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YRA1, an essential *Saccharomyces cerevisiae* gene, encodes a novel nuclear protein with RNA annealing activity

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ABSTRACT

The complexity of eukaryotic mRNA processing suggests a need for certain factors, called RNA chaperones, that can modulate RNA secondary structure as well as the interactions between pre-mRNA and *trans*-acting components. To identify factors that may fulfill this role in the yeast *Saccharomyces cerevisiae*, we fractionated whole-cell extracts and assayed for activity that could facilitate a specific RNA–RNA annealing reaction. We detected one strong RNA annealing activity and purified it to homogeneity. This previously undescribed factor, Yra1p, is localized to the nucleus; its sequence contains one RNP-motif RNA-binding domain. The *YRA1* gene contains a 766-nt intron, the second-largest identified in this organism, and Yra1p serves an essential, nonredundant function. Taken together, our findings indicate that Yra1p is likely to have an important role in *S. cerevisiae* nuclear pre-mRNA metabolism.

Keywords: hnRNP protein; RBD; RNA-binding; RNA chaperone; RNA processing; RNP-CS; RNP motif; splicing

INTRODUCTION

In eukaryotic organisms, the process of mRNA biogenesis encompasses the removal of introns, the formation of mature 5′ and 3′ ends, and nucleocytoplasmic transport of the mature mRNA. Many specific RNA–protein and RNA–RNA interactions occur during these processes, the most well-described being those between snRNAs and particular sites on the pre-mRNA (Moore et al., 1993; Madhani & Guthrie, 1994). The RNA cleavages and ligations of splicing and polyadenylation take place with single-nucleotide precision, and occur superimposed upon a background of 10^3–10^4 nt that have the potential for incidental secondary structure that could obscure critical sites. These processes are therefore likely to require factors that promote specificity and inhibit background or fortuitous interactions. Some of these factors may act as RNA chaperones (Coetzee et al., 1994; Portman & Dreyfuss, 1994; Herschlag, 1995), which modulate RNA structure, destabilizing weak, nonspecific RNA–RNA interactions so that stronger RNA–RNA or RNA–protein interactions may occur.

Using a variety of assays, it has been shown that the human hnRNP A1 protein is able to promote the specific interaction of complementary ssRNAs (Kumar & Wilson, 1990; Pontius & Berg, 1990, 1991; Munroe & Dong, 1992; Portman & Dreyfuss, 1994). Because A1 is an abundant nuclear pre-mRNA-binding protein (Dreyfuss et al., 1993; Kiledjian et al., 1994), is has been suggested that this property is important for its functions in mRNA biogenesis (Pontius & Berg, 1992; Pontius, 1993; Portman & Dreyfuss, 1994). Subsequently, using a purification-based strategy designed to identify the major RNA annealing activities in human HeLa tissue culture cells, many of the human pre-mRNA-binding hnRNP proteins were found to promote RNA annealing, and it has been suggested that, for some hnRNP proteins, this property results from a more general RNA chaperone function (Portman & Dreyfuss, 1994). The human hnRNP proteins include approximately 20 major polypeptides, designated hnRNP A1–hnRNP U, which associate with nascent pre-mRNAs. They have been implicated in diverse functions including splice-site choice (Swanson & Dreyfuss, 1988b; Caceres et al., 1994), nuclear retention of pre-mRNAs (Nakielny & Dreyfuss, 1996), and nuclear export of mature mRNAs (Piñol-Roma & Dreyfuss, 1992; Mi-

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chael et al., 1995). The RNA annealing activity of many of these proteins suggests that hnRNP proteins, as they bind to and coat nascent RNA polymerase II transcripts, facilitate specific and rapid intermolecular interactions, while diminishing hindrance from fortuitous RNA secondary structure.

These results suggest that a search for RNA annealing activities in other organisms may be a useful strategy for identifying hnRNP proteins or other factors involved in regulating the interactions of pre-mRNAs. Here we report such a study in the yeast *Saccharomyces cerevisiae*. Comparatively little is known about the complement of pre-mRNA binding proteins in this organism, although it is clear that there is not a one-to-one correspondence between vertebrate and yeast factors (Anderson et al., 1993a, 1993b; Matunis et al., 1993). However, a great deal has been established from genetic and molecular studies of yeast pre-mRNA processing mechanisms (Guthrie, 1991; Ruby & Abelson, 1991; Rymond & Rosbash, 1992). The maturation of pre-mRNAs in *S. cerevisiae* is significantly different than in vertebrate cells, occurring by a highly ordered pathway, with more rigid sequence and structural requirements than in higher eukaryotes (Woolford, 1989; Guthrie, 1991; Rymond & Rosbash, 1992). The majority of yeast genes do not contain introns; in those that do, there is generally only one intron of relatively small size (~50 nt) located near the 5′ end of the mRNA (Kalogeropoulos, 1995). Regulation of processing is rare; well-described examples of this include the splicing of the *RPL32* mRNA, which is directly inhibited by its own product (Vilardell & Warner, 1994; Li et al., 1996) and the meiosis-specific splicing of the *MER2* transcript (Engebrecht et al., 1991).

Despite these differences between mRNA processing in yeast and vertebrate systems, the same general concerns regarding the potential hazards of random RNA structure and the need to facilitate rapid, specific interactions with pre-mRNAs apply in *S. cerevisiae* as they do in vertebrates. Indeed, there may exist special requirements for specific pre-mRNA structure in yeast mRNA processing: it has been found that intramolecular pre-mRNA base pairing is important for promoting the efficiency of splicing (Deshler & Rossi, 1991; Goguel & Rosbash, 1993; Libri et al., 1995) and, in special cases, for the transcript-specific regulation of splicing (Eng & Warner, 1991). Factors that facilitate or hinder formation of such structures are therefore likely to be important for mRNA biogenesis.

Several nuclear RNA-binding proteins that may function as hnRNP proteins have been identified recently in *S. cerevisiae*. Some, such as Pub1p and the Nab proteins, have been isolated through biochemical means (Anderson et al., 1993a, 1993b; Matunis et al., 1993; Wilson et al., 1994); others (such as Npl3p and Hrp1p) have emerged from genetic screens (Bossie et al., 1992; Henry et al., 1996). However, specific functions have yet to be attributed to them. Here, we report the use of an assay for a biochemical function—RNA annealing activity—to identify a novel yeast protein. This factor, Yra1p, is nuclear and likely binds directly to RNA; furthermore, it is essential for *S. cerevisiae* viability. These results provide a new point of entry for the study of RNA-binding proteins in budding yeast.

RESULTS

Identification of an RNA annealing activity in *S. cerevisiae*

To systematically identify RNA annealing activities in yeast, we fractionated whole-cell lysate on an ssDNA-cellulose column [to enrich for RNA-binding proteins, many of which display an affinity for ssDNA in vitro (Piñol-Roma et al., 1990)] and assayed fractions using an RNA annealing assay, as reported previously (Portman & Dreyfuss, 1994). For this assay, two in vitro-transcribed RNAs were used: the "sense" RNA (454-nt long) and the probe RNA (internally ^{32}P-labeled, 99-nt long, 60 nt of which are the perfect complement to a central region of the sense RNA). These two RNAs were mixed together in the presence of protein fractions. After a brief incubation, RNAse T_1 was added to digest single-stranded RNAs, and the digestion products were analyzed by denaturing electrophoresis. The presence of a 66-nt protected, labeled fragment indicates that dsRNA was formed. Under these conditions, virtually no background annealing occurs in the absence of protein or with negative control proteins, such as *Escherichia coli* single-stranded binding protein (SSB) (Portman & Dreyfuss, 1994).

One prominent peak of RNA annealing activity was detected in the ssDNA column eluate, in fractions 38–40 (Fig. 1A). Further fractionation of the multiple proteins in this sample by cation-exchange chromatography (Fig. 1B) revealed a strong peak of activity (fraction 24). Other fractions had lower activity that was not detected consistently. The elution profile of RNA annealing activity correlated well with that of the only major protein in fraction 24, a 27-kDa protein, which we named Yra1p (for yeast RNA annealing protein). Yra1p appears to be a low-abundance factor, because it is barely visible in the initial fractionation (Fig. 1A), although this could also be an artifact of the silver-staining process. The detection of a single strong RNA annealing activity contrasts with the multiple peaks found in a similar fractionation from HeLa cell extracts (Portman & Dreyfuss, 1994).

Subcellular localization of Yra1p

Yra1p was purified on a preparative scale using the same procedures and was used to generate a murine polyclonal antiserum. By western blot, the obtained

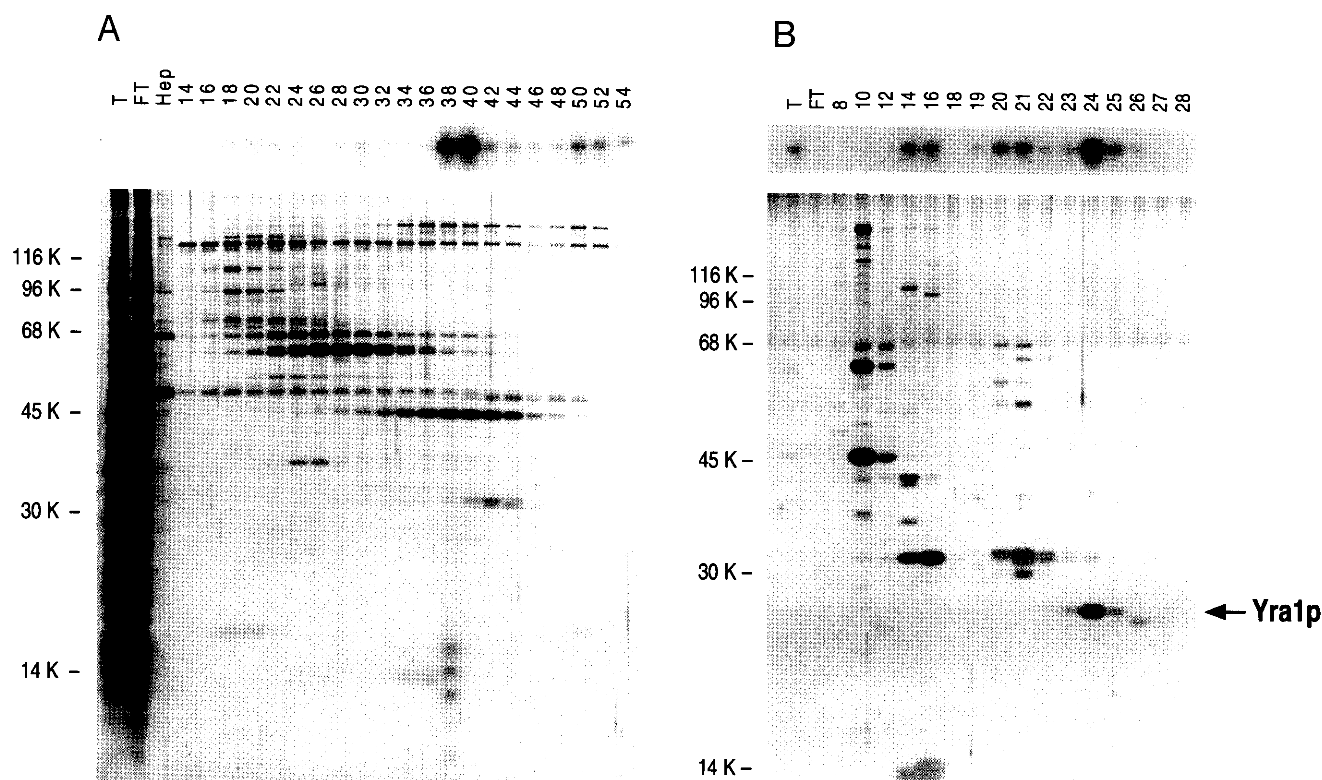

FIGURE 1. Identification of RNA annealing activities in *S. cerevisiae*. **A:** Total yeast lysate was fractionated on an ssDNA-cellulose column. Fractions were analyzed by SDS-PAGE and silver staining (below) and tested for RNA annealing activity (above). For the RNA annealing assay, only the region of the gel containing the 66-nt protected fragment is shown; the presence of a band is indicative of RNA annealing activity. **B:** Fractions 38–40 from the ssDNA-cellulose fractionation were subjected to cation-exchange chromatography and assayed again for RNA annealing activity. The activity identified in fraction 24 was the strongest detected; the 27-kDa protein in this fraction was designated Yra1p.

antiserum (but not the pre-immune serum; data not shown) recognized Yra1p strongly in enriched fractions and, although more weakly, detected one major band in total *S. cerevisiae* lysate (Fig. 2A). When this serum was used to detect Yra1p in situ by indirect immunofluorescence, the signal was found uniformly localized in the yeast nucleus (Fig. 2B), suggesting that Yra1p is a nuclear protein, and that its function may involve pre-mRNA metabolism.

Cloning of the *YRA1* gene

To further study Yra1p, amino acid sequence was obtained from two Yra1p fragments, called peptides *4* and *5*, generated by lysyl endopeptidase digestion (Table 1). Database queries (Altschul et al., 1990) using these sequences identified the *S. cerevisiae* expressed-sequence tag EST101677 (GenBank accession number T36443) as a candidate for the *YRA1* sequence. The conceptual translation of EST101677 contains a perfect copy of peptide *4*, and a partial match to the sequence of peptide *5*. It seems likely that EST101677 represents a *YRA1* transcript, despite several mismatches, for the following reasons. First, three of the residues that do not match peptide *5* were scored as histidines in the peptide sequence, but are alanines in the translation of EST101677. Visual inspection of the primary data (the HPLC chromatogram; data not shown) reveals that there was poor resolution of the alanine and histidine standards; it therefore seems likely that the automated analysis of our sequencing data was inaccurate in this regard. Second, two of the peptide *5* residues were judged to be modified amino acids, because they eluted between the positions of the tyrosine and arginine standards (data not shown). Because these positions are represented by codons for arginine in the EST sequence, it is possible that Yra1p contains the modified amino acid N^G,N^G-dimethylarginine (DMA), which is a common posttranslational modification in nuclear RNA-binding proteins, including many hnRNP proteins (Liu & Dreyfuss, 1995).

The EST sequence was used to design PCR primers (**YRA1-EST F** and **YRA1-EST R**) that amplified a 260-bp probe fragment from *S. cerevisiae* genomic DNA. Using this fragment to screen a *S. cerevisiae* genomic phage library resulted in the isolation of two clones, of ~1.3 kb and ~3.9 kb in length. During the sequencing of the longer clone, a database search found a perfect match of its sequence in a GenBank entry (accession number U28373) that contains 42 kb of genomic se-

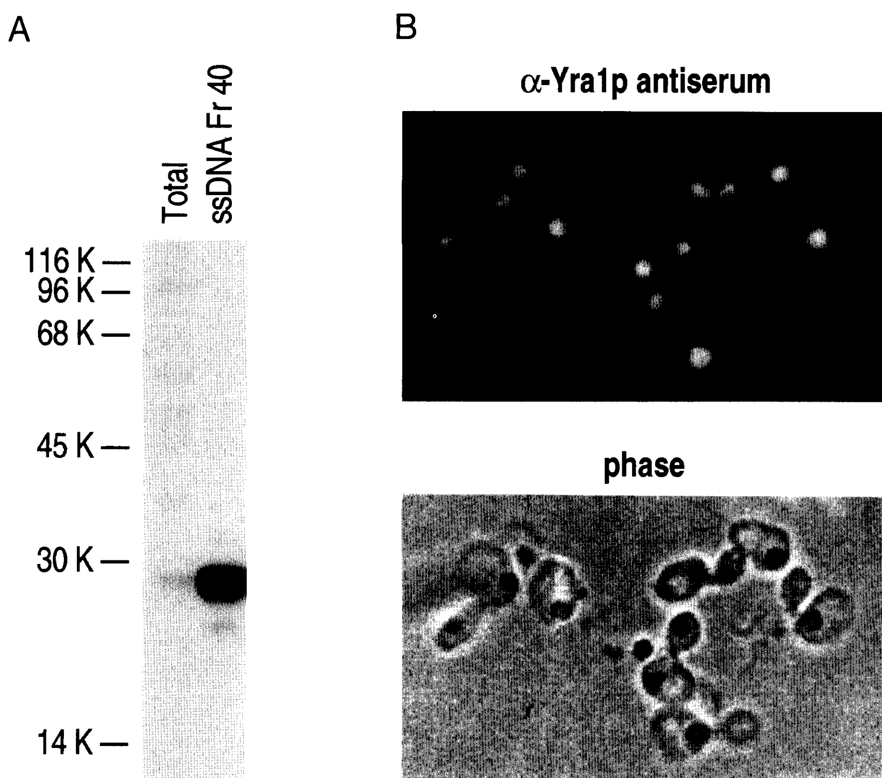

FIGURE 2. Yra1p antiserum recognizes Yra1p by western blot and decorates the nucleoplasm of *S. cerevisiae*. **A:** Western blot with the anti-Yra1p antiserum. Lane 1, total *S. cerevisiae* extract. Lane 2, fraction 40 from the ssDNA column fractionation shown in Figure 1A. **B:** *S. cerevisiae* spheroplasts (shown by phase contrast, below) were stained with the Yra1p antiserum. Antibody binding was detected using a fluorescent secondary antibody (above).

quence from a region of *S. cerevisiae* chromosome IV. Although the matching region was not part of an open reading frame as predicted by the Genome Sequencing Center, visual inspection of this region and its conceptual translation products (Fig. 3) identified an unannotated short open reading frame (ORF) containing both peptide sequences (nt 33916–34200) followed, 766-bp downstream, by another ORF of 392 bp. (Part of this latter ORF, starting from its most 5′ methionine codon, had been identified as gene D9481.2 by the Genome Sequencing Center.) Based on the data below, we propose that these two ORFs encode Yra1p.

A large intron

The region between these two ORFs, spanning nt 34201–34966 of the cosmid sequence, has the hallmarks of a

TABLE 1. Comparison of Yra1p peptide sequence with EST101677.

	Sequence[a]
Peptide *4*	QVGSQRRSLPNRRGP
EST101677	. . .QVGSQRRSLPNRRGP. . .
Peptide *5*	NTXPPPNHVHXVHK
EST101677	. . .NTRPPPNAVARVAK. . .

[a]Sequences are shown in standard one-letter code from the amino-terminal end. Underlined residues indicate mismatches; "X" represents a putative modified amino acid eluting between the positions of the alanine and histidine standards.

yeast intron: the putative 5′ and 3′ splice sites (highlighted in Fig. 3) conform to the consensus sequences for these regions (Rymond & Rosbash, 1992; Kalogeropoulos, 1995), and a potential branch point exists, although it is slightly divergent (UgACUAAC in *YRA1* versus the consensus UACUAAC). Because of the large size of this region and the unusual branch point sequence, we sought to determine experimentally whether it is excised in mature *YRA1* mRNA by using reverse-transcription (RT)-PCR. Using primers flanking the putative intron, fragments were amplified from both yeast genomic DNA and reverse-transcribed total yeast RNA. Figure 4 shows the presence of the predicted 205-bp fragment amplified from the spliced product in the cDNA sample, but not from genomic DNA, demonstrating that the 677-bp interruption is genuinely an intron. To our knowledge, this sequence represents the second-largest intron in *S. cerevisiae*, the largest being the 1,001-nt intron in *DBP2* (see Discussion).

Verification of the *YRA1* clone

To confirm that the cloned gene encodes the protein that was purified, a cDNA was constructed using RT-PCR (see Materials and Methods) and subcloned into an *E. coli* expression vector. Upon induction, the cells express a 27-kDa polypeptide that is recognized in a western blot with the polyclonal serum raised against

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acctacatctgctaa                                                          33915

atg tct gct aac tta gat aaa tcc tta gac gaa atc att ggc tct aac aaa gca  33969
 M   S   A   N   L   D   K   S   L   D   E   I   I   G   S   N   K   A      18
gga agt aat aga gcc cgt gtc ggt ggt act cgt ggt aac ggt cca aga aga gtt  34023
 G   S   N   R   A   R   V   G   G   T   R   G   N   G   P   R   R   V      36
ggt aag caa gtt ggt agc caa cgt agg agc ctt cca aac aga aga ggc cct atc  34077
 G   K   Q   V   G   S   Q   R   R   S   L   P   N   R   R   G   P   I      54
aga aaa aat act agg gca cct cca aac gca gtc gct aga gtt gcc aag ctc ttg  34131
 R   K   N   T   R   A   P   P   N   A   V   A   R   V   A   K   L   L      72
gac acc act aga gag gtc aag gtc aac gtc gaa ggt ttg cca agg gac att aag  34185
 D   T   T   R   E   V   K   V   N   V   E   G   L   P   R   D   I   K      90
cag gat gct gta aga                                                      34200
 Q   D   A   V   R                                                          95

gtatgttaatacgtgaaatgagagctatttgtttagttactcgcatcgtctcgtgtggatacgagaaatat  34271
tctttgtaaggaaggatatggcacacgcttttgatcaagaattctcttttagagatgaagaaagagtccg  34342
aaatacttcgctagagaaaatacaaaaagagtatcgttttcataagccaaccataccaatttttttcaata  34413
cttcaaatcatggcctataaggttggaattaccagctaccactgagaatgggtttgttcttattggctggg  34484
ttaactcaaatcaatctgactatctgctaatctgtcaatcttacattttaagctggcgtattgtgtcgcc  34555
tcgaccgtgatagttagttcctattttaataagcgtatcatccaatactgaagttgtggaaggatcaatt  34626
aattttgtatcctttcaagagggagtagctttcagtttcgaatggcaccattttcaaaaaatagctatggg  34697
aaacatgtctttcatgaacgttcgaaaagatatgagtgcatcgcttcatttttttattatgatagttacaa  34768
aaatatttgatttgagacacctaatgaaaccaaagaggaactcacccatcgtggtacatgtttatttgagg  34839
gtttatttttcctacgcaatggtatactttaatactctcaactccttgattgtttgtgtattgtcccttc  34910
cttctttgattttatatcatagagatatatgactaacttttttttttttattatag                  34966

gaa ttt ttt gca tct caa gta ggt ggt gtt caa aga gtt ttg ttg agt tat aac  35020
 E   F   F   A   S   Q   V   G   G   V   Q   R   V   L   L   S   Y   N     113
gaa agg ggc caa tct act ggt atg gct aac atc aca ttc aaa aac ggt gaa ttg  35074
 E   R   G   Q   S   T   G   M   A   N   I   T   F   K   N   G   E   L     131
gcc aga agg gct gtt gaa aga ttt aac ggc tct cca atc gat gga ggc aga tca  35128
 A   R   R   A   V   E   R   F   N   G   S   P   I   D   G   G   R   S     149
aga ttg aga ctt aac cta atc gtt gat cca aac caa cgc cca gtc aaa agt tta  35182
 R   L   R   L   N   L   I   V   D   P   N   Q   R   P   V   K   S   L     167
gcc gac agg atc aag gct atg cca caa aaa ggc gga aac gct cca aga cca gta  35236
 A   D   R   I   K   A   M   P   Q   K   G   G   N   A   P   R   P   V     185
aag agg ggt cca aat aga aaa gct gct atg gca aaa tcc caa aac aaa cca aag  35290
 K   R   G   P   N   R   K   A   A   M   A   K   S   Q   N   K   P   K     203
aga gaa aag cct gct aag aaa agt ctt gaa gat ctg gac aag gaa atg gcg gac  35344
 R   E   K   P   A   K   K   S   L   E   D   L   D   K   E   M   A   D     221
tat ttc gaa aag aaa                                                      35359
 Y   F   E   K   K                                                         226

taattaattgtcaatttttgtttgatttaatttggttttattaaatttaatttttccgagtttcattatg  35430
tattagtttccagaaaatttaaaggattaaaaaatgtattaataaaactttgatatgtttatgtaataata  35501
```

FIGURE 3. Sequence of the *YRA1* gene. The genomic DNA sequence of the region identified by the Yra1p peptide sequence is shown. Translated amino acid sequences are shown for the two predicted *YRA1* exons; the regions corresponding to the two sequenced Yra1p peptides are underlined. Features of the nucleotides sequence that are highlighted are an in-frame upstream stop codon and the predicted 5′ splice site, branch point, and 3′ splice site.

the yeast protein (Fig. 5A), strongly suggesting that the identity of the clone is *YRA1*.

To verify this, His_6-tagged protein from the cloned cDNA was overexpressed in *E. coli* and purified (data not shown); this preparation was then tested for RNA annealing activity. As shown in Figure 5B, the purified, recombinant protein displays strong RNA annealing activity, demonstrating that the cloned gene specifies a protein with the biochemical activity of the factor purified from yeast extract. The concentration-dependence of His_6-Yra1p RNA annealing activity is similar to that of the native Yra1p purified from *S. cerevisiae* (data not shown), suggesting that posttranslational modifications are not likely to be required for its activity in vitro. The specific activity of His_6-Yra1p is also very similar to that of purified human hnRNP A1 (Fig. 5B), which has been found previously to have strong RNA annealing activity (Kumar & Wilson, 1990;

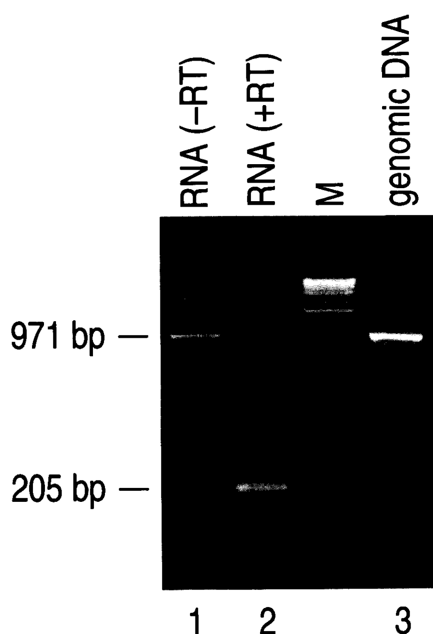

FIGURE 4. *YRA1* contains a large intron. Total yeast RNA (lane 1), reverse-transcribed total yeast RNA (lane 2), and yeast genomic DNA (lane 3) were PCR-amplified using primers flanking the putative intron. These primers amplified the full-length 971-bp intron-containing sequence in all samples (presumably due to genomic DNA contamination of the RNA samples); in addition, a 205-bp band (the expected size for a product lacking the intron) was detected only in the reverse-transcribed RNA. This fragment was excised from the gel, cloned, and sequenced, confirming the ligation of the putative exons (data not shown). Lane M contains size standards.

Munroe & Dong, 1992; Portman & Dreyfuss, 1994). Together, these findings establish that the cloned sequence is the *YRA1* gene.

Yra1p contains an RNP motif

Assembly of the two *YRA1* exons allows the conceptual translation of a 226-amino acid protein sequence (Fig. 6A) with a predicted molecular weight of 25.0 kDa, close to the observed size of the purified Yra1p ($M_r \sim 27{,}000$). The sequence contains one RNP-motif RNA-binding domain (RBD; also known as RNA-recognition motif or RRM) (Burd & Dreyfuss, 1994), suggesting that this protein binds RNA directly. The RNP2 hexamer submotif of the RBD matches its consensus; the RNP1 octamer, however, is somewhat divergent. Such atypical RNP1 sequences have been noted in other RBD-containing proteins (Birney et al., 1993), such as hnRNP I and L (Piñol-Roma et al., 1989b; Patton et al., 1991; Ghetti et al., 1992). The presence of an RBD is consistent with the RNA annealing activity of the purified protein, because a number of proteins with this domain exhibit RNA annealing activity, including the splicing factor SF2/ASF and the hnRNP proteins A1 and C1 (Krainer et al., 1990; Kumar & Wilson, 1990; Munroe & Dong, 1992; Portman & Dreyfuss, 1994). The *YRA1* sequence also encodes a cluster of basic amino acids that may serve as a nuclear localization signal (aa 201–210).

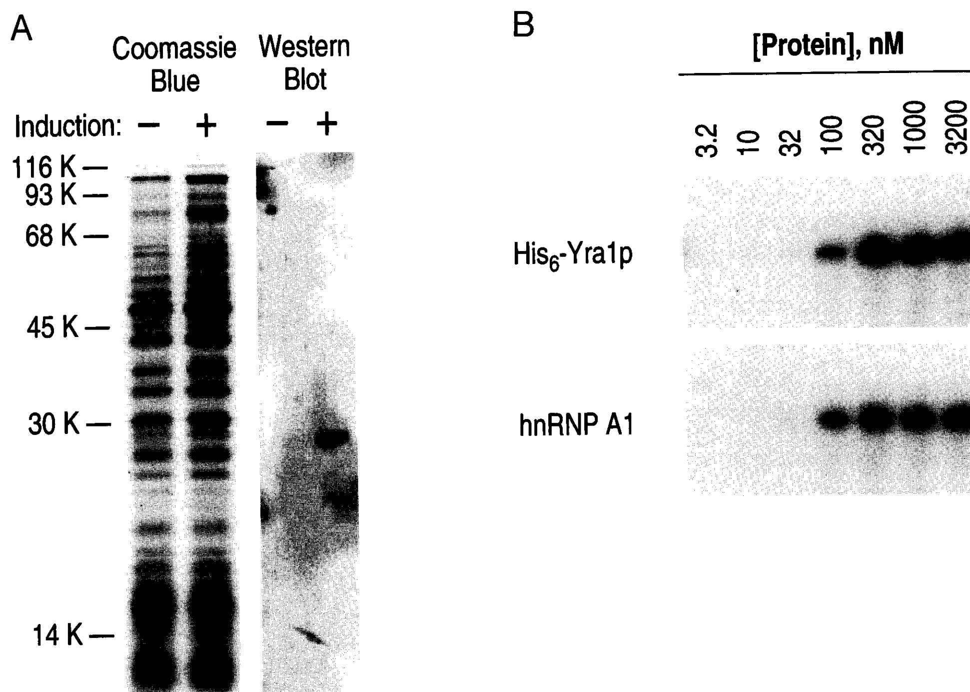

FIGURE 5. Properties of recombinant His$_6$-Yra1p. **A:** His$_6$-Yra1p was expressed in *E. coli*. Total cell lysates before (−) and after (+) induction are shown as Coomassie-blue-stained gels (left). Identical samples were run in parallel and transferred to a filter for western blotting with the anti-Yra1p antiserum (right). A 27-kDa protein is detected by the serum only in the induced lanes. **B:** RNA annealing assay using His$_6$-Yra1p and recombinant human A1 for comparison.

A

```
MSANLDKSLD EIIGSNKAGS NRARVGGTRG NGPRRVGKQV GSQRRSLPNR   50
RGPIRKNTRA PPNAVARVAK LLDTTREVKV NVEGLPRDIK QDAVREFFAS  100
QVGGVQRVLL SYNERGQSTG MANITFKNGE LARRAVERFN GSPIDGGRSR  150
LRLNLIVDPN QRPVKSLADR IKAMPQKGGN APRPVKRGPN RKAAMAKSQN  200
KPKREKPAKK SLEDLDKEMA DYFEKK                            226
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RNP2; RNP1; RNA-Binding Domain; RNP Core Motifs; Sequenced Peptides; Potential NLS

B

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                         RNP2                                             RNP1
Yra1p           78  VKVNVEGLPRDIKQDAVREFFASQVGGVQRVLLSYNERG.QSTGMANITFKNGELARRAVERFNGSPIDGGRSRLRLNLI
cDNA50          59  SKIIVSNLPTDVTEAQVKELFVKSIGPCKRVSLAYGPNG.RSKGIATIIFSRPGDATRAYEQYEGRLVDG.TRKMKVEII
PSF            297  CRLFVGNLPADITEDEFKRLFAK.YGEPGEVFINK......GKGFGFIKLESRALAEIAKAELDDTPMRG..RQLRVRFA
Nucleolin (2)  371  RTLFVKNLPYRVTEDEMKNVFEN...ALEVRLVLNKEGS..SKGMAYIEFKTEAEAEKALEEKQGTEVDG..RAMVIDYT
hnRNP M4 (1)    70  YRAFITNIPFDVKWQSLKDLVKEKVGEVTYVELLMDAEG.KSRGCAVVEFKMEESMKKAAEVLNKHSLSGRPLKVKEDPD
AZF              8  GKLFIGGLNRETNEKMLKAVFGK.HGPISEVLLIKDR.TSKSRGFAFITFENPADAKNAAKDMNGKSLHG..KAIKVEQA
Sex-lethal (2) 203  TNLYVTNLPRTITDDQLDTIFGK.YGSIVQKNILRDKLTGRPRGVAFVRYNKREEAQEAISALNNVIPEGGSQPLSVRLA
hnRNP G          8  GKLFIGGLNTETNEKALEAVFGK.YGRIVEVLLMKDRETNKSRGFAFVTFESPADAKDAARDMNGKSLDG..KAIKVEQA
                      β1          α1             β2                β3          α2                β4
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FIGURE 6. Yra1p protein sequence. **A:** Predicted primary structure of Yra1p. The RBD and its core RNP1 and RNP2 motifs are indicated, as are the sequenced peptides and a potential nuclear localization signal. **B:** Alignment of the Yra1p RBD with other proteins with similar domains. Residues that are highly conserved elements of the RBD are shown as white symbols on a black background; other conserved amino acids are shaded. Dots indicate some conservation at a particular position among the seven aligned domains. Structural motifs are shown below according to Wittekind et al. (1992). Sequences other than cDNA 50 (GenBank L42551) are from published reports (Lapeyre et al., 1987; Bell et al., 1988; Datar et al., 1993; Ma et al., 1993; Patton et al., 1993; Soulard et al., 1993).

Alignment of the RBD with other RNA-binding proteins containing this motif using the NCBI BLAST search service (Fig. 6B) (Altschul et al., 1990) suggests that the most similar sequence is "cDNA 50" from the yeast *Schizosaccharomyces pombe*. The GenBank entry for this sequence (accession number L42551) indicates that it was identified in a search for genes that disrupt chromosome segregation upon overexpression. Because some similarity between these two proteins extends beyond the RNP motif (data not shown), and both proteins have the same overall domain structure, it is possible that this protein is a Yra1p homologue. The other most closely related proteins (except nucleolin) are all thought to be pre-mRNA-binding proteins; this is consistent with the possibility that Yra1p may play a similar role in the cell. Aside from cDNA 50, no other database sequences show significant similarity to the *YRA1* auxiliary regions.

A *YRA1* null mutant

An insertion–deletion knockout of the *YRA1* gene was generated by homologous recombination (Rothstein, 1991). The resulting diploid *YRA1/yra1::trp1*$^+$ strain was sporulated and tetrads were dissected. Fourteen tetrads were dissected and analyzed for growth at 30 °C on rich medium. Eleven of the tetrads produced two viable spores; the remaining three tetrads produced only one viable spore. Examples are shown in Figure 7. All of the viable spore clones were tryptophan auxotrophs and therefore contained a wild-type *YRA1* gene, indicating that deletion of *YRA1* is lethal in *S. cerevisiae* under these growth conditions. Finally, transformation of a *YRA1/yra1::trp1*$^+$ diploid with a plasmid that conditionally expresses a *YRA1* cDNA clone rescued the null phenotype of *yra1::trp1*$^+$ spore clones, confirming that wild-type *YRA1* is sufficient to confer growth in a *yra1::trp1*$^+$ background (data not shown).

1
2
3
4
5

FIGURE 7. *YRA1* is an essential gene. Diploid *YRA1/yra1::trp1*$^+$ cells were sporulated and dissected. The pattern of spore growth from five diploid cells is shown.

DISCUSSION

Here we report the identification of a major RNA annealing activity, Yra1p, from the yeast *S. cerevisiae*. Because it was the only strong such activity we detected, Yra1p is likely to be a primary RNA annealing activity in this organism. This activity demonstrates that Yra1p can influence significantly the interactions of two RNAs in solution, and suggests that Yra1p plays an important role in RNA biogenic pathways in yeast. This could occur through the modulation of RNA secondary structure, or by promoting RNA-RNA or RNA-protein interactions in *trans*. However, the nature of the cellular RNA ligand(s) for Yra1p remains unclear, as does the way in which the protein-RNA interaction facilitates RNA annealing.

Yra1p has several of the characteristic features of hnRNP proteins (Dreyfuss et al., 1993): immunofluorescence staining reveals its nuclear localization; it contains an RNP-motif RNA-binding domain (RBD); and it has RNA annealing activity. Initial attempts to determine whether Yra1p indeed binds nuclear pre-mRNA in vivo, using ultraviolet light to covalently crosslink bound proteins to pre-mRNAs in intact cells (Piñol-Roma et al., 1989a), failed to detect any crosslinking (data not shown). This property is often considered diagnostic of hnRNP proteins (Dreyfuss et al., 1984b, 1993; Adam et al., 1986; Piñol-Roma et al., 1988; Swanson & Dreyfuss, 1988a, 1988b). However, some hnRNP proteins do not crosslink efficiently to pre-mRNA in vivo using this procedure [e.g., hnRNP A2 (Choi & Dreyfuss, 1984)]; our finding should therefore not be taken necessarily as excluding a pre-mRNA-binding function for Yra1p. However, it is also possible that Yra1p interacts with other nuclear RNAs, such as snRNAs, or nucleolar RNAs (Yra1p does not appear to be excluded from the nucleolus; see Fig. 2B). The identification of the RNA substrate of Yra1p will allow a better delineation of its function.

It will be particularly interesting to determine whether the defect in the *YRA1* null mutant can be complemented by known hnRNP proteins from other organisms, because this would suggest strongly a similar function for Yra1p. However, there do not exist specific homologues of vertebrate hnRNP proteins in yeast, and it seems that there is considerable structural (and possibly also functional) divergence between hnRNP proteins among these groups. The possibility that Yra1p exists in a macromolecular RNA-protein complex with other pre-mRNA-binding proteins, such as Pub1p and Nab2p, should also be investigated, because it is possible that the biochemical approaches that have identified these other factors overlooked Yra1p as a result of its lower abundance. It will also be of interest to determine whether the loss of *YRA1* function in yeast results in the accumulation of pre-mRNA or other intermediate RNA species, which would suggest a specific role for Yra1p during RNA processing. This phenotype has allowed the identification of the yeast *PRP* (pre-mRNA processing) genes, many of which have important roles in mRNA splicing (Guthrie, 1991; Ruby & Abelson, 1991; Rymond & Rosbash, 1992). *PRP* mutant screens are far from saturation, and it is possible that *YRA1* has a *PRP* phenotype.

It is noteworthy that the *YRA1* gene contains a 677-nt intron, the second-largest experimentally verified intron in *S. cerevisiae*. Its unusual size suggests that it may exert a function that is maintained through positive selection. The largest intron described to date (1,001 nt) is found in the gene *DBP2*, which encodes the yeast homologue of the p68 RNA helicase (Iggo et al., 1991). Notably, both of these large introns are in genes encoding factors that have the potential to modulate RNA secondary structure. Recently, it has been shown that negative autoregulation of the yeast *DBP2* gene occurs through sequences in its intron, and that functional Dbp2p protein is required for this regulation. These findings suggest that Dbp2p directly exerts an inhibitory effect on the excision of its own intron (Barta & Iggo, 1995). The YRA1 intron may also have a regulatory function: for example, Yra1p may interact with the *YRA1* intron to promote or disrupt secondary structure and thereby regulate the processing of its own pre-mRNA.

The essential nature of the *YRA1* gene is also of interest, particularly because it is possible that Yra1p is the major RNA annealing activity in *S. cerevisiae*. The null phenotype is therefore consistent with the notion that an RNA annealing activity, or perhaps a pre-mRNA chaperone, is required for yeast mRNA biogenesis. The potential requirement for a single such activity—in contrast with the many RNA annealing activities reported in vertebrate cells—may reflect the simpler RNA processing scheme used by *S. cerevisiae*. Although the precise in vivo function of Yra1p remains elusive, further biochemical studies in conjunction with the use of genetic tools are likely to result in a clear characterization of its roles.

MATERIALS AND METHODS

S. cerevisiae strains and oligonucleotides

The strains used were the protease deficient strain BJ5626 [MAT**a**/α, *ura3-52/ura3-52*, *trp1/+*, *+/leu2-Δ1*, *his3-Δ200/his3-Δ200*, *pep4::HIS3/pep4::HIS3*, *prb1-Δ1.6R/prb1-Δ1.6R*, *can/can*, GAL$^+$; obtained from the Yeast Genetic Stock Center (Berkeley, California)] and POC53 (MAT**a**/α, *ura3-52/ura3-52*, *leu2-Δ1/+*, *trp1-Δ63/trp1-Δ63*, *+/his3-Δ200*, *+/lys2-801*, GAL$^+$). The following oligonucleotides were employed: **YRA1 KO-U(F)**, 5′-GGGGGGGTCGACGGAAAGTATACT AATAG; **YRA1 KO-U(R)**, 5′-GGGGGGCTGCAGTTAGCAG ATGTAGGTATTTTC-3′; **YRA1 KO-D(F)**, 5′-GGGGGGGGAT CCTTAATTGTCAATTTTTTGTTTG-3′; **YRA1 KO-D(R)**, 5′-GGGGGGTCTAGAAGAGATGCTAAATTTATAC-3′; **YRA1-*Nde* I-F**, 5′-GGGGGGCATATGTCTGCTAACTTAGATAA

ATC-3′; **YRA1-*Bam*H I-R**, 5′-GGGGGGGGATCCTTATTTC TTTTCGAAATAGTCCG-3′; **YRA1-171(+)**, 5′-TACTAGGGCA CCTCCAAAC-3′; **YRA1-375(−)**, 5′-TGTGATGTTAGCCAT ACCAG-3′; **YRA1-EST F**, 5′-ACCTACATCTGCTAAATG TCTG-3′; and **YRA1-EST R**, 5′-ACGTTGACCTTGACCT CTC-3′.

Preparation and fractionation of *S. cerevisiae* extracts

For extract preparation, BJ5626 cultures were grown in YPD medium to a density of 1.0 OD_{600}. Cells were pelleted and resuspended in HEG buffer (50 mM Hepes, pH 7.6, 1 mM EDTA, 10% w/v glycerol) supplemented with 200 mM KCl, 0.5% aprotinin, 1 μg/mL leupeptin, 1 μg/mL pepstatin, 1 mM PMSF, and 10 mM $Na_2S_2O_5$. Cells were lysed at 4 °C with a French press. The resulting crude lysate was clarified (20 min at 15,000 × *g*, 4 °C) and supplemented with protease inhibitors as above. The supernatant was applied at 0.5 mL/min to a 10-mL ssDNA-cellulose (United States Biochemical) column (previously equilibrated in HEG with 200 mM KCl). Elution was performed using a linear gradient of 200–1,400 mM KCl in HEG; 2-mL fractions were collected and concentrated using Microcon filters (Amicon). Fractions containing RNA annealing activity (see below) were pooled, brought to 50 mM in KCl by dilution with HEG buffer, and loaded onto a TSK-SP column (TosoHaas) at a flow rate of 0.5 mL/min. Fractions of 2 mL were eluted with a gradient of 50–500 mM KCl in HEG, concentrated, and assayed as described below.

Peptide sequencing

Yra1p-containing fractions from a large-scale purification, deemed to be > 99% homogeneous by silver staining (data not shown), were pooled and concentrated; the sample was then subjected to proteolysis with lysyl endopeptidase. The resulting peptide fragments were separated and two of these were sequenced by the Protein Chemistry Facility of the University of Pennsylvania Medical Center.

Library screening

Using PCR on genomic *S. cerevisiae* DNA [prepared as described (Ausubel et al., 1994)], a probe was amplified with the primers **YRA1-EST F** and **YRA1-EST R** (derived from the sequence of EST101677; see Results), and end-labeled using a random priming method. This probe was used to screen an *S. cerevisiae* genomic λgt10 library by hybridization as described (Sambrook et al., 1989).

Cloning and expression of *YRA1*

Total *S. cerevisiae* RNA was prepared by the hot phenol method (Ausubel et al., 1994); 10 μg were used for reverse transcription, using random hexamers as primers. This cDNA sample was then used as the template for a PCR reaction using the oligonucleotides **YRA1-*Nde* I-F** and **YRA1-*Bam*H I-R** and the thermostable proofreading DNA polymerase *Pfu* (New England Biolabs). The amplified product, which was of the expected size, was digested with *Nde* I and *Bam*H I and cloned into the *E. coli* overexpression vector pET-11a (Novagen) to generate pET11a-YRA1. The same strategy was used to clone the *YRA1* cDNA into the pET-28c vector to generate pET28c-YRA1, which directs the expression of His_6-tagged Yra1p. For overexpression, pET11a-YRA1 or pET28c-YRA1 plasmid DNA was transformed into *E. coli* BL21(DE3) cells. Growth and induction were performed as described by Studier et al. (1990) and Novagen (Madison, Wisconsin). His_6-tagged protein expressed from the pET28c-YRA1 construct was purified on Ni^{+2}-agarose according to the manufacturer's protocols (Novagen).

Confirmation of the *YRA1* intron by RT-PCR

The yeast total cDNA sample (described above) and a control sample (in which no reverse transcriptase was added to the yeast RNA) were used as templates in PCR reactions containing primers **YRA1-375(−)** and **YRA1-171(+)**, which flank the putative intron. The 205-bp amplified fragment was cloned into pCRII (Invitrogen) and sequenced to confirm the precise ligation of the *YRA1* exons.

Targeted insertion–deletion mutagenesis

Genomic DNA fragments flanking the *YRA1* coding region were cloned into pKS-TRP1 after PCR amplification from genomic DNA using oligos **YRA1 KO-U(F)**, **YRA1 KO-U(R)**, **YRA1 KO-D(F)**, and **YRA1 KO-D(R)**, yielding pKS-TRP1-YRA1KO. Plasmid pKS-TRP1 was constructed by removing the *TRP1* gene from YCplac22 (Gietz & Sugino, 1988) (kindly provided by A. Sugino, National Institute of Environmental Health Sciences, Research Triangle Park, North Carolina) as an *Aat* II–*Bgl* II fragment, blunt-ending this fragment with T4 DNA polymerase and subcloning into the *Sma* I site of pBluescript II KS(+). pKS-TRP1-YRA1KO was linearized and used to transform the diploid yeast strain POC53 for one-step targeted deletion of *YRA1* as described by Rothstein (1991). Transformation was accomplished by electroporation using conditions described by the manufacturer (Bio-Rad). Transformants were selected for tryptophan heterotrophy. Diploid strains heterozygous for the *YRA1* deletion were sporulated and tetrads dissected using standard techniques (Sherman et al., 1986).

Protein and antibody methods

Unless noted otherwise, electrophoresis, staining, and blotting were performed as described (Dreyfuss et al., 1984a; Portman & Dreyfuss, 1994). For antiserum production, two mice were injected three times with 50 μg of purified Yra1p as described (Piñol-Roma et al., 1988); serum was collected upon sacrifice of the animal. Immunofluorescence on yeast was performed as described (Matunis et al., 1993), using the anti-Yra1p polyclonal antiserum at 1:200 dilution.

Sequence information

The nucleotide and protein sequences reported in this work are available in the GenBank database (accession number U72633).

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