisiae*/single-stranded DNA binding protein

Introduction

Genetic recombination is the molecular mechanism(s) which governs the precise and controlled rearrangement of homologous DNA sequences. The formation of hybrid DNA (hDNA) is central in this process (Meselson and Radding, 1975; Szostak *et al.*, 1983; as reviewed by Orr-Weaver and Szostak, 1985; Smith, 1988). A number of activities that can promote hDNA formation *in vitro* have been extensively purified from prokaryotic (Yonesaki *et al.*, 1985; as reviewed by Cox and Lehman, 1987) and eukaryotic (Kmiec and Holloman, 1982; Kolodner *et al.*, 1987; Sugino *et al.*, 1988) organisms. To date the best characterized proteins are the *Escherichia coli* recA and the bacteriophage T4 uvsX proteins. In both cases the *in vivo* role of the respective proteins is also clearly established as being essential for virtually all types of homologous recombination (Mosig, 1987; Smith, 1988). Both recA and uvsX are stimulated by their cognate single-strand DNA binding proteins (SSBs), *E.coli* SSB (Shibata *et al.*, 1980; McEntee *et al.*, 1980) and T4gp32 (Yonesaki and Minagawa, 1985; Formosa and Alberts, 1986) which had originally been discovered as being required for DNA replication. Whereas recA is stimulated non-specifically by many SSBs (Egner *et al.*, 1987) including the *Saccharomyces cerevisiae* SSB discussed here (Heyer and Kolodner, 1989), uvsX is dependent on the cognate T4 protein, gp32 (Formosa and Alberts, 1986). Other proteins are thought to interact with uvsX on the basis of biochemical (Formosa and Alberts, 1986) and genetic (reviewed in Mosig, 1987) properties leading to the concept developed by Alberts (1984) that complicated reactions like recombination are catalyzed *in vivo* by multi-protein complexes. Similar interactions between recA and SSB and recF have been detected by genetic analysis (see Smith, 1988).

We have been interested in understanding the molecular mechanisms(s) of genetic recombination in the yeast *S.cerevisiae*. Specifically, we have purified and characterized a strand exchange protein from mitotic *S.cerevisiae* cells (SEP1) that catalyzes many reactions in common with *E.coli* recA and T4 uvsX (Kolodner *et al.*, 1987; Heyer *et al.*, 1988). By analogy with the prokaryotic recombination proteins, we considered it possible that *S.cerevisiae* would have an accessory protein or proteins that stimulated the activity of SEP1. To identify such proteins we developed an *in vitro* stimulation assay containing suboptimal concentrations of SEP1. Thus far, two different proteins have been identified and purified using this assay. First, a M_r 34 000 protein that was characterized as an SSB (Heyer and Kolodner, 1989) and secondly, a M_r 33 000 protein termed SF1 (Stimulatory Factor 1) that markedly stimulates SEP1 (D.N.Norris and R.D.Kolodner, manuscripts in preparation).

A variety of SSB-like proteins have been identified in *S.cerevisiae* (Chang *et al.*, 1979; LaBonne and Dumas, 1983; Jong *et al.*, 1985; and see in Williams and Chase, 1989). Only a M_r 45 000 protein termed SSB1 has been studied in detail and these studies revealed a role in RNA metabolism rather than in DNA replicatiion or recombination as judged from the nucleic acid binding properties and cytological data (LaBonne and Dumas, 1983; Jong *et al.*, 1985; Jong *et al.*, 1987). Genetic experiments showed that the gene encoding SSB1 was not essential for mitotic growth (Jong and Campbell, 1986). For no other SSB-like protein is information about their *in vivo* function available. The M_r 34 000 SSB isolated in our laboratory is distinct from SSB1 in that it has no affinity for RNA and therefore it is unlikely that it is involved in RNA metabolism (Heyer and Kolodner, 1989).

Recently, a cellular protein, RP-A, which is essential for *in vitro* SV40 replication, has been purified to homogeneity (Fairman and Stillman, 1988; Wold and Kelly, 1988). RP-A is required for the localized unwinding of the origin, one

of the earliest steps in DNA replication (Wold and Kelly, 1988), and probably participates in chain elongation as well. The purified protein consists of three subunits of M_r 70 000, 32 000, and 14 000. The complex has single-stranded DNA binding activity, which resides in the M_r 70 000 subunit (Wold *et al.*, 1989). Although other single-stranded DNA binding proteins can substitute for RP-A in the origin unwinding reaction, they cannot replace RP-A in the complete DNA replication reaction.

Here, we describe the isolation of the gene (*RPA1*) that encodes the SSB protein previously identified as stimulating the *S.cerevisiae* SEP1 protein *in vitro* (Heyer and Kolodner, 1989). This gene is located near the centromere of chromosome I and is essential for viability. DNA sequence analysis revealed highly significant amino acid homology to the M_r 70 000 subunit polypeptide of human RP-A. Thus, RP-A is a highly conserved protein that appears to be involved in both genetic recombination and DNA replication. To our knowledge this is the first demonstration of an essential eukaryotic SSB.

Results

The S.cerevisiae gene is homologous to the large subunit of RP-A

The gene encoding the M_r 34 000 SSB was cloned by using degenerate oligonucleotides derived from the NH_2-terminal peptide sequence of several proteolytic fragments of the purified M_r 34 000 protein (see Table II) to probe a *S.cerevisiae* genomic library. Figure 1 shows the *S.cerevisiae* chromosomal insert cloned in YCp50 in the original clone pRPA1 along with some surrounding sequences and restriction sites that were mapped on chromosome I by Southern blotting using the *Bam*HI−*Mlu*I fragment of pRPA1 as a probe. Transcript analysis by Northern blotting using the same probe revealed a single 1950 nucleotide (nt) long mRNA with intermediate abundance (between the levels of the highly expressed *CYH2* and the housekeeping function *HIS4*; data not shown). The length of the mRNA is consistent with the 1861 bp long open reading frame (ORF) determined for the *RPA1* gene (see below). Since the translation product of the gene showed highly significant amino acid homology (see below) to the large subunit of human RP-A (termed RP-A I by Wold and Kelly, 1988), we propose to call the *S.cerevisiae* gene *RPA1*.

The DNA sequence of the entire 3436 bp chromosome I insert in pRPA1 was determined on both strands. It revealed one complete ORF with coding potential for a M_r 70 269 polypeptide that identified unambiguously the gene for the M_r 34 000 SSB. As shown in Figure 2, all sequenced peptides could be identified in the translated DNA sequence. It was possible to deduce the location of the NH_2-terminal amino acid sequence (as shown in Figure 2) even though sequencing of the native M_r 34 000 SSB gave multiple signals per cycle (see Table II). Evidently, the M_r 34 000 polypeptide was a proteolytic breakdown product of the primary translation product. We do not know whether this proteolytic reaction has any *in vivo* significance or is just a purification artefact.

Comparison of the amino acid sequence of the *S.cerevisiae* SSB with a partial sequence of the M_r 70 000 subunit of human RP-A, derived as described in Material and methods, revealed extensive and highly significant amino acid

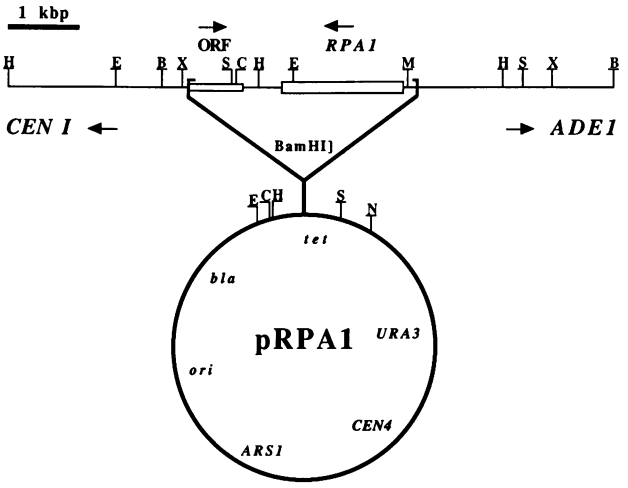

Fig. 1. Structures of pRPA1 and the *RPA1* region of chromosome I. The chromosomal sequence is drawn to scale and all the relevant restriction sites are shown. The positions of the *RPA1* reading frame and the open reading frame (ORF) that runs into the cloned fragment from the left hand site are shown as open boxes. The abbreviations are B, *Bam*HI; C, *Cla*I; E, *Eco*RI; H, *Hin*dIII; M, *Mlu*I; N, *Nru*I; S, *Sal*I, and X, *Xba*I. BamHI] indicates that a *Bam*HI site has been restored in pRPA1 that abuts the left hand site of the insert. This site arose from the fortuitous joining of the YCp50 *Bam*HI with the *Sau*3A site of the insert of the cloning process and does not correspond to a site in the *S.cerevisiae* genome. The vector (YCp50) is not drawn to scale and only the positions of the sites used in this study are indicated. The relative positions of the important features of YCp50 are shown based on the published map (Rose *et al.*, 1987). The orientation of the insert on chromosome I shown corresponds to the actual situation in the *S.cerevisiae* genome.

homology. As shown in Figure 3 the homology extends over the entire area sequenced with an overall identity of 41% (77 out of 189 amino acids) and a degree of homology (identity plus conservative substitutions) of 81% (155 out of 189 amino acids).

From our biochemical analysis of the M_r 34 000 proteolytic fragments we know that it contained a DNA binding domain. The amino acid sequence revealed a potential metal binding site of the zinc finger type (C - 4X - C - 13X - C - 2X - C) at positions 486−508 in the translated reading frame which is consistent with the biochemical properties of the protein. A potential metal binding domain was also predicted for T4gp32 (Berg, 1986) and other work (Giedroc *et al.*, 1986) corroborated this prediction. The entire translated *RPA1* amino acid sequence was used to search protein and DNA data banks using the Fasta program of Pearson and Lipman (1988). No significant homology was detected to any protein or DNA sequence in the library.

The DNA sequence (Figure 2) also revealed the presence of an ORF running into the cloned fragment from the left side (see also Figure 1). The translated amino acid sequence identified a highly charged polypeptide with 17% aspartate and glutamate residues (28 negative charges at neutral pH for the translated reading frame). The putative polypeptide showed homology to a variety of sequences in database searches on the basis of the presence of many consecutive aspartate and glutamate residues. We have demonstrated by transposon mutagenesis (see below) that an insertion in this part of the gene (see Figure 2 for the exact location of the

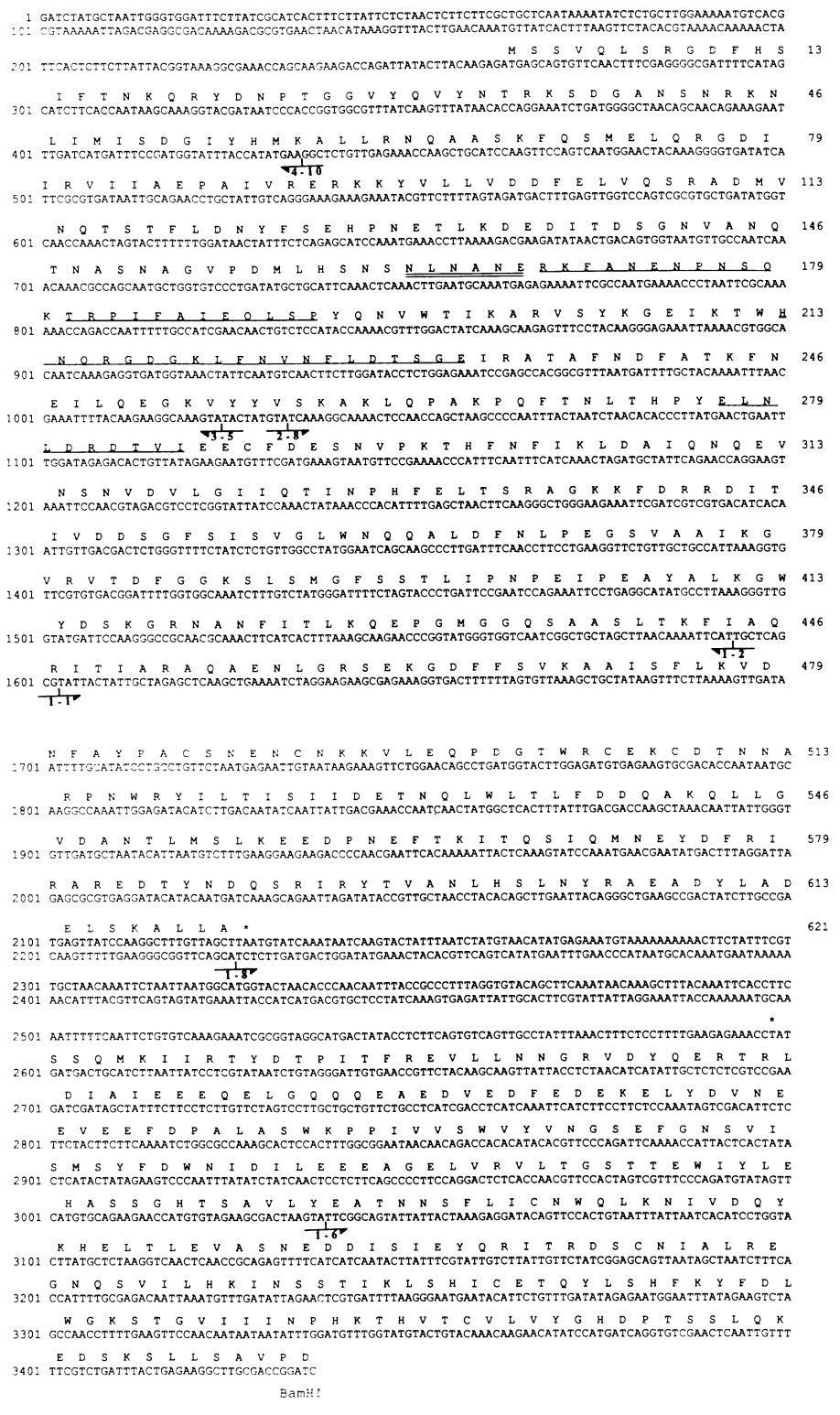

Fig. 2. DNA sequence of the *RPA1* region from chromosome I of *S.cerevisiae*. The entire insert of pRPA1 was sequenced on both strands and is shown here in inverted orientation compared with Figure 1 to facilitate the representation. The 5′ most ATG of the *RPA1* reading frame was chosen as the start codon and begins at nucleotide 263 and the TAA stop codon terminating the reading frame is at nucleotide position 2128. The ORF that runs into the cloned fragment (from the left hand site in Figure 1) is encoded by the opposite strand. It terminates with a TAG stop codon (CTA on the strand shown) at position 2597. For both reading frames the amino acid sequence is shown in single letter code above the DNA sequence. The underlined protein sequence of *RPA1* indicates the sequenced peptides (see Table II). The doubly underlined protein sequence denotes the inferred NH_2 terminus of the M_r 34 000 polypeptide. Beneath the DNA sequence are shown the position, orientation and number of the transposon Tn*10*LUK insertions that were generated in this fragment (4−10 after nt 435; 3−5 after nt 1024; 2−8 after nt 1033; 1−2 after nt 1593; 1−1 after nt 1602; 1−8 after nt 2225; 1−6 after nt 3037). The end of the arrow denotes the end of Tn*10*LUK containing the kanamycin resistance gene. Residues 486−508 of the *RPA1* ORF comprise a potential metal binding site. There are two possible polyadenylation signals (AATAA) in the 3′ flanking region of the *RPA1* reading frame.

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221  L F N V N F L D T S G E I R A T A F N D F A T K F N E I L Q E G K V Y Y V S K A K L Q P A K P Q
     : : . . . . . : : : : : : : : : : . . : :   . . . . : : : :   : : . . : .   : .  :
     L F S L E L V D E S G E I R A T A F N E Q V D K F F P L I E V N K V Y Y F S K G T L K I A N K Q

269  F T N L T H P Y E L N L D R D T V I E E C F D E S N V P K T H F N F I K L D A I Q N Q E V N S N
     : : . . .   : : . . . . . : .   : : .   . . : . . . : . : .   . : . . . : . .   . :
     F T A V K N D Y E M T F N N E T S V M P C E D D H H L P T V Q F D F T G I D D L E N K S K D S L

317  V D V L G I I Q T I N P H F E L T S R A G K K - F D R R D I T I V D D S G F S I S V G L W N Q Q
     : : . . : :   . .   . .   . . : : . . . .   . . : . : . . : . : : :   . . . . : : . . .
     V D I I G I C K S Y E D A T K I T V R S N N R E V A K R N I Y L M D T S G K V V T A T L W G E D

364  A L D F N L P E G S V A A I K G V R V T D F G G K S L S M G F S S T L I P N P E I P E A Y
     :   . : . .   . : : : : : . : : : : : . : : : .   : : : . : . : : . : : : : :
     A D K F D G S R Q P V L A I K G A R V S D F G G R S L S V L S S S T I I A N P D I P E A Y
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Fig. 3. Sequence comparison between *S.cerevisiae RPA1* and the large subunit of human RP-A. The *S.cerevisiae* sequence shown is the upper strand and only the homologous part of the protein is shown with numbering indicating the position of the sequence in Figure 2. Two dots between two residues indicate identity and one dot marks a conservative change as determined by the Pearson and Lipman rules (1988). The human sequence (lower strand) is derived from the translated ORF of a cloned DNA segment that has been synthesized using the polymerase chain reaction using the fully degenerate primers shown in Table II. The underlined residues were determined by analysis of tryptic peptides derived from the M_r 70 000 subunit of human RP-A.

insertion) conferred no apparent growth or lethal phenotype to the cell. This showed that the function encoded by this ORF is not essential.

RPA1 is essential for growth

To determine the phenotype of null mutations in the *RPA1* gene we isolated transposon Tn*10*LUK insertions in the cloned fragment. The insertion points were determined at the nucleotide level by DNA sequence analysis (see Figure 2 for the exact insertion points). We isolated many insertions and analyzed 10 insertions at seven positions in and around the *RPA1* gene. To determine the phenotype of the insertion mutations, each insertion mutation was integrated by homologous recombination into diploid *S.cerevisiae* strains, resulting in strains heterozygous for the insertion mutation at the *RPA1* locus. All diploids were sporulated and tetrads were dissected by micromanipulation. The germination pattern showed that *RPA1* is essential for spore germination, since all diploids with insertions in the *RPA1* ORF segregated 2:2 for viability (see Figure 4A for strain RKY1148). All viable spores lacked the Ura^+ marker carried by the transposon and were therefore Rpa^+. The terminal phenotype for the spores that could not form visible colonies was a multiply budded structure with 6–12 cells and buds that could not be separated by micromanipulation (Figure 5, C–F). Staining of these cells with the DNA-specific fluorescent probe 4′,6-diamidino 2-phenylindole (DAPI) revealed that they contained a single nucleus (Figure 6, C and D). A similar arrest phenotype is displayed by the cell division cycle mutant cdc4 at the restrictive temperature (Figure 6B), whereas at the permissive temperature no multiply budded cells were observed (Figure 6A). All insertion mutants showed this phenotype in a consistent manner (>90%). The untransformed wild type RKY1154 and a control diploid, RKY1182, that carried a heterozygous insertion (Tn*10*LUK1-8) 97 bp past the TAA stop codon of the *RPA1* reading frame (see Figure 2) showed perfect viability, 94% and 96% respectively. This showed that the disruption of the *RPA1* gene itself caused the haplolethal phenotype.

To extend this result, we constructed pRPA1-LEU2 which is an autonomously replicating centromere-containing plasmid carrying the *RPA1* gene as well as the *S.cerevisiae LEU2* gene and transformed heterozygous diploids carrying the insertion. Tetrad analysis after sporulation of the transformants demonstrated the occurrence of tetrads with

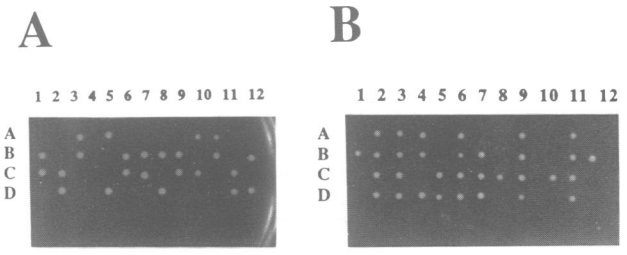

Fig. 4. Tetrad analysis of heterozygous diploids containing the *rpa1::Tn10LUK* disruption. **A**. Tetrad analysis of diploid RKY1148 carrying the heterozygous insertion Tn*10*LUK4-10 (see Figure 2). **B**. Tetrad analysis of a pRPA1-LEU2 transformed diploid (RKY1159) carrying the heterozygous insertion Tn*10*LUK2-8 (see Figure 2).

three and four viable spores (see Figure 4B for RKY1159). Not all tetrads are expected to have four viable spores since the two plasmid copies have to cosegregate with the two chromosomes carrying the *rpa1* insertion mutation to generate a fully viable tetrad. Tetrads with four viable spores were analyzed and every Ura^+ spore that formed a colony also contained the Leu^+ marker, and therefore contained the pRPA1-LEU2 plasmid. This shows that the plasmid borne copy of *RPA1* complemented the haplolethal phenotype of a *rpa1::Tn10LUK* mutation. These cells (*rpa1::Tn10LUK*/pRPA1-LEU2) had no apparent growth phenotype compared to wild type cells. Furthermore, the pRPA1-LEU2 plasmid was essential for growth in this genetic background (*rpa1::Tn10LUK*/pRPA1-LEU2), since Ura^+ Leu^- segregants were never detected during mitotic growth. Control transformants of wild type RKY1154 or RKY1182, which carry the insertion behind the *RPA1* gene, yielded segregants that could readily lose the plasmid (data not shown). Thus, *RPA1* is essential and continuously required for mitotic growth.

RPA1 is a previously unidentified function

The *RPA1* gene was genetically mapped in two steps. First, the gene was localized to chromosome I by Southern hybridization analysis of separated *S.cerevisiae* chromosomes (data not shown). Second, mapping was performed by tetrad analysis using the strain RKY1133 that carries the Ura^+ marker of the Tn*10*LUK closely linked (97 bp 3′ of the stop codon) to the *RPA1* gene. *RPA1* was mapped in a three-point cross (*LEU2–RPA1–ADE1*) using strains H330 and

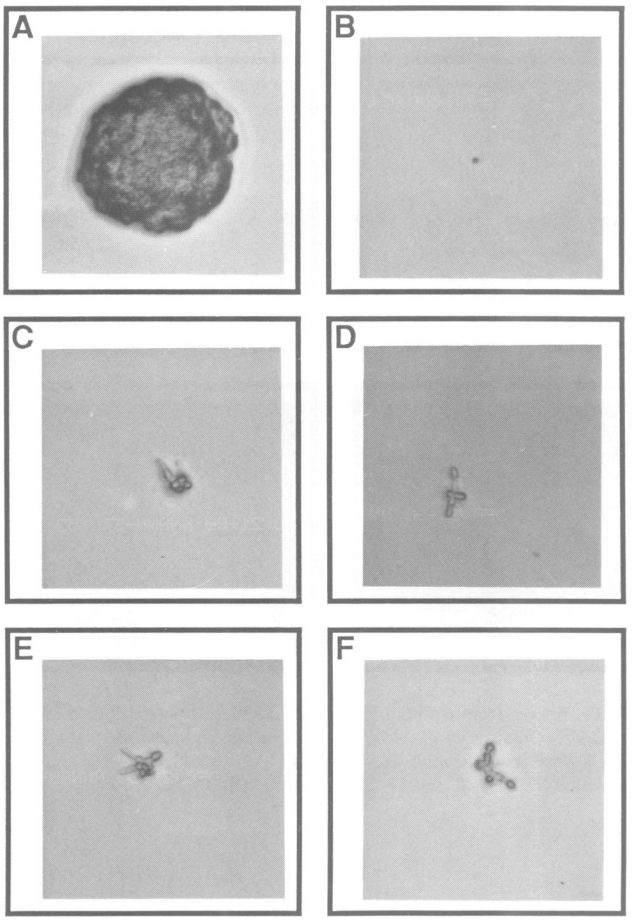

Fig. 5. Spores lacking *RPA1* form multiply budded cells. Tetrad analysis was performed and microphotographs were taken at 26× magnification after 24 h of incubation at 30°C. **A**. Wildtype spore that has formed a minicolony. **B**. Spore that failed to germinate. **C**–**F**. Examples of spores containing a disrupted copy of *RPA1* forming multiply budded cells.

RKY1133 to the region between centromere I and *ade1* on the right (small) arm of chromosome I (see Figure 7). This area of the *S.cerevisiae* genome is well characterized (Steensma *et al.*, 1987; Kaback *et al.*, 1989). By comparison with the published physical map (Steensma *et al.*, 1987) we believe that *RPA1* defines the uncharacterized transcript FUN3 and the ORF that runs into the cloned fragment is the origin for the transcript FUN16. The combination of our mapping data and the physical map of this area of chromosome I (Kaback *et al.*, 1989) unambiguously assigns *RPA1* between *ade1* and *CEN1*.

Discussion

Recently, we have identified a single-stranded DNA binding protein (SSB) from the yeast *S.cerevisiae* on the basis of its functional *in vitro* interaction with the cognate strand exchange protein (SEP1) (Heyer and Kolodner, 1989). Here we have shown that the gene, *RPA1*, encoding this protein has highly significant amino acid homology with the M_r 70 000 subunit of human RP-A, a cellular protein essential for SV40 *in vitro* DNA replication. Furthermore, we have demonstrated that mutations in the *S.cerevisiae RPA1* confer a recessive haplolethal phenotype and showed that this function is continuously required during mitotic growth.

Since the *RPA1* gene has a coding potential for a M_r 70 269 polypeptide, we believe that the SSB we have purified from *S.cerevisiae* is a proteolytic breakdown product of the primary translation product. The fragment that we have characterized had been identified by its *in vitro* function and clearly contains a DNA binding domain. In addition, the fragment was capable of specifically stimulating SEP1. Now, with the gene at hand, we can readdress the biochemical properties of the *RPA1* gene product using the purified full length protein.

We have shown that the *S.cerevisiae RPA1* gene codes for an essential function. The terminal phenotype of spores containing a disrupted copy of *RPA1* is consistent with a defect in DNA replication. These spores arrest as multiply budded cells resulting in a cluster of 6–12 cells and buds containing a single nucleus as shown by DAPI staining which suggests that they arrested prior to DNA synthesis (Pringle and Hartwell, 1981). This phenotype is reminiscent of the arrest phenotype of only one class of cell division cycle (cdc) mutants, *CDC4* (see Figure 6B) and *CDC34* (Hartwell, 1971; Hartwell *et al.*, 1973; for a review see Pringle and Hartwell, 1981), although *RPA1* is not allelic with either of these genes. *CDC4* and *CDC34* functions are required for the initiation of chromosomal DNA synthesis as well as for spindle pole body separation, although these events are mutually independent. The phenotype of arrest prior to the initiation of DNA synthesis is intriguing, in view of the essential role of RP-A in the very earliest steps of SV40 DNA replication *in vitro* (Fairman and Stillman, 1988; Wold and Kelly, 1988). This implies that RP-A may have similar roles in DNA replication *in vivo* to those it has *in vitro*. It will also be interesting to examine the function of RP-A in recombination *in vivo*. We are currently isolating conditional mutants in the *RPA1* gene in order to investigate more extensively the role of *RPA1* in DNA replication and recombination.

Database searches did not reveal any homologies between *RPA1* and any published gene or protein. In particular, we cannot find the similarity in the spacing of certain functional groups that has been revealed for some prokaryotic single-stranded DNA binding proteins including *E.coli* recA and SSB, T4gp32, and M13gp5 (Prashad and Chiu, 1987). The only other *S.cerevisiae* SSB studied in genetic detail is *SSB1* (Jong *et al.*, 1985, 1987; Jong and Campbell, 1986) which is believed to be involved in RNA metabolism and defines a non-essential function in *S.cerevisiae*. Our initial biochemical characterization of the M_r 34 000 SSB indicated a closer proximity of this protein to the archetypal SSBs like T4gp32, than SSB1. This difference is now accentuated by our finding that *RPA1* defines an essential function in *S.cerevisiae*, as should be expected for a functional equivalent of the prokaryotic SSBs.

S.cerevisiae RPA1 shows significant homology to the large subunit of human RP-A (>40% identity in amino acid sequence over 189 amino acids). The sequence information for human RP-A was obtained by sequencing a cloned segment of DNA synthesized using the polymerase chain reaction and from amino acid sequence information of two random trypsin fragments of the M_r 70 000 subunit of human RP-A. The striking homology observed suggests that it is unlikely to result from the identification of a functional domain that was conserved between two unrelated proteins. The degree of homology found between the *S.cerevisiae*

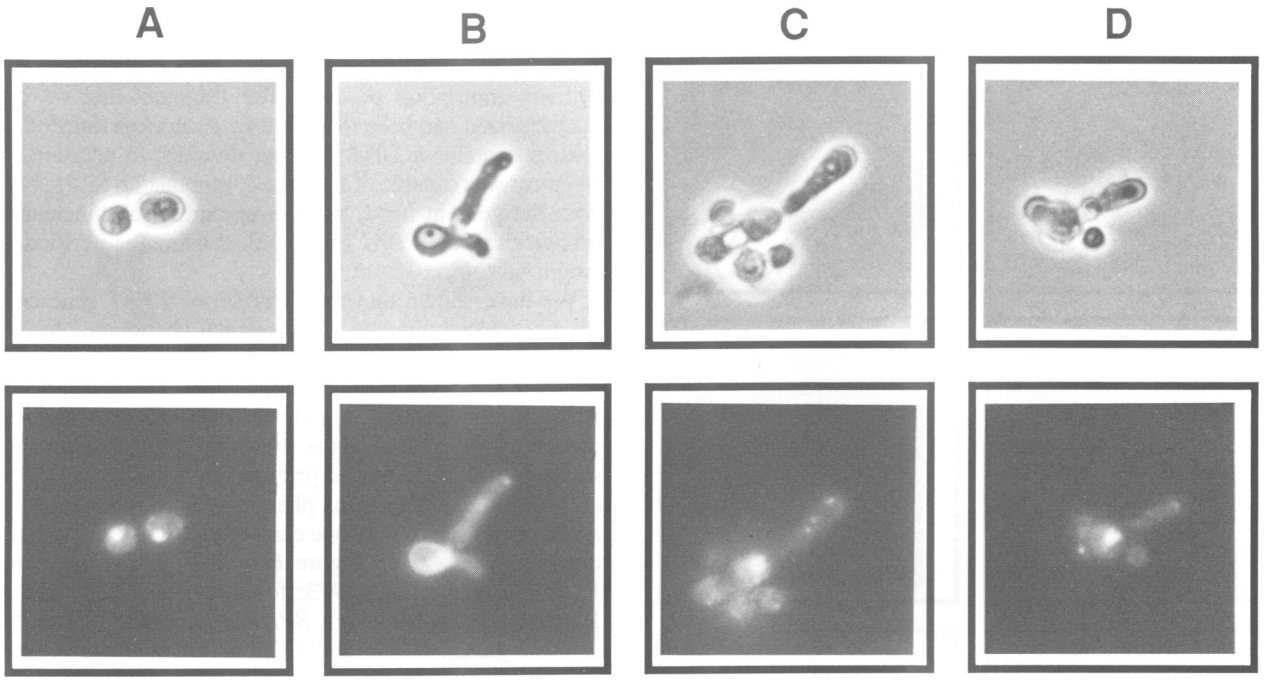

Fig. 6. Analysis of cells containing the disrupted *rpa1* gene by staining with DAPI. Spores from tetrads of RKY1159 were germinated for 48 h and stained with the DNA-specific fluorescent probe 4',6-diamidino 2-phenylindole (DAPI) (Johnson *et al.*, 1985). The upper panel shows the light microscopy pictures and the lower panel shows the fluorescence pictures taken at a 95× magnification. **A**. Strain 6433-4C (cdc4^{ts}) grown at the permissive temperature (22°C). **B**. Strain 6433-4C grown at the restrictive temperature (37°C). **C** and **D**. Examples of multiply budded cells containing the disrupted *rpa1* gene.

RPA1 and the large subunit of human RP-A is among the highest reported for proteins between these two organisms. Previously found examples for highly conserved proteins from yeasts and man include histones (Matsumoto and Yanagida, 1985), α- and β-tubulins (Toda *et al.*, 1984; Hiraoka *et al.*, 1984) and a cell cycle control protein, cdc2 (Lee and Nurse, 1987). The high degree of sequence homology between *RPA1* and the large subunit of human RP-A opens the intriguing possibility of the existence of a trimeric RP-A like complex in *S.cerevisiae*. In fact, Brill and Stillman (1989) recently reported the purification of an RP-A like protein from *S.cerevisiae* using the unwinding of the SV40 origin of replication as an assay. Their data indicated that the *S.cerevisiae* activity effectively substituted for human RP-A in the unwinding reaction, like other non-cognate SSBs can. However, the *S.cerevisiae* protein supported the SV40 *in vitro* replication reaction inefficiently. These observations combined with our work open up the possibility of using yeast genetics to help understand the functions of RP-A.

The structure of human RP-A and RP-A from other organisms suggests a complex role in cellular processes, one of which we believe is binding to single-stranded DNA. The DNA binding domain of RP-A has been assigned to the large subunit and we can further narrow one DNA binding domain to the region that is homologous to the M_r 34 000 polypeptide that we purified from *S.cerevisiae*. Furthermore, we have shown (W.-D. Heyer and L.Erdile, unpublished results) that human RP-A stimulated *E.coli* recA protein in the strand exchange reaction in the same way and to the same extent as other SSBs do. This provides additional evidence to identify RP-A as an SSB. However, human RP-A did not stimulate the strand exchange activity of *S.cerevisiae* SEP1 (W.-D.Heyer and L.Erdile, unpublished results) which is specifically stimulated by the *S.cerevisiae* M_r 34 000 polypeptide but not by *E.coli* SSB (Heyer and Kolodner, 1989). This might indicate species specificity similar to the observations by Brill and Stillman (1989) that the *S.cerevisiae* RP-A only inefficiently supported SV40 replication *in vitro*. Further biochemical analysis of *S.cerevisiae* RP-A will be required to determine its role in DNA replication and recombination.

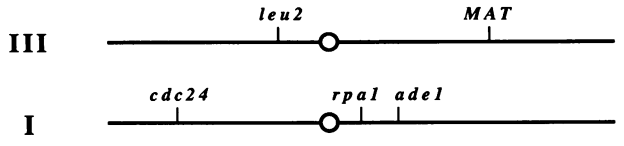

Fig. 7. Genetic map position of *RPA1*. The *RPA1* gene was mapped to the right arm of chromosome I as described in the text. The gene maps 5 cM centromere proximal from *ADE1* [49 parental ditypes [PD], 0 nonparental ditypes (NPD), and 7 tetratypes (T)] and between *CEN1* and ADE1 as measured against the centromere III linked marker *LEU2*. The distance *RPA1−CENI CENIII−LEU2* was 18.2 cM: 22 PD, 13 NPD, 20 T; and the interval *ADE1−CENI CENIII−LEU2* was measured as 21.8 cM: 18 PD, 13 NPD, 24 T. (Perkins 1949).

Materials and methods

Strains

The *S.cerevisiae* strains used in this study are derived from SK-1 (except H330, 6433-4C) and are listed in Table I. Standard genetic nomenclature is used: upper case letters denote a dominant allele; lower case letters indicate a recessive allele. *RPA1::Tn10LUKx−y* (where x indicates pool number and y the isolate number in the transposition protocol) denotes a transposon insertion outside the *RPA1* gene leaving its function intact, whereas *rpa1::Tn10LUKx−y* indicates an insertion in the *RPA1* gene which in all cases results in a recessive haplolethal phenotype. *E.coli* strain HB101 (Boyer and Roulland-Doussoix, 1969) was the host for the YCp50 library and

Table I. *Saccharomyces cerevisiae* strains

Strain	Genotype
NKY278	*a/α ho::LYS2/ho::LYS2 ura3/ura3 lys2/lys2 leu2::hisG/leu2::hisG*
H330	*α ura3-1 ade 1 can1 lys2-c met13-2 cyh2 trp5-1 leu2-k*
6433-4C	*a cdc4 leu2-3*
RKY1103	*a/α ho::hisG/ho::LYS2 lys2/lys2 ura3/ura3 LEU2/leu2::hisG*
RKY1123	as NKY278 but *RPA1/RPA1::Tn10LUK1-8*
RKY1133	*a ho::LYS2 lys2 ura3 RPA1::Tn10LUK1-8*
RKY1148	*a/α ho::hisG/ho::LYS2 ura3/ura3 lys2/lys2 LEU2/leu2::hisG RPA1/rpa1::Tn10LUK4-10*
RKY1154	*a/α ho::hisG/ho::hisG lys2/lys2 ura3/ura3 leu2::hisG/leu2::hisG*
RKY1156	as RKY1154 but *RPA1/rpa1::Tn10LUK3-5*
RKY1157	as RKY1154 but *RPA1/rpa1::Tn10LUK1-7*
RKY1159	as RKY1154 but *RPA1/rpa1::Tn10LUK2-8*
RKY1161	as RKY1154 but *RPA1/rpa1::Tn10LUK1-2*
RKY1181	as RKY1154 but *RPA1/RPA1::Tn10LUK1-6*
RKY1182	as RKY1154 but *RPA1/RPA1::Tn10LUK1-8*

pRPA1; RK1400 (Symington *et al.*, 1983) was used as host for all other plasmids. *E.coli* and phage lambda strains for the Tn10LUK mutagenesis were described in Huisman *et al.* (1987).

Media

Rich (YPD), minimal (SD), and synthetic complete (SC) media were prepared as described (Sherman *et al.*, 1982). YPAc is 1% potassium acetate, 2% peptone, 2% yeast extract, and SPM is 3% potassium acetate and 0.02% raffinose. For selection of Ura^+ *S.cerevisiae* transformants SC medium lacking uracil was used. For scoring nutritional requirements SC medium lacking the appropriate requirements was used. To confirm the genotype of strains, the absence of other requirements was verified by scoring on SD medium supplemented with the appropriate requirements.

Genetic methods

Mating, sporulation, and tetrad analysis were performed essentially as described (Sherman *et al.*, 1982). Diploid cells were sporulated in liquid SPM after growth in YPAc to a titer of $\sim 2 \times 10^7$ cells/ml. *S.cerevisiae* cells were transformed by the lithium acetate method (Ito *et al.*, 1983) and *E.coli* cells were transformed as described (Wensink *et al.*, 1974). Transposon Tn*10*LUK insertion mutagenesis was performed exactly as described (Huisman *et al.*, 1987). All *S.cerevisiae* insertion mutations were recombined into diploid strains to replace wild type sequences using the 9.2 kb *Sal*I restriction fragment of the respective pRPA1::Tn10LUK derivative by transforming with the entire restriction digest and selecting for Ura^+ transformants. All constructs were verified in *S.cerevisiae* by genomic restriction analysis using several restriction endonucleases.

Plasmids

Plasmids were constructed by standard procedures. Restriction fragments were purified from agarose gels using the glass elution method (Vogelstein and Gillespie, 1979). Restriction endonucleases were purchased from New England Biolabs and used according to the recommendations of the manufacturer. T4 DNA ligase was purified in this laboratory using an unpublished procedure of R.D.Kolodner. Small scale plasmid preparations were performed using the boiling method of Holmes and Quigley (1981), large scale plasmid preparations were performed by standard alkali lysis with subsequent purification of form I plasmid DNA on CsCl−ethidium bromide density gradients.

pRPA1 contains a 3.4 kb *Sau*3A insert from chromosome I of *S.cerevisiae* in the *Bam*HI site of YCp50 and was recovered from the library constructed by Rose *et al.* (1987). pRPA1::Tn10LUKx−y (where x denotes the pool number and y the isolate number in the insertion mutagenesis scheme) have transposon Tn*10*LUK inserted at various positions in the *S.cerevisiae* insert (see Figure 2 for the precise insertion points and orientation). pRPA1-LEU2 was constructed by replacing the 0.65 kb *Bam*HI−*Sal*I fragment of pRPA1 with the *Bam*HI−*Sal*I fragment of pLEU2 (Heyer *et al.*, 1986) containing the *S.cerevisiae LEU2* gene. The *Bam*HI site that was used in pRPA1 had been fortuitously created in the joining of the *Sau*3A site of the *S.cerevisiae* insert to the *Bam*HI site of YCp50. This *Bam*HI site does not correspond to a site in the *S.cerevisiae* genome. pJH227 contains the *S.cerevisiae HIS4* gene as a *Pst*I fragment (Donahue *et al.*, 1982) and pWO19 contains the *S.cerevisiae CYH2* gene as a *Bam*HI−*Hin*dIII fragment (Käufer *et al.*, 1983).

Cloning of the RPA1 gene

A homogeneous preparation of the M_r 34 000 SSB protein was purified as described (Heyer and Kolodner, 1989). The NH_2 terminus of the M_r 34 000 protein and of various proteolytic fragments were subjected to amino acid sequence analysis on an Applied Biosystems 470A protein sequencer. Proteolysis was performed in storage buffer [20 mM Tris−HCl pH 7.5, 0.1 mM EDTA, 10 mM β-mercaptoethanol, 60% (w/v) glycerol, 0.1 mM PMSF] with 3 μg SSB and 150 ng of protease per digest at 37°C for an optimized amount of time [*S.aureus* Endo Glu-C (V8) 2 h, trypsin 30 min, chymotrypsin 1 h]. Proteolytic products were separated on a 15% SDS−polyacrylamide gel (Laemmli, 1970), transferred to PVDF membrane and sequenced directly off the membrane as described (Matsudaira, 1987). Fully degenerate oligonucleotides were designed from amino acid sequence of several proteolytic fragments (see Table II).

Oligonucleotides were synthesized on an Applied Biosystems 380A DNA synthesizer using phosphoamidite chemistry and purified as described (Evans and Kolodner, 1987). The hybridization characteristics of the oligonucleotides were evaluated in hybridizations to chromosomal DNA and oligonucleotide No. 798 was chosen for the initial screen. Oligonucleotide No. 798 was kinased to 3.6×10^6 c.p.m./pmol using the protocol of Richet *et al.* (1986) and ~7500 colonies of the YCp50 library were probed using the protocol of Wood *et al.* (1985) employing the salt tetramethylammonium chloride for base composition independent hybridization. The initially positive clones were single-colony purified using the above probe and reprobed with oligonucleotides nos 1133, 1134, 1135, resulting in a single plasmid clone (pRPA1) with the expected hybridization pattern (positive for 798, 1133, 1134; negative for 1135).

DNA sequence analysis

DNA sequencing was performed on CsCl purified plasmid DNA using a Sequenase (TM) kit from US Biochemicals. The entire insert of pRPA1 was sequenced on both strands using appropriate oligonucleotide primers. Tn10LUK insertion points were determined by using the oligonucleotide 5′ CAAGATGTGTATCCACC-3′ that hybridizes to the *lacZ* end of Tn*10*LUK pointing outwards. The DNA sequence was analyzed using the Pearson−Lipman algorithm (Pearson and Lipman, 1988) available on the Intelligenetics program package for homology searches against the protein sequence data bases PIR (release No. 19.0) and SWISS (release No. 10.0) (using ktup values of 1 and 2) and against the Genbank (release No. 59.0) and EMBL (release No 18.0) DNA sequence libraries (ktup value of 4).

Southern hybridization analysis

Southern hybridization was performed by transferring the DNA from agarose gels to Genescreen membrane (TM; Dupont) in 25 mM Na_2HPO_4/NaH_2PO_4 pH 6.5 buffer and crosslinking the DNA to the membrane by irradiation with UV light (Church and Gilbert, 1984). Hybridization was done as described (Heyer *et al.*, 1986) with the purified *Bam*HI−*Mlu*I fragment of pRPA1 which was made radioactive by the method of Feinberg and Vogelstein (1983). Washes were done for 30 min with a solution containing 2 × SSC and 1% SDS at 65°C with constant agitation.

RPA1 was mapped to chromosome I by hybridization with the purified *Cla*I−*Nru*I fragment of pRPA1 which was made radioactive by the method of Feinberg and Vogelstein (1983). The filters used contained *S.cerevisiae* chromosomes from strain AB972 separated by orthogonal field alternating gel electrophoresis (OFAGE) (Carle and Olson, 1985).

Northern hybridization analysis

RNA was prepared from strain GRF167, subjected to electrophoresis and transferred to a Genescreen membrane as described (Osley and Lycan, 1987). Hybridization was performed as described above using equimolar (to available homologous RNA) radioactive DNA probes of the same specific activity from pRPA1 (*Bam*HI−*Mlu*I fragment), and pJH227 and pWO19 as size standards allowing a rough estimate of the relative steady state concentration of *RPA1* mRNA.

Purification and peptide sequence analysis of human RP-A

Human RP-A was purified as described (Wold and Kelly, 1988), except that the 1.3 M KSCN wash from the Affi-Gel Blue column was concentrated and desalted by passing it over a small (0.2 ml) hydroxyapatite column and eluting the protein with buffer F containing 70 mM potassium phosphate (Wold and Kelly, 1988). To obtain the sequence of the M_r 70 000 subunit, ~1 nmol (150 μg) of purified human RP-A was reduced in 100 mM Tris (pH 8.0), 1% SDS, 20 mM dithiothreitol for 60 min at 60°C. After cooling

Table II. Amino acid sequence of individual NH_2 termini from proteolytic fragments

Fragment	amino acid sequences	Oligonucleotide	
S.cerevisiae			
chy 1	?NQ?GDGKLFNVNFLDT?(F,G)E		
	position 213–232		
	FNVNF	1133	TT T/C AA T/C GTNAA T/C TT
	DGKLFN	1134	GA C/T GGNAA A/G CTNTT T/C AA
	DGKLFN	1135	GA C/T GGNAA A/G TTNTT T/C AA
chy 2	?LNLD(Y)DTVI		
	position 277–286		
try 1	??PIF?IEQ?(L)P		
	position 181–192		
V8 1	??FANENPN?(Q)?	798	TT T/C GCNAA T/C GA G/A AA T/C CC
	position 169–179		
V8 3	?(G,E)FANENPN?(Q)(Y)(T,F)	798	TT T/C GCNAA T/C GA G/A AA T/C CC
	position 169–181		
native	(N,T)(T,E,H,L)(N,R)(T,A,L,K)(N,F)(E,A)N(E,K)(N,F)(P,A)N(E,A)(N,Q)(P,E)N(S)(Q,P)IFA		
Human			
try 1	LFSLELVDESGEI	TK56	tcctgcagGA T/C GA G/A T/A C/G NGGNGA G/A
try 2	LSVLSSSTIIANPDIPEAY		
	DIPEAY (complement)	TK55	ctaagcttTA NGC T/C TCNGG A/G/T AT A/G TC
	TIIANP (complement)	TK64	GG A/G TTNGC A/G/T AT A/G/T AT NGT

The upper part displays sequences from the *S.cerevisiae* protein and the lower part from the large subunit of human RP-A. ? denotes a cycle without a clear signal, residues in parenthesis were weak or ambiguous signals. Fragment names give protease used (chy, chymotrypsin; try, trypsin, V8, *Staphylococcus aureus* Endo Glu-C) and number of the fragment. Position of the *S.cerevisiae* sequences corresponds to the translated ORF as numbered in Figure 2. The multiple signals per sequencing cycle obtained with the native *S.cerevisiae* protein are also shown in the table. The underlined amino acid sequences were used to design degenerate oligonucleotides. The difference between Nos. 1134 and 1135 is in the central leucine codon (CTN for 1134, TTN for 1135) to reduce the amount of degeneracy. The amino acid sequence from *S.cerevisiae* fragments chy2 and try1 did not allow the design of a promising probe. Oligonucleotides TK55 and TK56 were used for the polymerase chain reaction and carry linkers (lower case letters) which were attached to the fully degenerate sequence to facilitate cloning.

to room temperature, cysteine residues were carboxymethylated by the addition of 1/20 volume of 0.44 M iodoacetamide (freshly made) with incubation at room temperature in the dark for 30 min. Then 9 volumes of −20°C ethanol was added and the protein was allowed to precipitate overnight at −20°C. The protein was collected by centrifugation and then was subjected to SDS−PAGE in a Mini Protean II (Hoefer Scientific) using a 10% running gel and a 5% stacking gel (Laemmli, 1970). The protein was then transferred to nitrocellulose and portions of the filter containing each separated subunit were excised and individually blocked with polyvinylpyrrolidone (Aebersold *et al.*, 1987). Trypsin (3.75 μg for the M_r 70 000 subunit) freshly made up in Tris (100 mM, pH 8.2), 5% acetonitrile was added and digestion was allowed to proceed overnight at 37°C. The supernatant was frozen in liquid N_2 and stored at −70°C. The supernatant was loaded on a Brownlee 210 × 2.1 mm C_4 column and eluted with a gradient from 0% to 78.4% acetonitrile in 0.06% trifluoroacetate using a Hewlett-Packard 1090 HPLC. Well resolved peaks were directly sequenced in an Applied Biosystems 470 gas phase sequencer.

Cloning of human RP-A sequences

A λgt11 library containing HeLa cDNA inserts was obtained from Stratagene (a kind gift of Dr H.Ratrie, Johns Hopkins Medical School). This was the same library from which a full-length cDNA encoding the M_r 32 000 subunit of RP-A was obtained (Erdile *et al.*, 1990). DNA was prepared from the library by digesting the bacteriophage in 0.5% SDS with proteinase K, extracting with phenol and chloroform, and then precipitating the DNA with ethanol and resuspending in 10 mM Tris−HCl pH 8, 1 mM EDTA. The polymerase chain reaction (Saiki *et al.*, 1988) was performed in a Perkin Elmer Cetus DNA Thermal Cycler using ~5 ng of λgt11 library DNA and 1 μg of each of the oligonucleotide primers TK55 and TK56 (Table II). Thirty cycles of amplification were carried out with the following parameters: 30 s denaturation at 94°C, 1 min annealing at 55°C, and 1 min extension at 72°C. The products were electrophoresed and transferred to nitrocellulose. The membrane was probed with oligonucleotide TK64 (Table II), labeled at the 5′ end with T4 polynucleotide kinase. Hybridization and washing were performed by the method of Wood *et al.* (1985), with a final wash at 52°C. A single band of ~550 bp was recognized by TK64. This band was gel-purified by electrophoresis onto NA 45 paper (Schleicher and Schuell) and elution into 1 M NaCl, 50 mM arginine, 10 mM Tris−HCl pH 8, followed by extraction with phenol:$CHCl_3$ 1:1 and ethanol precipitation. The gel-purified fragment was subjected to an additional 30 rounds of PCR as described above, using the oligonucleotides TK55 and TK56. The product of this reaction was digested with *Hin*dIII and *Pst*I and cloned into the polylinker of the vector pBluescript KS+ (Stratagene), which had been digested with *Hin*dIII and *Pst*I. The insert was sequenced on both strands using primers homologous to the pBluescript polylinker.

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Note added in proof

Brill and Stillman (1989) have described an *S.cerevisiae* protein that has similar subunit structure and biochemical properties to human RP-A. We have compared the sequence of the *yRPA1* gene reported here with one region of protein sequence obtained by Brill and Stillman from sequencing proteolytic fragments of the 70 kd subunit of their protein (unpublished data of Brill and Stillman). There was 100% identity between this protein sequence and a region of the translated *yRPA1* gene indicating that this gene most likely encodes the 70 kd subunit described by Brill and Stillman (1989).

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