MOLECULAR AND CELLULAR BIOLOGY, May 1990, p. 2341–2348
0270-7306/90/052341-08$02.00/0

Vol. 10, No. 5

A Gene Family for Acidic Ribosomal Proteins in *Schizosaccharomyces pombe*: Two Essential and Two Nonessential Genes

MONICA BELTRAME[1]* AND MARCO E. BIANCHI[2]

Gene Expression Program, European Molecular Biology Laboratory, Meyerhofstrasse 1, D-6900 Heidelberg, Federal Republic of Germany,[1] and Dipartimento di Genetica e Microbiologia, Università di Pavia, I-27100 Pavia, Italy[2]

Received 4 November 1989/Accepted 26 January 1990

We have cloned the genes for small acidic ribosomal proteins (A-proteins) of the fission yeast *Schizosaccharomyces pombe*. *S. pombe* contains four transcribed genes for small A-proteins per haploid genome, as is the case for *Saccharomyces cerevisiae*. In contrast, multicellular eucaryotes contain two transcribed genes per haploid genome. The four proteins of *S. pombe*, besides sharing a high overall similarity, form two couples of nearly identical sequences. Their corresponding genes have a very conserved structure and are transcribed to a similar level. Surprisingly, of each couple of genes coding for nearly identical proteins, one is essential for cell growth, whereas the other is not. We suggest that the unequal importance of the four small A-proteins for cell survival is related to their physical organization in 60S ribosomal subunits.

The large ribosomal subunits of all organisms studied so far contain small proteins, designated A-proteins, with unusual characteristics. In contrast to most ribosomal proteins, they are present in multiple copies on the ribosome and are acidic. They share an exceptional amino acid composition: about 20% alanine, few aromatic residues, only one or two arginines, and generally no cysteine. A-proteins are also characterized by a high alpha-helical content.

Escherichia coli L7/L12 protein is the most studied component of this unique group of ribosomal proteins. Protein L7 differs from L12 only for an acetyl group at the N terminus, and both are transcribed from a single gene, *rplL* (35). L7/L12 is present in four copies in the ribosome: two L7/L12 dimers are attached to the 23S RNA via protein L10. This pentameric complex is thought to form the long stalk protruding from the 50S subunit (22). L7/L12 binds to several translation factors, most notably elongation factors Tu and G, and is important in factor-dependent GTP hydrolysis (21). Mutations in the *rplL* gene encoding L7/L12 are associated with an increased misincorporation in polypeptide synthesis, indicating that these proteins are implicated in the control of translational accuracy (12).

In contrast to eubacteria, which contain a single though modified polypeptide, eucaryotic cells usually have two A-proteins, whose functions and physical properties appear to be analogous to those of *E. coli* L7/L12. They also interact with soluble factors (elongation factor 2) (16, 31, 38) and have similar molecular weights, isoelectric points, the tendency to aggregate in solution, and solubility in 50% ethanol. Their structural arrangement in the ribosome is also conserved: it has been shown that the two A-proteins of the brine shrimp *Artemia salina* form two dimers that interact with protein P0 to form a pentameric unit analogous to the $(L7)_2(L12)_2L10$ unit of *E. coli* (37). Although there is little sequence similarity between bacterial and eucaryotic A-proteins, *E. coli* L7/L12 can form active hybrid ribosomes with yeast core ribosomes from which acidic proteins have been extracted by an ethanol treatment (31). The A-proteins from *Saccharomyces cerevisiae*, *A. salina* (15), *Drosophila melanogaster* (25, 42), and human cells (28) have been cloned. The sequences of the cDNAs demonstrate that the multicellular organisms contain two genes, coding for proteins that have been extremely conserved during evolution. *S. cerevisiae*, on the other hand, contains four independent genes for small acidic ribosomal proteins, which have been called A1 (19), L44′ (26), L45 (26), and L44 (26), alternatively named A2 (20). Proteins L44 and L45 are very similar to each other, as are proteins A1 and L44′, suggesting that each of the A-proteins of higher organisms has two counterparts in yeast cells.

It is not known whether the A-proteins of eucaryotes have a different and specific role or whether a single protein could ensure the functionality of ribosomes, as is the case for *E. coli*. The latter possibility is particularly appealing given the high degree of similarity between individual A-proteins and their interchangeability across vast phylogenetic distances. The presence of four A-proteins in *S. cerevisiae*, on the other hand, makes it likely that the maintenance of multiple genes even in an organism with a limited genome size is required by the diversification of roles for each type of acidic protein.

We have addressed the problem of the function(s) of acidic ribosomal proteins by cloning the A-protein genes of the unicellular yeast *Schizosaccharomyces pombe*, an organism whose phylogenetic distance from *S. cerevisiae* appears to be at least as great as the distance between *S. cerevisiae* and humans. *S. pombe* is shown to contain four individual A-proteins, which can be divided in two couples of very similar but not identical sequences. Each couple is distinctly related to one of the two A-proteins present in multicellular organisms but shows a degree of sequence divergence that suggests that each protein might have a unique function. Each protein is coded by a single gene; genetic analysis indicates that two of these genes are essential for cell survival and that two are not.

MATERIALS AND METHODS

Yeast strains and media. Growth and handling of *S. pombe* were as described by Gutz et al. (7). Gene disruptions were performed on a diploid strain (h^+/h^+, *ade6-704/ade6-704*

* Corresponding author.

leu1-32/leu1-32 ura4-Δ18/ura4-Δ18; kindly provided by D. Tollervey) and on a haploid strain with the same genotype. Cells were usually grown in YPD liquid medium at 30°C. Transformed strains were selected on minimal plates supplemented with adenine and leucine or uracil.

Isolation of cDNA and genomic clones for *S. pombe* acidic ribosomal proteins. Approximately 8×10^5 plaques from an *S. pombe* cDNA library in λgt11 (kindly provided by P. Nurse) were screened by the method of Young and Davis (43) with an antiserum from a patient affected by systemic lupus erythematosus. This antiserum (Kueng) is of the Sm class and was kindly provided by I. Mattaj. Positive areas of the plates were rescreened until single positive plaques were isolated. Bacteriophage DNA was prepared; the inserts were excised by *Eco*RI digestion and subcloned into the *Eco*RI site of plasmid pTZ18R (Pharmacia). Four different cDNA clones were mixed, uniformly labeled with ^{32}P, and used as probes to isolate clones from a λEMBL4 genomic library of *S. pombe* (kindly provided by M. Yanagido). The inserts were excised from positive clones by restriction with *Eco*RI and subcloned into the *Eco*RI site of plasmid pTZ18R, generating plasmids designated pTZrpa1 to pTZrpa4.

Determination of DNA sequences. Most of the sequences were determined by the dideoxy-chain termination method of Sanger et al. (32) either on single-stranded or on double-stranded DNA. A few uncertainties were resolved by chemical degradation (18). All of the sequences were at least double checked in different subclones.

Construction of null mutants. (i) *rpa1*. A 1-kilobase-pair (kb) *Nhe*I-*Nhe*I fragment containing the entire *RPA1* gene was cloned into the *Xba*I site of plasmid pHSS6 (34). The transposon mutagenesis procedure described by Seifert et al. for *S. cerevisiae* (34) was then strictly followed. The m-Tn*3*(*LEU2*) transposon carries the *S. cerevisiae LEU2* marker, which complements the *leu1* mutation of *S. pombe* (2, 27).

(ii) *rpa2*. The *S. cerevisiae LEU2* marker was recovered from plasmid pEVP11 (kindly provided by P. Nurse) as a 2.2-kb *Xho*I-*Sal*I fragment, filled in with DNA polymerase I large fragment, and used to substitute a small *Eco*RV-*Eco*RV fragment (83 base pairs) of pTZrpa2. The selectable marker thus interrupts the first exon of the *RPA2* gene.

(iii) *rpa3*. A unique *Nar*I site in the second exon was used to introduce the selectable *URA4* marker within the *RPA3* gene. The *S. pombe URA4* gene was excised from plasmid pURA4 (kindly provided by D. Tollervey) as a 1.3-kb *Acc*I-*Acc*I fragment, filled in with DNA polymerase I large fragment, and cloned into the blunted *Nar*I site of pTZrpa3.

(iv) *rpa4*. The *URA4* marker was also inserted in the first exon of the *RPA4* gene. An *Eco*RI-*Eco*RI fragment containing the *RPA4* gene was cloned into the *Eco*RI sites of plasmid pUC7 (39). The resulting plasmid was cut with *Pst*I, blunted by treatment with exonuclease III and DNA polymerase I large fragment, and ligated to the filled-in *Acc*I-*Acc*I fragment of pURA4.

Yeast transformation. The genes carrying the selectable markers were excised from the vectors and in some cases purified by gel electrophoresis. Remaining polylinker sequences were removed with a limited BAL 31 digestion. One to five micrograms of DNA was used to transform *S. pombe* cells, using a slight modification of the method of Ito et al. (11).

Southern blot analysis. Chromosomal DNA was extracted from spheroplasts. Samples of 0.5 to 1.5 μg were digested with the appropriate restriction enzymes, run on agarose gels, blotted on GeneScreen Plus filters, and hybridized with uniformly ^{32}P-labeled DNA probes. Hybridization and washes were carried out at 65°C according to standard procedures.

Northern (RNA) blot analysis. Total RNA was extracted from mid-exponential-phase cultures by heating cells at 65°C in sodium dodecyl sulfate-phenol (30). To each lane of a 1.5% agarose-formaldehyde gel, 15 μg of RNA or 15 ng of *Eco*RI-cut pTZrpa DNA was applied. The gel was then capillary blotted onto Hybond-N filters (Amersham Corp.). The filter was cut in strips; each group of three RNA lanes and the appropriate cDNA lane were hybridized together with ^{32}P-labeled oligonucleotides, autoradiographed, washed, and rehybridized to a probe for 7SL RNA (27). Hybridizations with oligonucleotides were performed in 6× SSC (SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–10× Denhardt solution–0.2% sodium dodecyl sulfate at room temperature for 2 h. The filters were briefly washed in 6× SSC three times at room temperature and once at 35°C, i.e., 5 to 7°C below the calculated melting temperatures of the 15-mers. Autoradiographic films were then scanned with a FA70B video camera (Grundig) connected to a Macintosh II computer (Apple Computer, Inc.), applying proprietary software.

RESULTS

Isolation and analysis of cDNAs for *S. pombe* acidic ribosomal proteins. All known eucaryotic A-proteins have almost identical C-terminal sequences, which constitute a conserved epitope with extended immunological cross-reactivity. Patients affected by systemic lupus erythematosus generally produce autoantibodies against nuclear components, but some also produce antiribosome antibodies that react almost exclusively against the conserved C-terminal epitope of A-proteins (5). We have used an antiserum from one such patient to screen a λgt11 cDNA library from *S. pombe*. Immunologically reactive clones were sorted into four groups on the basis of cross-hybridization. One clone from each class was selected for further studies.

The inserts contained in the phage clones were subcloned and completely sequenced. The corresponding amino acid sequences were derived and used to search protein sequence data bases. One sequence (A2; Fig. 1) could be attributed to an already characterized A-protein of *S. pombe*, designated SP-L40c. The first 40 amino acids of A2 are identical to the N-terminal sequence of SP-L40c obtained by Otaka et al. (24), and the overall amino acid compositions of A2 and SP-L40c also match well (3).

All four proteins we have cloned show amino acid compositions unique to A-type ribosomal proteins. They are acidic and alanine rich, and they contain few aromatic residues, one arginine as a maximum, and no cysteine. All have a calculated molecular mass of about 11 kilodaltons and are 110 amino acids long except for A3, which is composed of 109 amino acids. The amino acid sequences of the four proteins can be aligned by introducing only a few gaps (Fig. 1). A comparison of all four amino acid sequences at the same time gives 35% identity and 52% similarity, taking into account only strictly conservative changes. As expected, most of the identities are concentrated in the C-terminal portions of the proteins, which are recognized by the antiserum that we used; apart from the insertion of three amino acids in protein A4, the last 19 amino acids are identical.

The four proteins can be divided in two couples of very similar amino acid sequences; the percentage of identical residues is 79% within one couple (A2-A4) and 93% within

FIG. 1. Amino acid sequence of *S. pombe* acidic ribosomal proteins. The amino acid sequences of the four proteins were derived from the cDNA sequences and were aligned manually by introducing a few gaps. Identities within all four proteins are marked with an asterisk. The proteins have been grouped in two couples (A1-A3 and A2-A4) to highlight similarities.

the other (A1-A3). Each couple of *S. pombe* proteins appears to be homologous to one or the other of the two A-proteins of multicellular eucaryotes (Table 1). The four small acidic ribosomal proteins of *S. cerevisiae* also fall in the two overall groups of homology; it appears that yeasts (and possibly unicellular eucaryotes in general) contain two variants of what is a single ribosomal protein in several species of multicellular organisms.

In several organisms, including *S. cerevisiae* and *Xenopus laevis*, a certain number of ribosomal proteins are encoded by two different genes; in most cases, however, the two polypeptides produced by the duplicated genes are either identical or almost identical. The differences within the A1-A3 couple are only slightly more pronounced than is usual among duplicated ribosomal proteins, but the A2-A4 couple is sufficiently divergent to suggest that the two proteins may not be functionally indistinguishable.

Isolation and characterization of *RPA* genes. Southern blot analyses of total *S. pombe* DNA cut with several enzymes showed a few bands of hybridization to each of the cDNAs at low stringency but only one major band at high stringency (data not shown). Therefore, it appears that each cDNA corresponds to a single gene of *S. pombe*; duplicated genes for the same cDNA or pseudogenes are unlikely to exist.

A mixture of the four cDNAs was used as a probe to screen an *S. pombe* genomic library in λEMBL4. Positively hybridizing clones were partially mapped by restriction analysis and sorted in four groups. A member of each group was then subcloned and sequenced.

We called the four genes coding for A-proteins in *S. pombe* *RPA1* to *RPA4* (ribosomal protein type A). Their overall structures (Fig. 2) are quite similar. All four genes contain one or two introns, which occur in two conserved positions with respect to coding sequences. The structure of the last exon of genes *RPA2*, *-3*, and *-4* is unusual: it codes for only seven amino acids (usually the last exon is the largest) and begins with the triplet ATG, coding for methionine. Moreover, the intron interrupts the C-terminal sequence that is highly conserved throughout species.

TABLE 1. Comparison of *S. pombe* A-proteins with those of other eucaryotes

	% of amino acids identical with *S. pombe*				Reference(s)
	A1	A2	A3	A4	
S. cerevisiae L44′	55.0	33.3	58.2	37.4	26
S. cerevisiae A1	57.3	27.7	64.5	31.2	19
D. melanogaster rp21C	53.6	27.8	50.5	28.7	42
A. salina eL12′ (P1)	58.2	35.9	55.0	28.7	15
Human P1	53.1	29.8	53.1	26.7	28
S. cerevisiae L44 (A2)	32.8	56.8	33.3	57.7	20, 26
S. cerevisiae L45	24.6	55.9	28.3	55.9	26
D. melanogaster rpA1	33.7	54.9	31.9	52.2	25
A. salina eL12 (P2)	32.4	56.8	33.1	52.7	15
Rat P2	25.7	50.5	30.0	55.9	13
Human P2	34.5	57.4	32.0	55.7	28

The sequences at both splice sites and the TACTAAC motif near the 3′ end of the five introns are very conserved (Fig. 3). The consensus sequence that can be derived is also characteristic of *S. cerevisiae* introns, whereas it differs from the sequences for mammalian introns in several aspects, such as the absence of a polypyrimidine tract near the branch site.

The third position 5′ to first ATG of the coding sequence is consistently an A, as is the rule for highly expressed *S. cerevisiae* genes (10). We did not attempt to determine the exact site of transcription initiation or to identify possible regulatory regions in the 5′-flanking sequences of the genes. None of the *RPA* genes contain sites that fit well the consensus sequence for the HOMOL1/RPG box, which acts as a transcription activator of most ribosomal genes in *S. cerevisiae* (17, 41). A sequence with partial homology (14 of 19 nucleotides) to the UAS_T transcriptional activator of gene *TCM1* (9) is present at positions 154 to 172 of the *RPA3* gene (Fig. 2), whereas no homologies were found with the transcriptional activator for the *RPS33* gene (40).

Disruption of *RPA* genes. Since *S. pombe* contains four genes for A-proteins, we tested whether all were essential for the functioning of ribosomes. *RPA* genes were inactivated by gene disruption, and the viability of null mutants in the haploid state was examined. Mutated alleles of the four genes were constructed in vitro by introducing a selective marker within coding regions (Fig. 4A); the resulting null alleles were introduced into the genome by homologous recombination. Transformant Ura^+ or Leu^+ clones were selected, and the successful disruption of *RPA* genes was checked by Southern analysis (Fig. 4B).

Transformation of a haploid Leu^- strain with *rpa1*::m-Tn*3*(*LEU2*) or *rpa2*::*LEU2* genes gave rise to colonies on selective medium. Southern analysis of some Leu^+ transformants demonstrated the presence of the disrupted *rpa1* gene (fragment of 4.5 kb) and the absence of the *RPA1* wild-type allele (fragment of 1 kb) (Fig. 4B). Analysis of the clones arising from transformation with the mutated *rpa2* gene demonstrated the presence of the disrupted *rpa2*::*LEU2* gene (fragment of 5.8 kb) and the absence of the *RPA2* wild-type allele (fragment of 3.6 kb) (Fig. 4B). These results indicate conclusively that proteins A1 and A2 are not essential for cell viability, possibly because they can be substituted by their homologs A3 and A4, respectively.

The null alleles of *RPA3* or *RPA4* genes were introduced into a diploid Ura^- strain, and transformant Ura^+ colonies

rpa1

```
  1 TCAAGGAAATAGTTTTGTTAGAGAAAGAATTGGTTTATGGCAAGAATTTTCTTGCTTAGTAGGGATTGTGAAGGAATTAAAACGCGTCCGACGCGTTTTC
101 AATCAATTGTTACCTAGTACTAGCAAGGTAACACCTACTAAATAGACACACACATTAGGGTAGGGCTGTAAAAATAGTCACGAGATAGGGAACGAAACAA
201 AACACAGTTTTTTAACACTTGCATTTACAACAAGAGAACAGCACTGGTCAAAATGTCTGCATCTGAACTTGCTACCAGTTATTCAGCTCTCATTTTGGCT
                                                 M  S  A  S  E  L  A  T  S  Y  S  A  L  I  L  A
301 GACGAGGGTATTGAAATCACCGTACGTTATATTTTACTAGAAAAGTTGTGAATGTACTTCTACCGATACTGGTAAAAGATCTTTTCCATAGCGTGTAGTC
    D  E  G  I  E  I  T
401 GTCTTATTCCTTCTTATTCGGTTTTTGTTAAATTAATTTATACTAATTGTTGCGTAGTCTGACAAGCTCTTGTCTTTAACCAAGGCTGCCAACGTCGATG
                                                       S  D  K  L  L  S  L  T  K  A  A  N  V  D  V
501 TTGAACCCATTTGGGCTACCATTTTCGCTAAGGCTCTTGAGGGCAAGGACCTTAAGGAACTTTTATTAAACATTGGCTCTGGTGCTGGTGCTGCTCCTGT
      E  P  I  W  A  T  I  F  A  K  A  L  E  G  K  D  L  K  E  L  L  L  N  I  G  S  G  A  G  A  A  P  V
601 TGCTGGCGGTGCTGCTGCTCCCGCTGCCGCTGATGGCGAAGCTCCTGCTGAAGAGAAGGAGGAAGCCAAGGAAGAGGAGGAATCTGATGAAGATATGGGA
     A  G  G  A  A  A  P  A  A  A  D  G  E  A  P  A  E  E  K  E  E  A  K  E  E  E  E  S  D  E  D  M  G
701 TTCGGTTTGTTTGACTAATTTCCCTAATCTGCTAGCATGAACTAGTTGATCCAACTCAAAAAATAACCATTTTTCTATATCTCACTTAATATCTTCTTGT
    F  G  L  F  D
801 GTTTTGAAATTAAAAATTTAGACCGAGTAATCTTTATGAAACCGCCTTTGATCTGCGCCAACTATTTGTATGGCCAATGTTGGTGATTTTCAGACATATG
```

rpa2

```
  1 ACGCCCTTAACTAACAACTATTTAGGTTGATTTGAACTTGTAGAAAAAGCTTGTAAATATCGTGCTCGTCAACTAAACGAATATAACAAACCTTAGAAAT
101 CATCGCAGCATTCCACTCAAACACATACCCTAACCTAACTCGAAAACCAGTCACAAAAGTTTCGAGTCCCTTAGTGTTGTGAATGCTTAATACTTTCTGC
201 CATCAACACGCACTGCACTCCTCGTCAAAATGAAGTACCTTGCAGCTTATTTACTCTTGACCGTTGGTGGAAAGGACTCCCCTTCCGCTTCTGATATCGA
                          M  K  Y  L  A  A  Y  L  L  L  T  V  G  G  K  D  S  P  S  A  S  D  I  E
301 GTCTGTTTTGTCTACTGTCGGTATTGAAGCTGAATCTGAGCGCATTGAAACTTTGATCAACGAGCTTAACGGTAAGGATATCGACGAGCTCATCGCTGCT
     S  V  L  S  T  V  G  I  E  A  E  S  E  R  I  E  T  L  I  N  E  L  N  G  K  D  I  D  E  L  I  A  A
401 GGTAACGAGAAGCTTGCTACTGTCCCCACTGGTGGTGCTGCTTCTGCTGCTCCTGCCGCCGCTGCTGGCGGTGCTGCCCCTGCTGCTGAGGAGGCTGCTA
    G  N  E  K  L  A  T  V  P  T  G  G  A  A  S  A  A  P  A  A  A  A  G  G  A  A  P  A  A  E  E  A  A  K
501 AGGAGGAAGCCAAGGAAGAGGAGGAATCTGATGAAGACGTAAGTTAATTTTAGATAAGAAGCATTCTTTGTTTCTCAAAAGCATCTTACTAACAATTTTA
      E  E  A  K  E  E  E  E  S  D  E  D
601 GATGGGTTTCGGTCTTTTCGACTAAACCAAATGATCCCTTGTTTTCATTACGCTTTCATAAAAATAAATCGCCGTGTTGGTTTTATTTTACCAATTTTTT
     M  G  F  G  L  F  D
701 TTTAGTGCTCCATTCGTAATAGTATTTTTCGTTTGATAAATGTAATTTTAGCTTACTTTAAATGTTAATGTACCACTGGTGCCCCGTGTTGTTGATTTGA
```

rpa3

```
  1 AGTGTAATCACGACGGTTAACAGTGTCAAGAATAACTGGTCGATACAAAGCAATAACAAGGTTTTCCGCCATCGTCTTCTCTTTTAATGCTTCTAAAAAA
101 GTGGGGTCACCAAACCAACCGGTCGTAATCAGTGGAAATAACTTTCGTTCTCGTAAAATACGGCCTAAACGATGATATAACGTAATGAGAAAGCGAATGT
201 TAGGAGATGCTAATCTCCAAGACCCATTGATATCTTTACCAACACCAAAATGTCTGCCTCCGAACTTGCTACAAGTTACTCTGCTCTCATCTTGGCCGAC
                                                  M  S  A  S  E  L  A  T  S  Y  S  A  L  I  L  A  D
301 GAGGGTATTGAGATCACCGTAAGTTAACACATTTATGGAGGTGCACGCGTTGTACACATAGAGTCATCAAAAATGATTACTGAAATCCTACCACATTAAA
    E  G  I  E  I  T
401 TAATGACGCGTCATCATGTAGATTTTGCCTAAAGGGGATTATTAACAGTATTTCTAGTCAGATAAACTCTTGTCTTTGACTAAGGCTGCCAACGTCGATG
                                                        S  D  K  L  L  S  L  T  K  A  A  N  V  D  V
501 TTGAGCCTATCTGGGCAACCATCTTTGCTAAGGCTTTGGAGGGCAAGGACTTGAAGGAACTCCTTTTGAACATTGGCTCTGCTGCTGCCGCTCCTGCTGC
      E  P  I  W  A  T  I  F  A  K  A  L  E  G  K  D  L  K  E  L  L  L  N  I  G  S  A  A  A  A  P  A  A
601 TGGTGGCGCCGGTGCTCCTGCCGCTGCTGCTGGTGGTGAAGCCGCTGCTGAAGAACAGAAGGAAGAGGCTAAAGAAGAAGAGGAGTCTGATGAAGATGTA
     G  G  A  G  A  P  A  A  A  A  G  G  E  A  A  A  E  E  Q  K  E  E  A  K  E  E  E  E  S  D  E  D
701 AGTATAAAATAGCGGTAGTAATTACTACAACTAGTGTTAAGTTATTTTTACTCATTATTTATTCCTTACTATATTGTTATTTTATTACGCAATTAGGTAC
801 TAACTTTTTTATAGATGGGATTCGGATTGTTTGATTAAAATTTTTGTCAATGAGAATCTATGTATCTTTTTAATTGTTCATCAAATAGCTGGCATTTGAT
                  M  G  F  G  L  F  D
901 TGGCATCTCTAAAGTGTAGAAAGGATTTTTGAGTACATAGTCGGAGTAAAGTTCATAAAACTTTTGTAAT
```

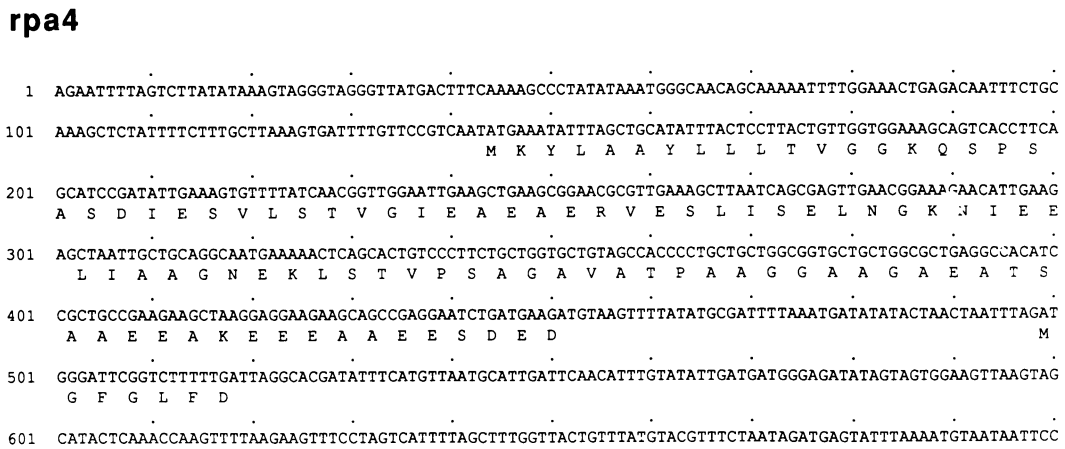

FIG. 2. Sequences of the *RPA1*, *RPA2*, *RPA3*, and *RPA4* genes. The coding regions have been translated into amino acids (one-letter code). The introns were identified by comparing genomic and cDNA sequences.

were selected. The genotypes of transformant colonies were checked by Southern analysis (Fig. 4B). We chose one Ura$^+$ transformant displaying the pattern of one disrupted and one wild-type allele for the *RPA3* gene (bands of 5.1 and 3.8 kb, respectively) and one demonstrating one disrupted and one wild-type allele for the *RPA4* gene (bands of 2.7 + 2.3 and 3.7 kb, respectively). These transformant clones were induced to sporulate, and random spore analysis was performed. Colonies grown on germination medium were streaked both on complete and on selective plates. Of 110 colonies obtained from the sporulation of *RPA3*/*rpa3*::*URA4* heterozygotes, only 22 were capable of growing on selective medium lacking uracil. These colonies were shown to be unsporulated diploids heterozygous for the *RPA3* gene. Growth on selective medium was not observed for any of 103 colonies obtained from sporulation of *RPA4*/*rpa4*::*URA4* heterozygotes. These results indicate that haploids carrying the Ura$^+$ marker, and hence a disrupted *rpa3* or *rpa4* allele, are inviable. A few tetrads were also dissected, confirming the inviability of *rpa3* and *rpa4* haploids.

To assess whether the *rpa1* and *-2* mutants have any phenotype at all, we measured the growth rates of the different haploid strains in rich liquid medium at different temperatures (Table 2). The disruption of the nonessential genes was shown to cause a small increase in duplication times at all temperatures tested. A slow-growth phenotype is also observed when nonessential ribosomal genes are disrupted in *S. cerevisiae* (1, 14, 29).

Expression of *RPA* genes in wild-type and mutant strains. Total RNA was prepared from exponentially growing haploid cells of the wild-type strain and of disrupted mutants *rpa1*::m-Tn*3*(*LEU2*) and *rpa2*::*LEU2*. Equal amounts of RNA from the three strains were run on agarose gels, blotted, and hybridized against oligonucleotides specific for each one of the four genes. To maximize the sequence difference between probes, we used oligonucleotide corresponding to nonconserved sequences 3′ to the termination codon of the four genes. As an internal control, the filters were also probed for 7LS RNA (27); in addition, the oligonucleotide probes were tested against standard amounts of cDNA to check their hybridization efficiencies.

rpa1	/GTACGTT	112bp	TACTAAT	7bp	TAG/
rpa2	/GTAAGTT	41bp	TACTAAC	5bp	TAG/
rpa3-1	/GTAAGTT	114bp	TATTAAC	8bp	TAG/
rpa3-2	/GTAAGTA	93bp	TACTAAC	7bp	TAG/
rpa4	/GTAAGTT	24bp	TACTAAC	5bp	TAG/
consensus	/GTAaGTt		TAcTAAc	5-8bp	TAG/

FIG. 3. Comparison of intron sequences. Conserved sequences in all five introns are outlined, and the derived consensus sequences are shown at the bottom. A slash indicates both 5′ and 3′ exon-intron borders.

The *RPA1*, *-2*, *-3*, and *-4* transcripts showed similar but not identical mobilities in formaldehyde gels, corresponding to lengths of about 400 nucleotides (data not shown). The wild-type strain expressed all four *RPA* genes at the same time, although at different levels (Table 3). The *RPA1* transcript was about fivefold more abundant than the *RPA3* transcript, whereas the transcripts for *RPA2* and *RPA4* had similar, intermediate abundances. No signal was detected from the *RPA1* transcript in the *rpa1*::m-Tn*3*(*LEU2*) strain or from the *RPA2* transcript in the *rpa2*::*LEU2* strain, confirming that the corresponding genes were indeed interrupted before the sequence to which the probes hybridized. There was no significant alteration in the steady-state levels of the other three *RPA* transcripts when either the *RPA1* or *RPA2* gene was disrupted. Transcription of the four *RPA* genes therefore appears to be controlled independently.

DISCUSSION

It had been shown previously that multicellular organisms (a crustacean, an insect, and a vertebrate) contain two small acidic ribosomal proteins (15, 25, 28, 42), whereas the budding yeast *S. cerevisiae* contains four distinct proteins (19, 20, 26). We report here that the distantly related yeast *S. pombe* contains four genes for small A-type ribosomal proteins, confirming the suggestion that this might be a characteristic of lower eucaryotes (26).

The four small A-proteins of *S. pombe* are all very similar to each other, and they can be divided in two couples of more closely related amino acid sequences. The sequence of the two proteins of each couple are related to one of the two A-proteins of multicellular organisms. The structures of the four *RPA* genes of *S. pombe* are consistent with a common origin from an ancestral gene containing two introns, which duplicated twice and lost one or the other of the introns after the second duplication.

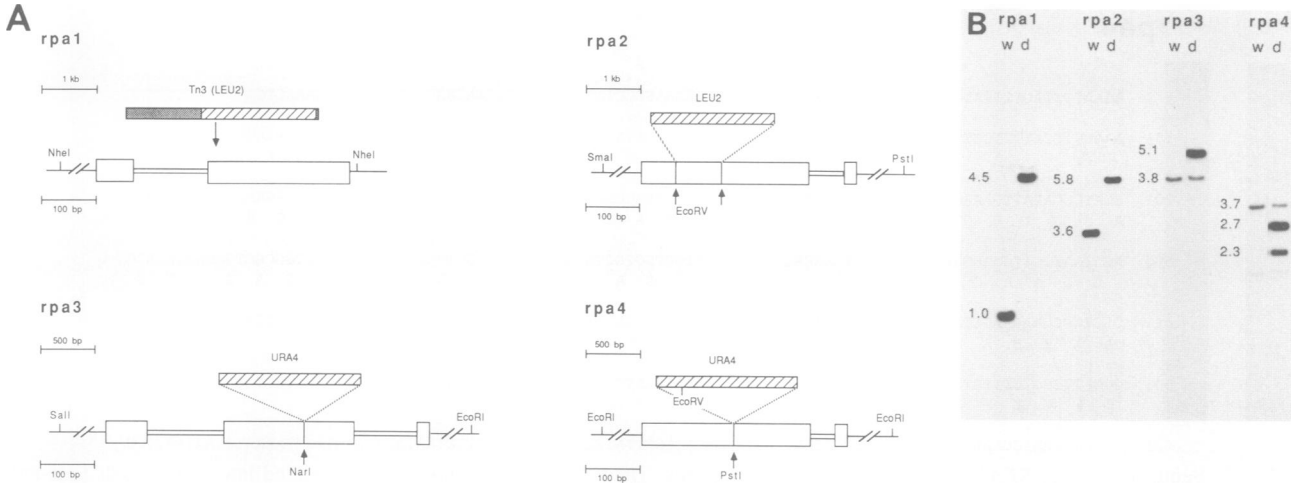

FIG. 4. Gene disruptions. (A) Scheme of insertion of selectable markers within the *RPA* genes (see Materials and Methods for a description). Symbols: ▭ and ═, exons and introns, respectively; ▨, selectable markers (*LEU2* or *URA4*); ▩, transposon sequences. Scales of inserted fragments and genomic sequences are given at the top and bottom, respectively, of each scheme. Arrows identify sites of insertion of the selectable markers. Other restriction sites used for cleaving genomic DNA and making probes for Southern analysis are also shown. (B) Southern analysis. The restricted genomic DNAs of a wild-type strain (w) and a disrupted strain (d) hybridized with a labeled probe are shown for each gene; *rpa1* and *rpa2* were introduced in haploid strains; *rpa3* and *rpa4* were introduced in diploid strains, which therefore contain one wild-type and one allele. Restriction enzymes used to cut the chromosomal DNAs: *Nhe*I (*rpa1*), *Sma*I and *Pst*I (*rpa2*), *Sal*I and *Eco*RI (*rpa3*), and *Eco*RI and *Eco*RV (*rpa4*). The probes used span the following genomic sites: *Nhe*I-*Nhe*I (*rpa1*), *Sma*I-*Pst*I (*rpa2*), *Sal*I-*Eco*RI (*rpa3*), and *Eco*RI-*Eco*RI (*rpa4*). Insertion of m-Tn*3*(*LEU2*) produces a size increase of 3.5 kb, insertion of *LEU2* produces an increase of 2.2 kb, and insertion of *URA4* produces an increase of 1.3 kb. In the case of the *rpa4*::*URA4* allele, an additional *Eco*RV site is introduced by the *URA4* marker.

Many ribosomal protein genes in the yeast *S. cerevisiae* are duplicated, and the same situation applies to other eucaryotes. Usually the two copies of a particular duplicated gene give rise to indistinguishable protein products, but this does not appear to be the case for *S. pombe* A-proteins. The sequence divergence within the two couples of proteins is somewhat more than is usual for products of duplicated genes and suggests that each protein might have a specific function. A clue for the explanation of this puzzling observation may be the unique stoichiometry of A-proteins. Both procaryotes and eucaryotes contain four copies of A-proteins per large subunit. *E. coli* contains four copies of the unique *rplL* gene product, although they are differentiated by the presence or the absence of acetylation at their N termini (22, 35). On the other hand, *A. salina* ribosomes contain two copies each of the products of two different genes, eL12 and eL12′ (also called P1 and P2), each forming a homodimer (37). It is quite possible that the products of the four genes of *S. pombe* are present in stoichiometric amounts on 60S subunits, possibly forming A1-A3 and A2-A4 heterodimers. The couple A1-A3 shows special features that support the hypothesis of dimer formation: the N-terminal part of each protein is predicted by computer analysis to adopt an alpha-helical conformation, the ratio of polar to apolar residues is close to 1, and both peptide sequences contain a leucine (or another small hydrophobic residue) every seventh residue. Peptides with such characteristics have a strong tendency to form coiled-coil structures and dimerize, often forming het-

TABLE 2. Growth rates of *S. pombe* strains carrying wild-type and mutant *RPA* genes[a]

Relevant genotype	Temp (°C)	Doubling time	
		Min	Normalized[b]
RPA1 RPA2	25	254	100
	31	140	100
	37	136	100
rpa1 RPA2	25	281	90
	31	175	80
	37	175	78
RPA1 rpa2	25	296	86
	31	175	80
	37	169	80

[a] Haploid wild-type and mutant strains were grown in YEL medium (0.5% yeast extract [Difco Laboratories], 3% glucose) under rotary agitation. The A_{595} of samples taken every 2 h was determined for at least 14 h. The growth curve was fitted with a least-squares algorithm.

[b] Number of cell divisions per hour of indicated strain divided by number of cell divisions per hour of wild-type strain at the same temperature.

TABLE 3. Expression of *RPA* genes[a]

Genotype	Amt of transcript[b]			
	RPA1	*RPA2*	*RPA3*	*RPA4*
RPA1 RPA2	2.6	1.2	0.5	1.0
rpa1 RPA2	0.0	1.0	0.5	0.9
RPA1 rpa2	2.3	0.0	0.6	1.0

[a] Total RNAs extracted from wild-type, *rpa1*, and *rpa2* haploid strains were run on an agarose-formaldehyde gel alongside known amounts of *Eco*RI-cut pTZrpa DNAs. The nucleic acids were transferred to nylon filters and hybridized with 15-mers corresponding to unique 3′ sequences of the four genes (oligonucleotide rpa1, TCATGCTAGCAGATT; oligonucleotide rpa2, GAAAACAAGGGATCA; oligonucleotide rpa3, GATACATAGATTCTC; oligonucleotide rpa4, CATGAAATATCGTGC). The filters were then rehybridized to a probe for 7SL RNA. The autoradiographic films were subjected to densitometric analysis. The relative amount of transcript for each gene in the three strains was determiend by normalizing the Northern signal obtained by hybridization to each of the oligonucleotides with respect to the total amount of RNA loaded in the gel (the 7SL RNA signal) and to the hybridization efficiencies of the oligonucleotides (the signals obtained from the standard amounts of cDNAs).

[b] Expressed as arbitrary units. The amount of *RPA4* mRNA in the wild type is taken to be 1 U.

erodimers with peptides of like structure (4). Such is the case, for example, of the dimer formed by the Jun protein with members of the Fos protein family (23, 36). The couple A2-A4 does not have leucine heptads, but given its high similarity to the dimerizing *A. salina* P2 protein, it might also form a dimer. The evolution from one protein in four copies per ribosome to two proteins in two copies and eventually to four proteins in one copy may allow for subtle refinements in the functioning of an important site of the 60S ribosomal subunit, involved in multiple contacts with translation factors and in GTP hydrolysis.

The genetic analysis we performed clearly indicates that two genes, *RPA3* and *RPA4*, are essential for cell survival, whereas *RPA1* and *RPA2* are not. This finding is somewhat surprising given the high similarity between the products of each couple of essential and nonessential genes. Duplicated genes are usually not essential: disruption of either gene of a couple often produces only a rather mild phenotype of reduced growth rates, and only the disruption of both genes is lethal to the cell (1, 14, 29). On the other hand, if the four *RPA* genes of *S. pombe* have effectively diverged to serve separate functions, one might expect all of them to be essential. In our view, two possible explanations of the data are most likely. The first is that the four gene products are in fact interchangeable but two of them constitute the bulk of small acidic ribosomal proteins available in the cell, whereas the other two are much less abundant. A precedent for such a situation is that of ribosomal protein rp28 of *S. cerevisiae*, which is encoded by two genes expressed at different levels. The amount of rp28-1 mRNA exceeds that of rp28-2 mRNA by about a factor of 6; deletion of the rp28-1 gene is a lethal event, whereas the rp28-2 gene is dispensable for growth (17). The second possibility is that the four *RPA* genes are at an intermediate stage of divergence and specialization, where one member of each couple is sufficient to carry out a basic function (in this case, contribute to a functional ribosome) but the other member adds an extra feature or a refinement to the process.

Our present data do not allow us to distinguish conclusively whether the specific primary sequences of proteins A1 and A2 are inadequate to ensure the correct assembly or functioning of the 60S subunit of the ribosome or whether the amounts of A1 and A2 proteins present in the cells are not sufficient to support life. The direct measurement of protein levels is complicated by the extreme physical and immunological similarity of *S. pombe* A-proteins and by alternative states of phosphorylation (data not shown). However, analysis of the steady-state *RPA* mRNA levels indicates that there is no dramatic difference between the transcripts for the essential proteins and those for the nonessential ones; at any rate, the most abundant transcript is that for A2, which is dispensable. Therefore, A1 and A2 might be much scarcer than A3 and A4 only if their mRNAs are translated with very low efficiency or if these proteins are very rapidly degraded.

The alternative hypothesis of a qualitative difference between essential and nonessential A-proteins is favored mainly by default; however, it fits rather nicely with the idea that A-proteins are present in stoichiometric amounts as heterodimers on *S. pombe* ribosomes. Proteins A3 and A4 would be able to produce functional homodimers, in addition to heterodimers with A1 and A2, whereas homodimers of A1 and A2 would be either unstable or nonfunctional. There is at least one example of a similar situation, again involving proteins Jun and Fos. Jun and Fos, or peptides corresponding to their dimerization domains, preferentially form heterodimers. Jun can also form a homodimer, although at reduced efficiency, whereas there has been no evidence for Fos dimerization (6, 8, 23, 33, 36).

In conclusion, our data suggest that *S. pombe* A-proteins are present in the 60S ribosomal subunits as A1-A3 and A2-A4 heterodimers, but that A3-A3 and A4-A4 homodimers apparently contain all of the minimum structural information required for ribosome assembly and function. A1-A1 and A2-A2 homodimers are not adequate to ensure a sufficient functionality of the translation machinery. We believe that the uneven relevance for cell survival of the four A-proteins is due to their specific amino acid sequences rather than to their bulk availability in *S. pombe* cells. In addition, one A-protein dimer apparently cannot substitute for the other, suggesting that each of the two A-proteins of multicellular eucaryotes may be essential for cell growth.

ACKNOWLEDGMENTS

We thank Lennart Philipson for continuing support, Riccardo Cortese, Iain Mattaj, and David Tollervey for laboratory space and much advice, and all of our colleagues at the Gene Expression Program for helpful discussions.

This work was partially supported by a grant from Ministero della Pubblica Istruzione (Italy). M.B. was the recipient of a biotechnology training contract from the Commission of European Communities and of a short-term fellowship from Fondazione Anna Villa Rusconi.

LITERATURE CITED

1. **Abovich, N., and M. Rosbash.** 1984. Two genes for ribosomal protein 51 of *Saccharomyces cerevisiae* complement and contribute to the ribosomes. Mol. Cell. Biol. **4**:1871–1879.
2. **Beach, D. H., and P. M. Nurse.** 1981. High-frequency transformation of the fission yeast *Schizosaccharomyces pombe*. Nature (London) **290**:140–142.
3. **Beltrame, M., and M. E. Bianchi.** 1987. Sequence of the cDNA for one acidic ribosomal protein of *Schizosaccharomyces pombe*. Nucleic Acids Res. **15**:9089.
4. **Cohen, C., and D. A. D. Parry.** 1986. α-Helical coiled coils—a widespread motif in proteins. Trends Biochem. Sci. **11**:137–140.
5. **Elkon, K., S. Skelly, A. Parnassa, W. Moeller, W. Danho, H. Weissbach, and N. Brot.** 1986. Identification and chemical synthesis of a ribosomal protein antigenic determinant in systemic lupus erythematosus. Proc. Natl. Acad. Sci. USA **83**: 7419–7423.
6. **Gentz, R., F. J. Rauscher III, C. Abate, and T. Curran.** 1989. Parallel association of Fos and Jun leucine zippers juxtaposes DNA binding domains. Science **243**:1695–1699.
7. **Gutz, H., H. Heslot, U. Leupold, and N. Loprieno.** 1974. *Schizosaccharomyces pombe*, p. 395–446. *In* R. C. King (ed.), Handbook of genetics, vol. 1. Plenum Publishing Corp., New York.
8. **Halazonetis, T. D., K. Georgopoulos, M. E. Greenberg, and P. Leder.** 1988. c-Jun dimerizes with itself and with c-Fos, forming complexes of different DNA binding affinities. Cell **55**:917–924.
9. **Hamil, K. G., H. G. Nam, and H. M. Fried.** 1988. Constitutive transcription of yeast ribosomal protein gene *TCM1* is promoted by uncommon *cis*- and *trans*-acting elements. Mol. Cell. Biol. **8**:4328–4341.
10. **Hamilton, R., C. K. Watanabe, and H. A. de Boer.** 1987. Compilation and comparison of the sequence context around the AUG start codons in *Saccharomyces cerevisiae* mRNAs. Nucleic Acids Res. **15**:3581–3593.
11. **Ito, H., Y. Fukuda, K. Murata, and A. Kimura.** 1983. Transformation of intact yeast cells treated with alkali cations. J. Bacteriol. **153**:163–168.
12. **Kirsebom, L. A., and L. A. Isaksson.** 1985. Involvement of ribosomal protein L7/L12 in control of translational accuracy. Proc. Natl. Acad. Sci. USA **82**:717–721.
13. **Lin, A., B. Wittman-Liebold, J. McNally, and I. G. Wool.** 1982.

The primary structure of the acidic phosphoprotein P2 from rat liver 60 S ribosomal subunits. Comparison with ribosomal 'A' proteins from other species. J. Biol. Chem. **257**:9189–9197.

14. **Lucioli, A., C. Presutti, S. Ciafré, E. Caffarelli, P. Fragapane, and I. Bozzoni.** 1988. Gene dosage alteration of L2 ribosomal protein genes in *Saccharomyces cerevisiae*: effect on ribosome synthesis. Mol. Cell. Biol. **8**:4792–4798.
15. **Maassen, J. A., E. N. Schop, J. H. G. M. Brands, F. J. Van Hemert, J. A. Lenstra, and W. Moeller.** 1985. Molecular cloning and analysis of cDNA sequences for two ribosomal proteins from *Artemia*. The coordinate expression of genes for ribosomal proteins and elongation factor 1 during embryogenesis of *Artemia*. Eur. J. Biochem. **149**:609–616.
16. **MacConnell, W. P., and N. O. Kaplan.** 1982. The activity of the acidic phosphoproteins from the 80 S rat liver ribosome. J. Biol. Chem. **257**:5359–5366.
17. **Mager, W. H.** 1988. Control of ribosomal protein gene expression. Biochim. Biophys. Acta **949**:1–15.
18. **Maxam, A. M., and W. Gilbert.** 1980. Sequencing end-labeled DNA with base-specific chemical cleavages. Methods Enzymol. **65**:499–560.
19. **Mitsui, K., and K. Tsurugi.** 1988. cDNA and deduced amino acid sequence of acidic ribosomal protein A1 from *Saccharomyces cerevisiae*. Nucleic Acids Res. **16**:3574.
20. **Mitsui, K., and K. Tsurugi.** 1988. cDNA and deduced amino acid sequence of acidic ribosomal protein A2 from *Saccharomyces cerevisiae*. Nucleic Acids Res. **16**:3575.
21. **Moeller, W.** 1974. The ribosomal components involved in EF-G- and EF-Tu-dependent GTP hydrolysis, p. 711–731. *In* M. Nomura, A. Tissieres, and P. Lengyel (ed.), Ribosomes. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
22. **Moeller, W., and J. A. Maassen.** 1986. On the structure, function, and dynamics of L7/L12 from *Escherichia coli* ribosomes, p. 309–325. *In* B. Hardesty and G. Kramer (ed.), Structure, function, and genetics of ribosomes. Springer-Verlag, New York.
23. **O'Shea, E. K., R. Rutkowski, W. F. Stafford III, and P. S. Kim.** 1989. Preferential heterodimer formation by isolated leucine zippers from Fos and Jun. Science **245**:646–648.
24. **Otaka, E., K. Higo, and T. Itoh.** 1983. Yeast ribosomal proteins. VII. Cytoplasmic ribosomal proteins from *Schizosaccharomyces pombe*. Mol. Gen. Genet. **191**:519–524.
25. **Qian, S., J. Y. Zhang, M. A. Kay, and M. Jacobs-Lorena.** 1987. Structural analysis of the *Drosophila* rpA1 gene, a member of the eucaryotic 'A' type ribosomal protein family. Nucleic Acids Res. **15**:987–1003.
26. **Remacha, M., M. T. Saenz-Robles, M. D. Vilella, and J. P. G. Ballesta.** 1988. Independent genes coding for three acidic ribosomal proteins of the large ribosomal subunit from *Saccharomyces cerevisiae*. J. Biol. Chem. **263**:9094–9101.
27. **Ribes, V., P. Dehoux, and D. Tollervey.** 1988. 7SL RNA from *Schizosaccharomyces pombe* is encoded by a single copy essential gene. EMBO J. **7**:231–237.
28. **Rich, B. E., and J. A. Steitz.** 1987. Human acidic ribosomal phosphoproteins P0, P1, and P2: analysis of cDNA clones, in vitro synthesis, and assembly. Mol. Cell. Biol. **7**:4065–4074.
29. **Rotemberg, M. O., M. Moritz, and J. L. Woolford, Jr.** 1988. Depletion of *Saccharomyces cerevisiae* ribosomal protein L16 causes a decrease in 60 S ribosomal subunits and formation of half-mer polyribosomes. Genes Dev. **2**:160–172.
30. **Rubin, G. M.** 1975. Preparation of RNA from yeast. Methods Cell Biol. **12**:45–64.
31. **Sanchez-Madrid, F., F. J. Vidales, and J. P. G. Ballesta.** 1981. Functional role of acidic ribosomal proteins. Interchangeability of proteins from bacterial and eukaryotic cells. Biochemistry **20**:3263–3266.
32. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74**:5463–5467.
33. **Sassone-Corsi, P., L. J. Ransone, W. W. Lamph, and I. M. Verma.** 1988. Direct interaction between fos and jun nuclear oncoproteins: role of the 'leucine zipper' domain. Nature (London) **336**:692–695.
34. **Seifert, H. S., E. Y. Chen, M. So, and F. Heffron.** 1986. Shuttle mutagenesis: a method of transposon mutagenesis for *Saccharomyces cerevisiae*. Proc. Natl. Acad. Sci. USA **83**:735–739.
35. **Terhorst, C., W. Moeller, R. Laursen, and B. Wittman-Liebold.** 1973. The primary structure of an acidic protein from 50 S ribosomes of *Escherichia coli* which is involved in GTP hydrolysis dependent on elongation factors G and T. Eur. J. Biochem. **34**:138–152.
36. **Turner, T., and R. Tjian.** 1989. Leucine repeats and an adjacent DNA binding domain mediate the formation of functional cFos-cJun heterodimers. Science **243**:1689–1694.
37. **Uchiumi, T., A. J. Wahba, and R. R. Traut.** 1987. Topography and stoichiometry of acidic proteins in large ribosomal subunits from *Artemia salina* as determined by crosslinking. Proc. Natl. Acad. Sci. USA **84**:5580–5584.
38. **van Agthoven, A. J., J. A. Maassen, and W. Moeller.** 1977. Structure and phosphorylation of an acidic protein from 60 S ribosomes and its involvement in elongation factor 2 dependent GTP hydrolysis. Biochem. Biophys. Res. Commun. **77**:989–997.
39. **Vieira, J., and J. Messing.** 1982. The pUC plasmids, an M13mp7-derived system for insertion mutagenesis and sequencing with synthetic universal primers. Gene **19**:259–268.
40. **Vignais, M.-L., L. P. Woudt, G. M. Wassenaar, W. H. Mager, A. Sentenac, and R. J. Planta.** 1987. Specific binding of TUF factor to upstream activation sites of yeast ribosomal protein genes. EMBO J. **6**:1451–1457.
41. **Warner, J. R.** 1989. Synthesis of ribosomes in *Saccharomyces cerevisiae*. Microbiol. Rev. **53**:256–271.
42. **Wigboldus, J. D.** 1987. cDNA and deduced amino acid sequence of Drosophila rp21C, another A-type ribosomal protein. Nucleic Acids Res. **15**:1064.
43. **Young, R. A., and R. W. Davis.** 1983. Efficient isolation of genes using antibody probes. Proc. Natl. Acad. Sci. USA **80**:1194–1198.