Replication factor-A (RF-A) is a three-subunit protein complex originally purified from human cells as an essential component for SV40 DNA replication in vitro. We have previously identified a functionally homologous three-subunit protein complex from the yeast Saccharomyces cerevisiae. Here we report the cloning and characterization of the genes encoding RF-A from S. cerevisiae. Each of the three subunits is encoded by a single essential gene. Cells carrying null mutations in any of the three genes arrest as budded and multiply budded cells. All three genes are expressed in a cell-cycle-dependent manner; the mRNA for each subunit peaks at the G1/S-phase boundary. A comparison of protein sequences indicates that the human p34 subunit is 29% identical to the corresponding RFA2 gene product. However, expression of the human protein fails to rescue the rfa2::TRP1 disruption.

[Key Words: DNA replication; replication factor-A; S. cerevisiae; single-stranded DNA-binding protein]

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The study of eukaryotic DNA replication has relied heavily on the use of biochemistry to identify factors involved in the replication of eukaryotic chromosomes. For example, studies on the replication of SV40 DNA in vitro have been particularly useful in identifying human cell proteins required for viral DNA replication. However, the issue of whether these proteins function to replicate the chromosomal DNA of the cell cannot be addressed by using biochemistry alone; therefore, we have turned to the yeast Saccharomyces cerevisiae. The yeast system provides the genetics to address this question, as well as the only known chromosomal origins of DNA replication for future biochemical studies.

The SV40 system is based on the observation that plasmids containing the SV40 origin replicate in the presence of a protein extract from human cells and the viral initiator protein large T antigen (T-Ag) (Li and Kelly 1984, 1985; Stillman and Gluzman 1985; Wobbe et al. 1985). Human replication factors have been identified by biochemical fractionation of the cellular extract (Challberg and Kelly 1989; Stillman 1989; Hurwitz et al. 1990), and many aspects of the replication process can now be reconstituted by using purified proteins (Matsumoto et al. 1990; Tsurimoto et al. 1990; Weinberg et al. 1990). Because T-Ag performs multiple initiation functions, including origin-specific DNA-binding and DNA helicase activities, most of the human components are involved in the elongation stage of DNA replication with DNA polymerase α (Polα), DNA polymerase δ (Polδ), proliferating cell nuclear antigen (PCNA), replication factor-A (RF-A), replication factor-C (RF-C), and DNA topoisomerases I and II (Murakami et al. 1986; Prlich et al. 1987; Yang et al. 1987; Lee et al. 1989; Tsurimoto and Stillman 1989a; Weinberg and Kelly 1989; Melendy and Stillman 1991).

Human RF-A (also called RP-A and human SSB) is a single-stranded DNA (ssDNA)-binding protein complex composed of 70-, 34-, and 11-kD subunits (Wobbe et al. 1987; Fairman and Stillman 1988; Wold and Kelly 1988). RF-A is unique among the cellular proteins in that it has been shown to cooperate with T-Ag prior to the onset of DNA synthesis. Kinetically, preincubation of the template DNA with T-Ag, DNA topoisomerase I, and RF-A overcomes the lag in synthesis that is normally required for the establishment of a prepriming complex. The result of this preincubation is an extensively unwound DNA template (form U; Dean et al. 1987; Wold et al. 1987; Bullock et al. 1989; Tsurimoto et al. 1989) that can be primed by Polα. Subsequent elongation and switching of the polymerase complex to separate leading (Polδ) and lagging (Polβ) strand polymerases represents the elongation stage of DNA replication (Tsurimoto et al. 1990; Tsurimoto and Stillman 1991).

A role for RF-A in elongation has been inferred from its stimulatory effect on DNA polymerase activity with simple primer-template assays. At low levels of polymerase, RF-A stimulates Polα activity four- to sixfold (Kenny et al. 1989; Tsurimoto and Stillman 1989b). In the presence of RF-C and PCNA, RF-A stimulates Polδ activity 10-fold (Kenny et al. 1989; Tsurimoto and Stillman 1989b). Escherichia coli single-stranded DNA-binding protein (SSB) is unable to substitute for RF-A in some of these experiments, suggesting that the DNA polymerase
stabilization is due to specific protein–protein interactions and not due simply to the effect of RF-A on the secondary structure of the template DNA. Indeed, monoclonal antibodies directed against individual subunits of RF-A inhibit these stimulatory effects (Kenny et al. 1990). RF-A also functions to inhibit reinitiation by Polα on existing primers. As a result, Polα synthesizes 300- to 400-bp products typical of lagging strand products (Tsurimoto et al. 1990).

RF-A was identified in yeast extracts as a three-subunit protein complex consisting of 69-, 36-, and 13-kD subunits that cooperated with T-Ag and DNA topoisomerase I to unwind template DNA containing the SV40 origin of DNA replication. The yeast protein does not cross-react with antibodies raised against human RF-A, and it does not substitute for the human protein in the complete SV40 DNA replication reaction [Brill and Stillman 1989]. Like human RF-A, however, the yeast protein possesses a potent ssDNA-binding activity that is localized to the largest subunit [Brill and Stillman 1989; Wold et al. 1989; Kenny et al. 1990]. In addition, the 36/34-kD subunit of RF-A from both species is phosphorylated in a cell-cycle-dependent manner [Din et al. 1990]. The phosphorylated form of this protein appears in S phase and persists until mitosis, at which time the protein becomes dephosphorylated. Phosphorylation of RF-A may function to regulate DNA replication, origin unwinding experiments performed with T-Ag and crude human cell extracts indicate that G1 extracts have a lower specific unwinding activity than do extracts prepared from cells in the S or G2 phases of the cell cycle [Roberts and D'Urso 1988]. G1 extracts can be activated by a fraction, RF-S, that contains a kinase homologous to the p34cdc2 kinase [D'Urso et al. 1990]. These results suggest a regulatory role for RF-A, potentially linking DNA replication to cell-cycle control mechanisms.

Although conclusive proof for a role for RF-A in chromosomal DNA replication is lacking, it is thought that RF-A may be the eukaryotic counterpart to such well-characterized SSBs as Escherichia coli SSB or T4 gene 32 protein. As a step toward obtaining genetic confirmation of the role of RF-A in chromosomal DNA replication, as well as its role in cell-cycle control, we have isolated and characterized the genes encoding RF-A from S. cerevisiae. All three subunits of RF-A, including the 36- and 13-kD subunits, which apparently do not bind DNA, are required for viability. Cells lacking any of the three genes arrest as budded and multiply budded cells. mRNA for all three genes is expressed in a cell-cycle-dependent manner similar to genes known to be involved in DNA synthesis in S. cerevisiae. Finally, the RFA2 gene is unusual in that it contains an intron.

**Results**

**Cloning the genes encoding RF-A**

Tryptic peptides from each of the individual subunits of RF-A from S. cerevisiae were used to obtain internal amino acid sequence. Peptide sequences were used to design degenerate oligonucleotides as probes to obtain the genomic clones encoding each of the RFA subunits (see Materials and methods). Figure 1 shows the genomic organization of the genes RFA1, RFA2, and RFA3 encoding the 69-, 36-, and 13-kD subunits, respectively. Protein-coding regions are boxed, and the direction of transcription is indicated by arrows. The chromosomal location of each of the RFA genes was obtained by physical mapping and is indicated in Figure 1. The position of the RFA genes with respect to known genetic markers was made possible by aligning the physical and genetic maps: RFA1 maps to the CEN1- ADE1 interval on the right arm of chromosome I, RFA2 maps distal to RAD50 on the left arm of chromosome XIV, and RFA3 maps distal to SRA3 on the left arm of chromosome X. There are no markers in these regions of the current genetic map that are likely to represent alleles of any of the RFA genes.

**Sequences of the RFA genes**

Nucleotide sequences were obtained for each of the genes encoding the subunits of RF-A. These DNA sequences are shown in Figure 2, along with the predicted translation products. Each translation product contains amino acid sequences (underlined) that are identical to those obtained by direct amino acid sequencing.

![Figure 1](genesdev.cshlp.org)
Figure 2. Nucleotide sequence of the RFA1, RFA2, and RFA3 genes and flanking DNA. The predicted amino acid sequence is shown in the single-letter code. Underlined peptides correspond to sequences obtained from direct amino acid sequencing of tryptic peptides. Bold nucleotides are used to represent consensus splice site sequences, and bold underlined nucleotides represent the oligonucleotide primer sequences used to isolate the genes and flanking DNA. The predicted amino acid sequence is shown in the single-letter code. Underlined peptides correspond to sequences obtained from direct amino acid sequencing of tryptic peptides. Bold nucleotides are used to represent consensus splice site sequences, and bold underlined nucleotides represent the oligonucleotide primer sequences used to isolate the genes and flanking DNA.
**RFA1** contains a single open reading frame (ORF) encoding 622 amino acids. The predicted protein has a molecular mass of 70.4 kD and is very rich in asparagine (N) and charged residues. The DNA sequence of **RFA1** is identical to that of a previously identified gene, **RFA1**. Only the restriction map upstream of the gene differs from that obtained by Heyer et al. (1990). As originally noted, this gene encodes a zinc finger motif [amino acids 486–508] that may mediate its DNA-binding activity.

In keeping with the original identification of this protein in yeast, we refer to the genes encoding the subunits of RF-A as **RFA** genes. The use of this nomenclature, rather than **RPA**, has the advantage of avoiding confusion with the established gene symbols for the subunits of RNA polymerase I and acidic ribosomal proteins (Riva et al. 1986; Memet et al. 1988; Mitsui and Tsurugi 1988).

**RFA2** contains an intron with consensus splice-site signals ([Fig. 2, bold nucleotides]). To confirm that the message encoded by **RFA2** was spliced, a cDNA product was obtained by reverse transcriptase chain reaction (RT). Total RNA was first reverse-transcribed with random hexamer primers and reverse transcriptase, and then subjected to PCR with oligonucleotide primers flanking the putative intron [bold, underlined nucleotides in Fig. 2]. Polyacrylamide gel electrophoresis of the reaction products revealed two specific bands at 291 and 183 bp (Fig. 3A). The 291-bp band is predicted from the genomic sequence of **RFA2**. It was probably due to genomic DNA present in the RNA preparation, because it was obtained even in the absence of reverse transcriptase [Fig. 3A, lane 4]. The reverse transcriptase-dependent band of 183 bp was the size expected if it was derived from the spliced message. The sequence of this fragment, shown in Figure 3B, confirmed the exon/intron boundaries suggested by the splice-site consensus sequences.

The predicted **RFA2** protein is 273 amino acids with a molecular mass of 29.9 kD. The protein is rich in asparagine and acidic residues [calculated pI 4.6]. **RFA2** has no significant similarity to any protein in the current sequence data bases except for its human homolog (see below). The **RFA3** gene contains a single ORF encoding 121 amino acids. The predicted protein has a molecular mass of 13.8 kD and, like **RFA2**, is rich in asparagine and acidic residues [calculated pI 4.5]. **RFA3** has no significant similarity to any protein in the current sequence data bases.

Conclusive proof that these genes encode the subunits of RF-A was provided by immunoblotting bacterially produced proteins with antibodies against yeast RF-A. The gene for each subunit was expressed in E. coli with the T7 expression system [Studier et al. 1990]. Total cellular extracts from bacteria carrying recombinant plasmids or the T7 vector alone were subjected to SDS-PAGE and immunoblotted with affinity-purified antibodies raised against the individual subunits of RF-A from S. cerevisiae. Purified RF-A was included as a control. The 50-kD band recognized by the o69K antibody is a prominent breakdown product of the 69-kD subunit.

**Figure 3.** The **RFA2** gene contains an intron. Total yeast RNA was reverse transcribed with random primers and subjected to PCR. (A) PCR reaction products following polyacrylamide gel electrophoresis. [OLIGO] Oligonucleotides used as primers in the PCR reaction, shown as bold underlined nucleotides in Fig. 2; (I) the 21-bp amino-terminal primer, (2) the 24-bp carboxy-terminal primer. (RT) Reverse transcriptase, (M) pBR322 HpaII digest. (B) Sequence and translation product of the 183-bp band following XbaI digestion. Underlined bases indicate the XbaI recognition sequence.

**Figure 4.** **RFA1**, **RFA2**, and **RFA3** genes encode the subunits of RF-A. Total bacterial cell extract from induced cells carrying either the expression vector alone or a recombinant plasmid containing the indicated gene was separated by 17% SDS-PAGE and transferred to nitrocellulose. Strips were separated and probed with the respective affinity-purified rabbit antibody raised against the individual subunits of RF-A from S. cerevisiae. Purified RF-A was included as a control. The 50-kD band recognized by the o69K antibody is a prominent breakdown product of the 69-kD subunit.
authentic RF-A subunits. The protein encoded by RFA1 appears as two strong bands, perhaps due to internal translation start sites or to proteolytic degradation (Fig. 4, second lane). The identification of the RFA genes by a method (immunoblotting) that is independent of the method used to clone them [protein sequencing] proves their identity.

**RFA genes are essential**

To determine whether the RF-A protein performed an essential function, most of the coding region for each of the RFA1, RFA2, and RFA3 genes was replaced by the TRP1 gene, as shown in Figure 1. These three chromosomal deletion alleles were used individually to translocate the corresponding wild-type genes in a diploid yeast strain. Tetrad dissection of each of the three heterozygous diploids yielded no more than two viable spores, and all viable haploid spores exhibited a Trp- phenotype (13, 11, and 15 tetrads, respectively, for rfa1::TRP1, rfa2::TRP1, and rfa3::TRP1; data not shown). Lethality was caused by the disruption of the RFA genes because the same diploid strains gave three or four viable spores when they carried the respective gene on single or multicopy plasmids prior to sporulation (data not shown). In these cases Trp+ haploids were obtained that were always prototrophic for the plasmid marker. The rescuing DNA fragments are those shown in Figure 2, suggesting that all essential coding and promoter elements are contained on these fragments. Haploid strains carrying the disrupted RFA2 gene were rescued by a “cDNA” version of the gene in which the 279-bp XbaI genomic fragment was replaced by the 171-bp XbaI cDNA fragment obtained by PCR. These haploids had no observable phenotype, suggesting that the intron of RFA2 was not part of an important regulatory mechanism.

Inspection of the terminal phenotype by light microscopy revealed that all germinated spores, regardless of the specific gene disrupted, arrested as budded and multiply budded cells (Fig. 5). Often the dead cells were larger than viable siblings; they appeared swollen and occasionally contained buds on buds. Dissection of individual microcolonies from each disruption revealed approximately equal numbers of budded and aberrantly budded cells [data not shown]. Figure 5 indicates, however, that the number of divisions that each germinated spore was capable of undergoing differed, depending on which gene was disrupted. Loss of RFA1 resulted in the most severe arrest, usually at the 2- to 4-cell stage. Loss of RFA2 resulted in arrest at the 8- to 16-cell stage, whereas the loss of RFA3 resulted in arrest at the 16- to 32-cell stage. The simplest interpretation of these results is that the RF-A complex is essential for viability and that the individual subunits of the complex are either not present in equal amounts or they turn over at different rates.

**Regulation of RFA gene expression**

In *S. cerevisiae* most, if not all, of the genes encoding enzymes involved in DNA synthesis are expressed in a cell-cycle-dependent manner, with mRNA levels peaking at S phase. This regulation has been shown to occur at the level of transcription and to be mediated by promoter elements containing the restriction enzyme-recognition sequence for MluI (Pizzagalli et al. 1988; Lowndes et al. 1991). MluI recognizes a 6-bp sequence containing 4 G/C residues and, consequently, rarely cleaves in the A/T-rich DNA of yeast. We observed that this sequence occurs twice in each promoter for RFA1 and RFA2 (Fig. 2, boxed nucleotides). Although the cleavage site is not found in the RFA3 gene, a degenerate form of the sequence is found in the RFA3 promoter.

![Figure 5. Terminal phenotype of the rfa1::TRP1, rfa2::TRP1, and rfa3::TRP1 gene disruptions. Microcolonies from tetrads exhibiting 2:0 segregation were photographed 48 hr after dissection using DIC optics. Magnification, 400x.](https://genesdev.cshlp.org/)
[boxed in Fig. 2]. Again the sequence appears to be duplicated and in each case is a 5 out of 6 match to the authentic ACGCGT.

To determine whether the RFA genes were expressed in a cell-cycle-dependent manner, yeast cells were synchronized by release from α-factor block and samples were harvested at 15-min intervals for Northern analysis. Inspection of the budding pattern [Fig. 6A] indicates that synchrony was maintained for at least two generations. Northern blot analysis shows fluctuations in the abundance of all three RFA messages. The message for each gene peaks at 30, 90, and 150 min after release [Fig. 6B]. Each of these time points corresponds to the appearance of small budded cells. As bud emergence is taken to be a landmark for the onset of DNA replication, the expression of the three RFA genes is coordinate and appears to occur in late G1 and S phase. This expression pattern is consistent with a complex involved in DNA replication.

**Similarity to human RF-A**

The cDNA encoding the 34-kD subunit of human RF-A (RP-A) has been cloned and sequenced (Erdile et al. 1990). A comparison of the human gene product to that of the yeast RFA2 gene is shown in Figure 7. The proteins are approximately the same size (the yeast protein is only 3 amino acids larger), however, gaps have been introduced to maximize the alignment. Overall, the proteins are 29% identical and 44% similar, allowing for conservative amino acid substitutions. The similarity extends equally throughout the length of the protein except for one segment near the center (bold in Fig. 7). This segment, which comprises 39 residues uninterrupted by gaps, is 49% identical and 59% similar allowing for conservative substitutions. This region may identify a functionally important domain of the protein, perhaps mediating interactions with the other subunits of RF-A.

Attempts to rescue the rfa2::TRP1 deletion strain with the human cDNA failed. Diploids heterozygous for the rfa2::TRP1 allele were transformed with multicopy plasmids carrying either the human cDNA encoding the 34-kD subunit of RF-A [RP-A, provided by L. Erdile and T. Kelly] or the yeast RFA2 cDNA under the control of the inducible galactose promoter. Following growth on galactose the diploids were sporulated and dissected onto galactose-containing plates. The strain carrying the human clone yielded no more than two viable spores while the strain carrying the yeast clone yielded three or four viable spores [data not shown]. Immunoblotting with monoclonal antibodies directed against the human protein indicated that the human p34 protein was expressed when induced in wild-type yeast. Furthermore, expression of the human protein had no phenotypic effect on wild-type yeast. Interestingly, yeast strains overexpressing the RFA2 gene from this inducible promoter produced smaller colonies than cells carrying the expression vector alone [data not shown]. This result suggests that improper expression of an RFA subunit is harmful to cell growth, perhaps due to interference with regulated pathways or to an imbalance in the RFA subunits.

**Discussion**

Several lines of evidence suggest that RF-A is the primary eukaryotic single-stranded DNA-binding protein involved in DNA replication. The biochemistry of SV40 DNA replication has shown that RF-A is an essential factor involved in both the initiation and elongation stages of viral DNA replication. Studies from both human and yeast cells indicate that RF-A is a highly conserved protein in its subunit structure, DNA-binding activity, and post-translational modification. The current study describing the characterization of the genes encoding RF-A from S. cerevisiae confirms the essential nature of this protein complex in vivo. The requirement for all three genes argues that RF-A functions as a complex; the absence of any single subunit results in lethality.

The terminal phenotype of budded and multiply budded cells suggests that entry into the cell division cycle is unaffected by the loss of RF-A activity. This arrest phenotype is consistent with an S-phase defect disconnected from the budding and growth cycles of the cell. It was found that the number of divisions that each null mutant was capable of undergoing before arrest differed,
Proteolytic fragment of the 69-kD subunit of yeast RF-A, for RF-A in these reactions, suggesting that specific protein–protein reactions are required (Coverley et al. 1991). It was suggested that even the use of multiple different mutagens fails to the loss of an auxiliary function associated with a specific subunit.

The specific functions of the individual subunits are still unclear. The observation that the 69-kD subunit may be a zinc finger protein is consistent with its biochemical activity of binding ssDNA, and it is likely that at least one essential role of the 69-kD subunit is to stabilize ssDNA. Interestingly, if the only role of the RF-A complex was to bind to ssDNA, this should be accomplished by the 69-kD subunit alone because SSB activity is detected in the isolated subunit (Brill and Stillman 1989; Wold et al. 1989; Kenny et al. 1990). The requirement for both the 36- and 13-kD polypeptides indicates that RF-A is a multifunctional complex and that the smaller subunits play other essential roles.

The protein sequences of the 36- and 13-kD subunits have not shed light on what these additional functions might be. They do not contain any obvious DNA-binding motifs nor do they share any obvious sequence motifs that might mediate their own protein–protein interactions. The interactions that maintain the subunits in a stable complex are extremely strong; the complex from both species is resistant to high concentrations (6 M) of urea. It seems reasonable that the two smaller subunits are involved in mediating protein–protein interactions with other components involved in DNA replication, repair, and recombination. It has been shown that antibodies directed against the human 34-kD subunit, as well as the 70-kD subunit, inhibit SV40 DNA replication in vitro (Kenny et al. 1990; S. Din and B. Stillman, in prep.). Antibodies directed against both subunits also inhibit DNA excision repair in vitro (Coverley et al. 1991). Although antibody binding to the 34-kD subunit may affect the DNA-binding activity of the complex, the effects of the anti-34-kD antibody may be explained more easily by the inhibition of protein–protein interactions involving the 34/36-kD subunit of RF-A. Candidates for such interacting proteins include the DNA polymerases (α and δ), as well as other proteins involved in DNA replication such as primase, RF-C, and PCNA. In the cell, RF-A may also interact with initiation factors that function prior to the onset of DNA synthesis. The in vitro mutagenesis of RFA2 and RFA3 provides a genetic method of identifying components of the DNA replication, repair, and recombination machinery that interact with RF-A through these subunits.

Given the requirement for three intact genes, it is odd that none of the RFA genes correspond to known temperature-sensitive mutations or cdc genes in yeast. One possible explanation is that the subunits exist as individual functional domains that are so structurally restricted that single point mutations and, hence, temperature-sensitive phenotypes, are not tolerated. More likely, however, the RF-A genes are part of the “gene-number” paradox. Harris and Pringle (1991) have shown that RF-A may perform additional functions that contribute to its null phenotype. Human RF-A has been shown to be involved in the process of DNA excision repair with a cell-free system (Coverley et al. 1991). It was suggested that RF-A may aid in the recognition of ssDNA at sites of DNA damage, as well as in the recruitment of DNA polymerase to the gapped template. E. coli SSB and adenovirus ssDNA-binding protein could not substitute for RF-A in these reactions, suggesting that specific protein–protein reactions are required (Coverley et al. 1991).

RF-A may also function in the process of genetic recombination similar to E. coli SSB and T4 gene 32 protein. A proteolytic fragment of the 69-kD subunit of yeast RF-A, which retained ssDNA-binding activity, was purified on the basis of its stimulation of a yeast-strand exchange protein (Heyer et al. 1990). Differences in the colony sizes of the null mutants may therefore be affected by the specific subunit associated with a particular subunit.
uncover essential genes that have been identified by molecular techniques. These workers suggest that differential repair processes may play a role in limiting the number of mutations that can be identified by classical genetics. On the other hand, if the arrest phenotype of the temperature-sensitive mutants resembles that of the null mutants, these mutants would be overlooked in a Cdc screen because of their complex phenotype: a mixture of budded and multiply budded cells as well as cells with buds on buds. The construction of temperature-sensitive alleles of the RFA genes by in vitro mutagenesis and reverse genetics should circumvent these problems.

The RFA genes are expressed periodically with mRNA levels peaking at the G1/S phase boundary. We believe that this regulation is mediated by known promoter elements and therefore occurs at the level of transcription. The promoters of most genes encoding proteins involved in DNA synthesis in yeast contain a single MluI site, but additional CGCG core elements have been found to be important (Pizzagalli et al. 1988; Bauer and Burgers 1990; Lowndes et al. 1991). The promoters of both rfa1 and rfa2 contain two MluI recognition sequences. The rfa3 gene is unique in that no MluI sequence is found in its promoter, yet it maintains its S-phase regulation. Therefore, the reiteration of a more degenerate motif such as ACGCGX may be sufficient for S-phase transcriptional regulation.

In contrast to the periodic transcription of these genes, previous cell-cycle studies in yeast have shown that the levels of RFA2 protein do not fluctuate throughout the cell cycle (Din et al. 1990). Thus, one must question whether the transcriptional regulation seen in this family of genes is important for driving the cell cycle or whether it is simply fortuitous. Conceivably, the regulation may be important for maintaining the stoichiometry of essential replication components. In support of this hypothesis, we noticed that when the RFA2 gene was overexpressed on a galactose-inducible promoter, the cells were viable but formed small colonies. This effect could be due to an imbalance of subunits. The importance of balanced gene expression in protein complexes has been demonstrated previously in yeast. Mecks-Wagner and Hartwell (1986) have shown that when either pair of the yeast histone genes (H2A/H2B and H3/H4) was overexpressed relative to the other pair, cells displayed an increase in chromosome loss. Their data suggest that proper stoichiometry is required in the assembly of the nucleosome core. Similarly, the overexpression of one subunit of RF-A may result in incorrect assembly of the complex. Alternatively, the overexpression of a subunit involved in protein–protein interactions may result in the sequestration of essential factors. Further experiments will be necessary to distinguish between these possibilities.

A comparison of the human and yeast RFA2 genes indicates that little amino acid sequence is conserved between the yeast and human protein. Genetically, it is found that the human p34 subunit does not substitute for the homologous yeast protein even though the synthesis of human protein was observed by immunoblot. Given the structural and functional similarity of RF-A from both yeast and human cells, these results were unexpected. The subunits have closely maintained their relative sizes and post-translational modifications while tolerating significant changes in amino acid sequence. This suggests that there is selective pressure to maintain the overall subunit structure of RF-A. We consider the structural and functional similarity of yeast and human RF-A to be a strong argument for a common mechanism of initiation and elongation in eukaryotic DNA replication.

The SV40 system has been used as a model to biochemically identify conserved replication factors in *S. cerevisiae*. These factors will aid in the establishment of a yeast in vitro system for the study of eukaryotic DNA replication with chromosomal origins. The identification of RFA genes in yeast presents a complementary approach to solving this problem, a genetic analysis of RF-A should identify new factors involved in the mechanism and regulation of eukaryotic chromosomal DNA replication.

Materials and methods

**Yeast strains and RF-A purification**

All DNA cloning and expression experiments were performed with the standard wild-type yeast strain W303-1a (Wallis et al. 1989). Disruption experiments were performed with the isogenic diploid strain W303 by using the method of Rothstein (1983).

RF-A was purified essentially as described (Brill and Stillman 1989) except that the starting material for the purification was dried *S. cerevisiae* from Sigma (YSC-1). Dried yeast (100 grams) was first rehydrated and washed once in distilled water (4°C), yielding 220 grams of yeast cells wet weight. The yield from this amount of material (400 µg of RF-A) was essentially the same as that obtained from laboratory strains. Because the cells had been grown to saturation before drying (all cells were unbudded) the 36-kD subunit conveniently migrated in SDS-PAGE gels as a single band at the unphosphorylated position. This is expected for RF-A isolated from G1 phase cells (Din et al. 1990). Attempts to perform amino-terminal protein sequencing on the subunits of RF-A failed, suggesting that their termini were blocked.

**Isolation of tryptic fragments and protein sequencing**

Purified RF-A (250 µg) was precipitated by the addition of TCA to 10% and incubation at 0°C for 30 min. The sample was pelleted in a microcentrifuge for 10 min and washed twice with acetone (−20°C). The visible pellet was dried briefly and 80 µl of 0.1 M Tris (pH 8.0) was added. The protein was solubilized by the addition of 80 µl of 2x SDS-PAGE sample buffer, heated at 60°C for 10 min, and separated electrophoretically in three lanes (6-mm wide) of a 1.5-mm-thick 12% SDS-PAGE gel. The protein was transferred to nitrocellulose (Schleicher & Schuell, BA85) with a Hoeffer wet transblotter and 25 mm Tris/200 mM glycine/20% methanol as buffer. Transfer conditions were 0.5 amp for 2 hr at 4°C. Following transfer, the nitrocellulose was stained with Ponceau S (0.1% in 1% acetic acid) for 1 min and destained in 1% acetic acid for 2 min. Transfer of the 36- and 13-kD subunits was estimated to be essentially complete while ~30% of the 69-kD subunit transferred out of the gel. The
The advantage of the use of PCR within a single peptide is that information from each end of a single long peptide, KWSEDAND- the size of the correct PCR product can be predicted precisely. LAAGNDDSSGK, that was obtained by amino acid sequencing. Deletions were made with exonuclease and S 1 nuclease. was performed with Sequenase (U.S. Biochemical). Directional sequencing localized the partial genomic plasmid library constructed from strain SP-1 was identified by using the insert to pJM101 to screen 46°C/6 x SSC/0.1% SDS). This 5.3-kb band on low-stringency Southern blots (final wash, RFAI gene to a 2.6-kb band was obtained from a size-selected genomic plasmid library (pJM201) and found to contain a large ORF with matches to additional peptide sequences. However, the ORF that was extended past the EcoRI site by a Southern blotting revealed that the ORF was contained on a 5.2-kb HindIII fragment. This fragment was isolated from a size-selected genomic HindIII plasmid library (pJM205) and found to contain the entire RFA2 gene within the Psrl and XmnI sites.

Cloning the RFA genes

**RFA1** The amino acid sequence of peptide VLEQDQGTVWR was used to design the 64-fold degenerate 21-mer oligonucleotide RFA1-1 [5'-GARCA RCCWG AYGGW ACWTG G-3']. Abbreviations: [R] A/G, [W] A/T, [Y] C/T, [N] A/C/G/T, [D] A/G/T. Oligonucleotide RFA1-1 hybridized strongly to a single 5.3-kb EcoRI band on low-stringency Southern blots (final wash, 46°C/6 x SSC/0.1% SDS). This 5.3-kb EcoRI band was obtained from a size-selected genomic EcoRI plasmid library in plburescript SK− (plasmid pMJ101). DNA sequencing revealed an ORF, containing an amino acid sequence corresponding to that of the original peptide. However, the ORF continued past the EcoRI site. A large (13.3-kb) DNA fragment containing RFA1 was identified by using the insert to pMJ101 to screen a HindIII partial genomic plasmid library constructed from strain SP-1 (provided by M. Wigler). Extensive restriction mapping and sequencing localized the RFA1 gene to a 2.6-kb Psrl–HindIII fragment. This fragment was isolated from a size-selected Psrl–HindIII genomic library from strain W303-1a in SK+ (pMJ112) and used as template for DNA sequencing. All DNA sequencing was performed with Sequenase [U.S. Biochemical]. Directional deletions were made with exonuclease and S1 nuclease.

**RFA2** PCR primers were designed by using sequence information from each end of a single long peptide, KWSEDAND- LAAGNDSSGK, that was obtained by amino acid sequencing. The advantage of the use of PCR within a single peptide is that the size of the correct PCR product can be predicted precisely. The use of the 128-fold degenerate 28-mer oligonucleotide, 36-1W1 [5'-ATCTC GAGAA RTGGT CNGAR GAYGC NAA-3'], and the 512-fold degenerate 29-mer oligonucleotide, 36-1C1 [5'- TAGGA TCCGT TWCNN GANGA RTCRT CRTT-3'], predicted a 73-bp product following amplification of genomic DNA. All PCR reactions were performed under the following conditions: 50-μl reaction volume containing 100 pmoles of each primer, 2.5 units of AmpliTaq (Perkin-Elmer) polymerase, and buffer conditions according to the manufacturer. The PCR reaction [95°C for 30 sec, 45°C for 1 min, 65°C for 1 min, 30 cycles] was carried out on 25 ng of yeast DNA and resulted in two major bands, one of which was ~73 bp as judged by an 8% PAGE gel. This band was eluted from the gel. Following digestion with Xhol and BamHI, the fragment was cloned and sequenced and found to contain an ORF with the same internal amino acid sequence as that of the original peptide. When the 73-bp band was used as a probe on Southern blots, it detected a single 3.8-kb EcoRI band [final wash, 55°C/0.5 x SSC/0.1% SDS]. This fragment was obtained from a size-selected genomic EcoRI plasmid library (pJM201) and found to contain a large ORF with matches to additional peptide sequences. However, this ORF that was extended past the EcoRI site by a Southern blotting revealed that the ORF was contained on a 5.2-kb HindIII fragment. This fragment was isolated from a size-selected genomic HindIII plasmid library (pJM205) and found to contain the entire RFA2 gene within the Psrl and XmnI sites.

**RFA2 cDNA** Fifteen micrograms of total yeast nucleic acid, isolated as described (Brill and Sternglanz, 1988), was reverse-transcribed in a 75-μl reaction containing 0.1 mg/ml of random hexamer primers [Pharmacia], 1 x RT buffer (BRL), 0.5 mm dNTPs, 60 units of RNasin (Promega), and 400 units of Moloney murine leukemia virus [MoMLV] reverse transciptase (BRL). The reaction proceeded for 60 min at 37°C before the nucleic acids were precipitated by the addition of 225 μl of TE, 1 μg of tRNA, 100 μl of 2.3 M NaClO4, and 200 μl of isopropanol. The precipitate was centrifuged for 15 min, washed with 70% ethanol, dried, and resuspended in 50 μl of TE. Ten microliters of the first-strand reaction products were subjected to PCR (94°C for 0.5 min/50°C for 1 min/72°C for 1 min/30 cycles) by using as primers the 21-mer oligonucleotide, RFA-1 [5’-CTATC TAGAA CAGGC TAGTT T-3’], and the 24-mer oligonucleotide, RFA-2-2 [5’-GACTC TAGAA TTTGT TTGAT CGTC-3’]. The products from this reaction, 183- and 291-bp fragments, were isolated from a 5% PAGE gel, digested with Xhol, and subcloned into pSK+ (pJM220 and pJM221, respectively). Plasmid pJM214 contains the 1.2-kb Clal–XmnI fragment of RFA2 ligated into the Clal and EcoRV sites of pSK+ in which the genomic Xhol fragment is replaced by the cDNA Xhol fragment from pJM220.
sequenced and found to contain an ORF with the same internal amino acid sequence as that of the original peptide. When the 67-bp band was used as a probe on Southern blots it detected a single 7-kb EcoRI band (final wash, 55°C/0.1 x SSC/0.1% SDS). This fragment was obtained from a size-selected genomic EcoRI plasmid library (pJM301), and the region of hybridization narrowed to a 1.0-kb HindIII fragment. Sequence of this region revealed an ORF with matches to additional peptide sequences. The complete gene was found to be contained on a PvuII–EcoRV fragment (pJM318).

Physical mapping
A set of DNAs on nylon filters from ~1000 overlapping yeast clones in phage λ was provided by Linda Riles and Maynard Olson (Washington University, St. Louis). Random hexamer-labeled probes were made from the fragments shown in Figure 2. The RFA1 probe detected clones 4236 and 4239, the RFA2 probe detected clones 5413 and 6742, and the RFA3 probe detected clone 6699.

Plasmid constructions
Plasmids pJM118, pJM223, and pJM329 contain, respectively, the RFA1, RFA2, and RFA3 genes or cDNA under the control of the phage T7 promoter and are described below. Plasmid pJM106, which contains the 2.6-kb PstI–HindIII fragment of RFA1 cloned into the polylinker of pVT102L (Vernet et al. 1987), was digested with MluI and subjected to Bal31 exonuclease, Xhol digestion, Klenow flushing, and self-ligation. The plasmid pJM106Δ10 was recovered, in which nucleotide number 450 of RFA1 is fused to the polylinker of pVT102L. The 1.9-kb Xbal–Scal fragment of pJM106Δ10 was ligated into the Nhel site and the filled-in BamHI site of pET11a (Studier et al. 1990) to create pJM118. The protein encoded by this construct replaces the first 4 amino acids of RFA1 with the amino acids MARRAP encoded by the polylinker.

Plasmid pJM214 (0.2 μg) containing the RFA2 cDNA was used as template for PCR using as primers the 22-mer oligonucleotide, RFA2T7-1 (5′-ATGCA TATGG CAAAC TATCA AC-3′), and the reverse sequencing primer (5′-AACAG CTATG AC-3′). Following PCR (94°C for 0.5 min/50°C for 1 rain/72°C for 2 min/10 cycles), the 1.1-kb product was digested with Ndel and BamHI and ligated into the Ndel and BamHI sites of pET11a to create pJM223.

Plasmid pJM318 (0.2 μg) was used as template for PCR by using as primers the 22-mer oligonucleotide, RFAA3T7-1 (5′-ATGCA TATGG CCAGC GAAC AC-3′), and the reverse sequencing primer (5′-AACAG CTATG ACCAT G-3′). Under the conditions described above, a 0.73-kb product was obtained, digested with Ndel and BamHI, and ligated into the Ndel and BamHI sites of pET11a to create pJM329.

Plasmids pJM111, pJM211, and pJM317 contain the alleles rfa1::TRPI, rfa2::TRPI, and rfa3::TRPI, respectively, and are described below. Plasmid pJM111 contains the 5.1-kb Xhol–BamHI fragment of RFA1 in pSK– in which the 1.45-kb EcoRV–EcoRI fragment is replaced by the 0.83-kb EcoRI–Stul fragment of TRPI. The allele was translocated by transformation of 2.5 μg of pJM111 DNA following Xhol–BamHI digestion. Plasmid pJM211 is the 4.4-kb Scal of RFA2 cloned into pSK+ in which the 530-bp HpaI–EcoRI is replaced by the 0.83-kb EcoRI–Stul fragment of TRPI. The allele was translocated by transformation of 2.5 μg of pJM211 DNA following Scal digestion. Plasmid pJM317 was constructed by replacing the 0.3-kb MscI–HindIII of pJM301 with the 0.83-kb TRPI gene on a Smal–HindIII fragment. The allele was translocated by transformation of 2.5 μg of pJM317 DNA following EcoRI digestion. All disruptions were verified by Southern blotting.

Plasmids pJM217 and pJM403 are galactose expression plasmids. Plasmid pJM217 contains the 1.1-kb SalI–BamHI fragment of pJM214 cloned into the SalI and BamHI sites of YEp51. Plasmid pJM403 was constructed by moving the EcoRI fragment of the human RP-A 32-kg subunit (pLEI, Erdile et al. 1990) into YEp51 on a SalI–BamHI fragment.

Bacterial expression
The vector plasmid pET11a and the recombinant plasmids pJM118, pJM223, and pJM329 were transformed into bacterial strain BL21(DE3) in which T7 RNA polymerase is under the control of the lacUV5 promoter (Studier et al. 1990). When the cultures reached an OD600 of 0.6 the cells were induced by the addition of IPTG to 0.4 mM for 2 hr, pelleted, and resuspended in one-tenth volume of TE. An equal volume of 2× SDS-PAGE sample buffer was quickly added, and the sample was heated at 100°C for 5 min. Samples were sonicated for 20 sec to shear chromosomal DNA and 5 μl (~10 μg of protein, RFA1, and RFA3) or 1 μl (~2 μg of protein, RFA2) was subjected to electrophoresis on a 1.5-mm-thick 17% SDS-PAGE gel and transferred to nitrocellulose. As a control, ~0.5 μg of RF-A was run alongside each sample. The nitrocellulose was stained with Ponceau S to visualize the lanes that were separated and incubated with a 1:100 dilution of the appropriate affinity-purified rabbit antibody raised against the individual subunits of yeast RF-A. The nitrocellulose strips were washed, incubated with a 1:1000 dilution of 125I-labeled goat anti-rabbit (ICN) as secondary antibody, washed again, and exposed to film.

Yeast cell-cycle expression
Yeast was grown at 30°C in 150 ml of YPD (1% yeast extract, 2% peptone, 2% dextrose) to an OD600 of 1.0 and was arrested by the addition of α-factor (Sigma) to 2.4 μM. After 100 min, the cells were pelleted in a clinical centrifuge, washed once with 10 ml of YPD, and resuspended in 200 ml of YPD at 30°C. Thirteen-milliliter samples were taken every 15 min into 15-ml polypropylene tubes, quickly pelleted, and frozen at −70°C. Simultaneously, 25 μl of each aliquot was fixed with an equal volume of 3.7% formaldehyde/0.15 M NaCl for microscopy. Isolation of nucleic acids and Northern analysis was performed essentially as described (Brill and Sternaglanz 1988).

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Note added in proof
The sequence of the gene encoding the human 70-kg subunit has been published by Erdile et al. (J. Biol. Chem. 266: 12090–12098, 1991.)
Sequence data described in this paper have been submitted to the EMBL/GenBank Data Libraries under accession numbers X59748 [RFA1], X59749 [RFA2], and X59750 [RFA3].

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