

Overexpression of the *SPP2* Gene of *Saccharomyces cerevisiae* and Production of Antibodies to Spp2p

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We have previously reported that *SPP2* gene product of yeast *Saccharomyces cerevisiae* is involved in the pre-mRNA splicing. To investigate the role in the splicing pathway of the Spp2p protein, the *SPP2* gene was overexpressed in *Escherichia coli* and polyclonal antibodies to Spp2p were generated from rabbits. First, a DNA fragment containing the *SPP2* gene without its promoter was subcloned into an *E. coli* expression vector, pKK223-3. The resulting recombinant plasmid pBQ14 contained an IPTG inducible *tac* promoter and the *SPP2* structural gene. Overexpression of the *SPP2* gene was achieved by addition of 0.1 to 1.0 mM IPTG to a logarithmic culture of *E. coli* JM103(pBQ14) for 90 min at 37°C. Sequence of N-terminal 15 amino acids of the overproduced protein was well matched to the deduced one from the *SPP2* reading frame. Then, polyclonal antibodies were generated from rabbits immunized with gel-purified Spp2p protein. These antibodies reacted specifically with the Spp2p protein extracted from yeast cells expressing the *SPP2* gene to a great extent. The antibodies could also block the activity of yeast splicing extracts.

Key words: *Saccharomyces cerevisiae*, *SPP2*, overexpression, polyclonal antibodies

Primary transcripts of most eukaryotic genes contain nontranslated introns as well as translated exons (16, 17). Thus pre-mRNA splicing in which introns are removed from nuclear mRNA precursors (pre-mRNAs) and remaining exons are spliced accurately is essential for the eukaryotic gene expression. The pre-mRNA splicing involves two consecutive *trans*-esterification reactions and takes place in a multimolecular complex called the spliceosome. At least four small nuclear ribonucleoproteins (U1, U2, U4/U6, and U5 snRNPs) and the pre-mRNA are assembled into a spliceosome through an ordered multistep pathway requiring ATP and additional extrinsic factors (8).

In the budding yeast *Saccharomyces cerevisiae*, more than 20 *PRP* (pre-mRNA processing) gene products have been identified to be involved in the pre-mRNA splicing. Many of the *PRP* gene products are protein components of snRNPs in the spliceosome or are involved in the assembly and disassembly of the spliceosome (1, 3, 6, 10, 27, 39). Five of the *PRP* products, however, have been identified as a family of putative ATP-dependent RNA helicase, termed DEAD/H box proteins (5, 7, 10, 11, 36).

Prp5p and Prp28p contain the DEAD motif, while Prp2p, Prp16p and Prp22p contain the DEAH one (35). The Prp2p, Prp16p and Prp22p share additional sequence similarity in their carboxy termini (7, 10).

The Prp2p is required for the first *trans*-esterification reaction. An active spliceosome can be assembled in the absence of Prp2p (42) and the Prp2p can bind to this spliceosome. After subsequent hydrolysis of ATP, the Prp2p is released from the spliceosome (21, 40). The Prp2p has an RNA dependent-ATPase activity, and this might cause some conformational changes in the spliceosome, which are required for the first *trans*-esterification reaction (32). Purified Prp2p has no helicase activity *in vitro*, though it belongs to the DEAD/H family (22). However, it is still possible that *in vivo* it functions as a helicase requiring some co-factors or specific substrates for its activity (14, 35, 41).

The *SPP2* (suppressor of pre-mRNA processing) gene, which had been isolated as a multicopy suppressor of temperature-sensitive *prp2* mutations (20, 25), is essential for cell viability and its gene product is involved in pre-mRNA splicing *in vivo* and *in vitro* (33). In this study, we overexpressed the *SPP2* gene in *E. coli* and generated polyclonal antibodies to the *SPP2* gene product as

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a first step to understanding the biological role of *SPP2* gene product in pre-mRNA splicing.

Materials and Methods

Strains, plasmids and media

The *Escherichia coli* and *Saccharomyces cerevisiae* strains, and plasmids used in this study, are listed in Table 1. The *E. coli* strains HB101 and JM103 were routinely grown in Luria-Bertani broth (LB broth: 1% tryptone, 0.5% yeast extract, 0.5% NaCl). M9 minimal medium (30) was used for the maintenance of *F'*(*traD36 proA⁺B⁺ lacI^a lacZΔM15*) within the JM103. For solid and soft agar media, 1.5% and 0.7% agar were included, respectively. Ampicillin (50 mg/l) was added to the LB media, when necessary. *Saccharomyces cerevisiae* strains were cultivated in YEPD (1% yeast extract, 2% peptone, 2% glucose), YEPG broth (1% yeast extract, 2% peptone, 2% galactose) or synthetic complete broth without a particular amino acid or base (13).

Manipulation of plasmid DNA

Plasmid DNA was prepared from overnight culture of *E. coli* by the boiling method of Holmes and Quigley (18) or by the large scale alkaline extraction method of Marko *et al.* (29). Various restriction endonucleases, T4 DNA ligase, and Sequenase T7 DNA polymerase were purchased from Boehringer Mannheim or United States Biochemical. Enzyme reactions were performed according to the supplier's instructions. Agarose or polyacrylamide gel electrophoresis of DNA was performed by the procedure of Sambrook *et al.* (34). Restriction fragments of plasmid DNA were eluted from agarose gels (12).

Transformation

E. coli and *S. cerevisiae* were transformed with plasmid DNA according to the procedures of Cohen *et al.* (9) and Ito *et al.* (19), respectively.

Electrophoresis of denatured proteins

Total cell extracts were prepared from *E. coli* by boiling cell pellets for 5 min in Laemmli sample buffer (24). For the extraction of polypeptides from yeast, cell pellets from 5 ml of logarithmically grown cells were resuspended in 0.4 ml lysis buffer [0.1 M Tris (pH 8.0), 20% glycerol, 1 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride (PMSF)] containing 0.2 g glass beads, vortexed vigorously for 2 min, and spun for 1 min at 14,000×g. One fourth volume of 5× Laemmli sample buffer was added to the supernatant, and boiled for 5 min before loading on an 11% SDS-polyacrylamide

Table 1. Strains and plasmids used in this study.

Strains or plasmids	Relevant properties	Sources
<i>Saccharomyces cerevisiae</i> KY5	<i>a leu2 ura3 his3 trp1 lys2</i>	(33)
<i>Escherichia coli</i> HB101	<i>hsdS hsdM supE44 ara14 galK2 lacY1 proA2 rpsL20 xyl-5 mtl-1 recA13</i>	ATCC
<i>Escherichia coli</i> JM103	<i>endA hsdR supE sbcBC thi strA Δ(lac pro) F'(traD36 lacI^aZΔM15 proA⁺B⁺)</i>	ATCC
pJDB207-SPP2	2μ replicon, <i>LEU2 SPP2</i>	(33)
YCp50-SPP2	<i>CEN4 ARS1 Amp^r URA3 SPP2</i>	(33)
YCpGal-SPP2	<i>CEN4 ARS1 Amp^r URA3 GAL1-SPP2</i>	(33)
pKK223-3	ColE1 replicon, Amp ^r <i>ptac</i>	(4)
YEpl357	2μ replicon, <i>URA3</i>	(31)
pBC2	2μ replicon, <i>URA3</i> promoterless <i>SPP2</i>	this study
pBQ14	ColE1 replicon, Amp ^r <i>ptac-SPP2</i>	this study

gel (28). Polypeptide size markers for the sodium dodecyl sulfate (SDS)-polyacrylamide gels (lysozyme, 14 kDa; β-lactoglobulin, 18 kDa; carbonic anhydrase, 31 kDa; ovalbumin, 43 kDa; bovine serum albumin, 68 kDa; phosphorylase B, 97 kDa; myosin, 200 kDa) were purchased from Bethesda Research Lab.

Electroelution of the *SPP2* product

Total protein extracts from *E. coli* JM103(pBQ14) induced by isopropylthiogalactoside (IPTG) were displayed on a preparative SDS-polyacrylamide gel, stained with Coomassie blue. The gel slice containing the *SPP2* product band was excised from the gel, put into a dialysis bag containing 50 mM NH₄HCO₃ and 0.05% SDS, and the protein was electroeluted from the slice.

N-terminal sequencing

The amino-terminal sequence of the *SPP2* product extracted from *E. coli* was determined by automatic microsequencer equipped with on-line HPLC and data module (Applied Biosystems, Inc.).

Preparation of anti-Spp2p serum

About 0.2 mg of the electroeluted *SPP2* product was suspended in 1 ml of complete Freund's adjuvant and injected intradermally into a New Zealand white female rabbit. At 2 weeks intervals, two booster injections were made with about 0.1 mg of the *SPP2* product suspended in 1 ml incomplete Freund's adjuvant. After 2 weeks from the second booster injection, the rabbit was bled for the preparation of the immune serum (26).

Affinity purification of antiserum

A piece of nitrocellulose filter paper (0.5×1.5 cm) was incubated overnight in 0.8 ml of the electroeluted Spp2p protein solution (0.04 mg/ml). The anti-Spp2p antibodies in the immune serum were adsorbed onto the Spp2p protein bound to the filter paper by immersing the filter paper in 1 ml of the immune serum overnight. The filter was washed with 20 ml of 20 mM Tris, pH 7.2 and incubated with 0.5 ml of 0.2 M glycine, pH 2.3. The eluate containing the anti-Spp2p antibodies was neutralized with the same volume of 1 M Tris, pH 8.0 (38).

Western blotting

Bacterial or yeast cell extracts prepared as described above were run on an 11% SDS-polyacrylamide gel. Proteins were electroblotted to polyvinylidene difluoride (PVDF) membrane. The blots were probed with the anti-Spp2p antibodies followed by incubation with alkaline phosphatase-conjugated goat anti-rabbit IgG, and developed with nitro blue tetrazolium (NBT) and X-phosphate (26).

Results

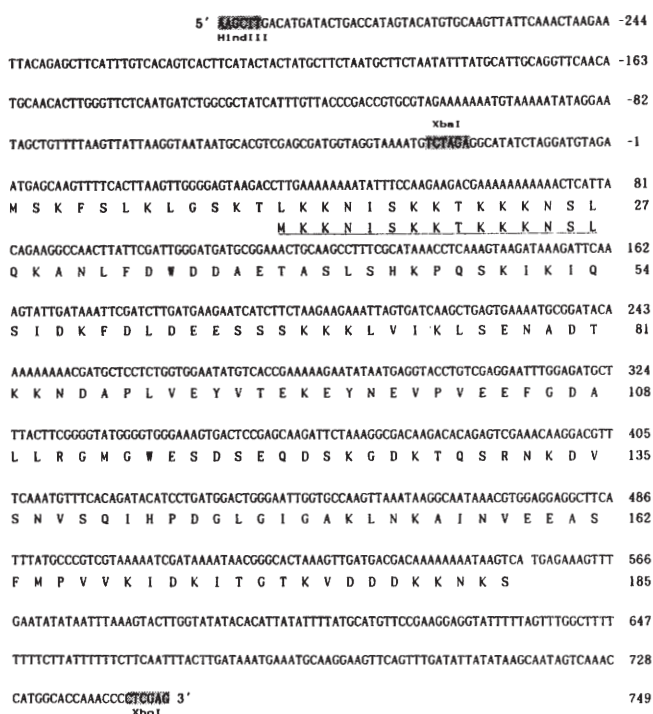
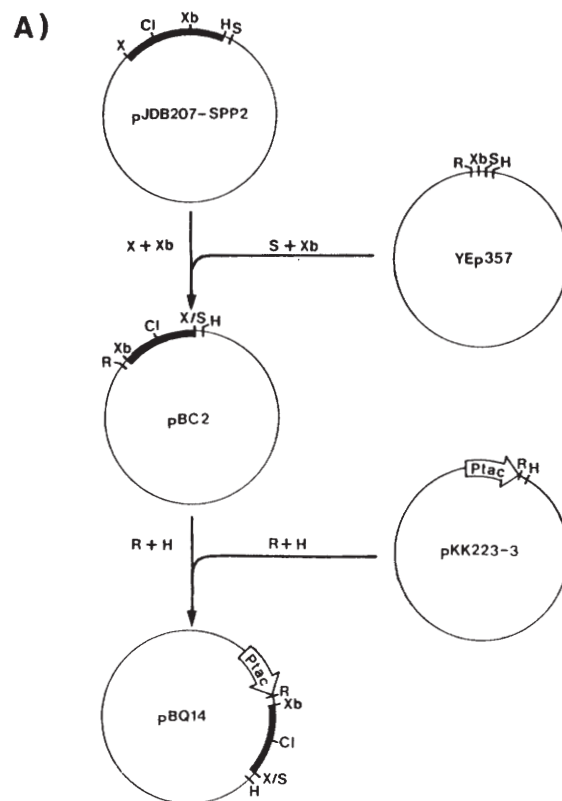


Fig. 1. DNA base sequence of *SPP2* from *S. cerevisiae* (33). Several unique restriction sites were shadowed. *SPP2* open reading frame and predicted amino acid sequence of Spp2p are shown. Determined sequence of N-terminal 15 amino acids of the overproduced *SPP2* product in *E. coli* was underlined.

Construction of an *SPP2* expression vector

Plasmid pJDB207-SPP2 retained a 1.1 Kb *HindIII*-*XhoI* fragment containing the yeast *SPP2* gene (33) (Fig. 1 and Table 1). One open reading frame corresponding to the *SPP2* gene and encoding a protein of 185 amino acids is found in this fragment (Fig. 1). There is one recognition sequence for *XbaI* restriction enzyme 19 bp upstream of the *SPP2* reading frame (Fig. 1), indicating



B)



Fig. 2. (A) Scheme for the construction of a *ptac-SPP2* fusion plasmid. (B) Restriction analysis of recombinant plasmids. Lane 1, *XhoI*-*XbaI* fragment of pJDB207-SPP2; lane 2, *SalI* plus *XbaI*-digested YEp357; lane 3, *HindIII* plus *XbaI*-digested pBC2; lane 4, *EcoRI*-*HindIII* fragment of pBC2; lane 5, *EcoRI* plus *HindIII*-digested pKK223-3; lane 6, *EcoRI* plus *HindIII*-digested pBQ14; lane 7, *HindIII*-digested lambda DNA.

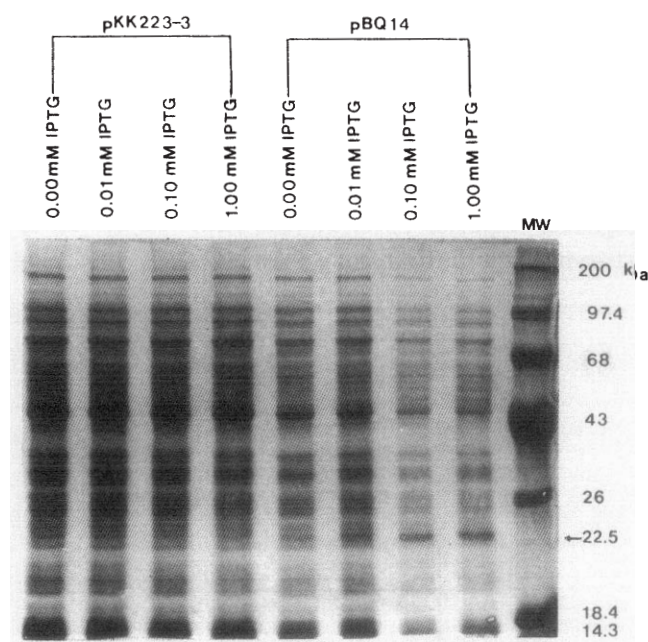


Fig. 3. SDS-polyacrylamide gel electrophoresis of extracts from JM103 containing pKK223-3 or pBQ14. Cultures were harvested 60 min after the addition of IPTG.

that the *XbaI-XhoI* fragment in the *SPP2* DNA contains the *SPP2* structural gene without its promoter (Fig. 1). If this *XbaI-XhoI* fragment were fused to a strong promoter like *ptac* (2), overexpression of the *SPP2* gene could be achieved. To do this, we first replaced the *XbaI-SalI* fragment of YEp357 (31) with the 0.8 Kb *XbaI-XhoI* fragment of pJDB207-SPP2 to generate a recombinant plasmid designated pBC2 (Fig. 2). In plasmid pBC2, the *SPP2* structural gene within the *XbaI-XhoI* fragment was flanked by *EcoRI* and *HindIII* sites. We therefore put the *EcoRI-HindIII* fragment of pBC2 just 3' downstream of the *ptac* promoter of pKK223-3 (4) to generate a *ptac-SPP2* fusion plasmid designated pBQ14 (Fig. 2).

Overexpression of the *SPP2* gene in *E. coli*

Appropriate amounts of IPTG were added to cultures of JM103(pKK223-3) and JM103(pBQ14) logarithmically grown on LB plus ampicillin liquid to induce the *SPP2* product. Total cellular proteins were prepared from the cultures 60 min after the induction, and displayed on an 11% SDS-polyacrylamide gel (Fig. 3). As shown in Fig. 3, no new band was detected in JM103(pKK223-3) after the addition of IPTG. However, a new band of 22.5 kDa was shown after the addition of IPTG to JM103 (pBQ14) (Fig. 3). The size of this polypeptide is approximately the same as that (20.5 kDa) deduced from the *SPP2* open reading frame. Though the amount of the new polypeptide induced by adding 0.01 mM IPTG to

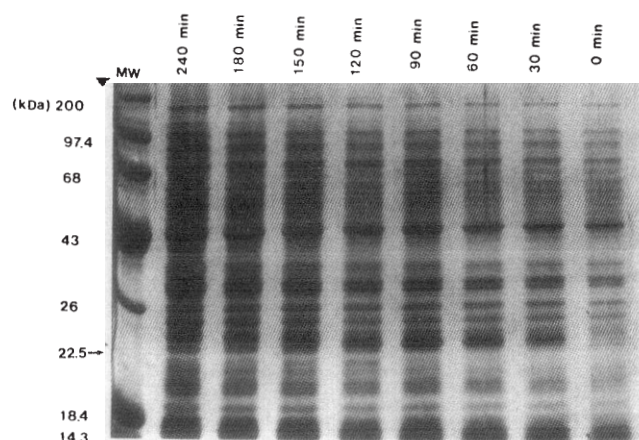


Fig. 4. SDS-polyacrylamide gel electrophoresis of extracts from JM103 containing pBQ14. 0.2 mM IPTG was added to a logarithmic culture of *E. coli* JM103(pBQ14) and the culture was incubated at 37°C for 0 to 240 min.

the culture was appreciable enough, the amounts of the peptide reached to about 10% of total cellular protein when 0.1 mM IPTG and 1 mM IPTG were added (Fig. 3). In addition, IPTG was added to a logarithmic culture of JM103(pBQ14) at a concentration of 0.2 mM, and then total cellular proteins were prepared from the culture every 30 min after the induction and analyzed on an SDS-polyacrylamide gel (Fig. 4). The new polypeptide was not detected right after the addition of IPTG, but it began to appear after 30 min. Relative amount of the polypeptide to total cellular protein increased until 90 min after the induction, and then it decreased slowly afterwards (Fig. 4). Accordingly we concluded that overexpression of the *SPP2* gene could be achieved when the logarithmically grown JM103(pBQ14) was incubated with 0.1 mM to 1 mM IPTG for 90 min at 37°C.

The induced polypeptide was isolated from 11% preparative SDS-polyacrylamide gels by electroeluting the corresponding polypeptide band from gel slices. N-terminal amino acid sequencing of the electroeluted polypeptide yielded the sequence MKKNISKKTKKKNSL (Met-LysLysAsnIleSerLysLysThrLysLysLysAsnSerLeu). Except the first amino acid methionine, this sequence is perfectly matched to the deduced one from a portion (from nucleotides 37 to 81) of the *SPP2* reading frame (Fig. 1). Thus the polypeptide must have contained many epitopes to yield polyclonal antibodies to the Spp2p protein.

Antiserum to the *SPP2* product made in *E. coli* recognizes an *SPP2*-specific protein from yeast

Rabbit immune sera were raised against the *SPP2* product made in *E. coli*. As expected, the immune serum

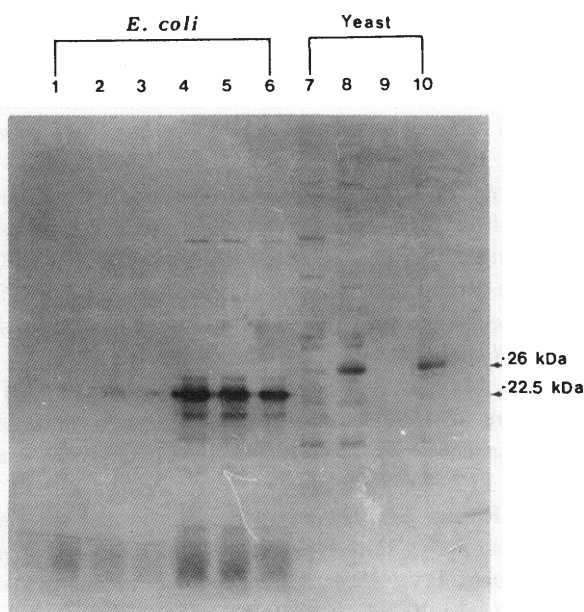


Fig. 5. Western blot analysis of *E. coli* and yeast culture extracts. Anti-Spp2p rabbit antibody was used as a probe. Lanes 1 to 3, JM103(pKK223-3) treated with 0.2 mM IPTG; lanes 4 to 6, JM103 (pBQ14) treated with 0.2 mM IPTG; lane 7, *S. cerevisiae* KY5(YCp 50-SPP2); lane 8, *S. cerevisiae* KY5(pJDB207-SPP2); lane 9, *S. cerevisiae* KY5(YCpGal-SPP2) grown on glucose; lane 10, *S. cerevisiae* KY5(YCpGal-SPP2) grown on galactose.

strongly and specifically reacted to the 22.5 kDa polypeptide on an immunoblot containing total cellular proteins of IPTG-induced JM103(pBQ14) cells (Fig. 5). The serum was absorbed to and eluted from the polypeptide bound to a piece of nitrocellulose filter paper. Using this purified serum as a probe, immunoblots were analyzed on the Spp2p polypeptide from yeast (Fig. 5). The serum specifically recognized a 26 kDa polypeptide from yeast cells *S. cerevisiae* KY5 (Table 1) expressing *SPP2* from a high copy plasmid pJDB207-SPP2 (Fig. 5). The serum was also able to recognize the same polypeptide from galactose-induced yeast cells *S. cerevisiae* KY5 containing a single-copy plasmid YCpGal-SPP2 which expresses *SPP2* to a great extent upon induction with galactose (Fig. 5). However, the serum did not recognize any specific polypeptide from either yeast cells containing a single copy plasmid YCp50-SPP2 or glucose-grown yeast cells containing YCpGal-SPP2 (Fig. 5). When the same blot used for Fig. 5 was probed with rabbit preimmune serum, no specific polypeptide was recognized (data not shown). The polyclonal antiserum to *SPP2* product was also able to block the splicing activity by immunoprecipitating the Spp2p protein from the yeast splicing extract, whereas the preimmune serum showed no immunoprecipitating activity (33).

Discussion

The *SPP2* gene product of yeast *S. cerevisiae* has been shown to be involved in the pre-mRNA splicing (33). As an approach to understanding the role in the splicing pathway of the Spp2p protein, we attempted to overexpress the *SPP2* gene in *Escherichia coli* and generate polyclonal antibodies to the overproduced Spp2p from rabbits. First, we have subcloned a 0.8 Kb *Xba*I-*Xho*I fragment of the *SPP2* DNA into an *E. coli* expression vector, pKK223-3. The resulting recombinant plasmid pBQ14 contained the *SPP2* structural gene just downstream of an IPTG inducible *ptac* promoter. By adding 0.1 to 1.0 mM IPTG to the logarithmic culture of *E. coli* JM103 (pBQ14) and subsequently incubating the culture for 90 min at 37°C, we could overexpress the *SPP2* gene to the extent that about 10% of the total cellular protein was the *SPP2* product. The *SPP2* product was eluted from preparative SDS-polyacrylamide gels and the sequence of 15 amino acids at the amino terminal of the polypeptide was determined. Except the first amino acid methionine, this sequence is perfectly matched to the deduced one from a portion (from nucleotides 37 to 81) of the *SPP2* reading frame (Fig. 1). This result was surprising because apparently in *E. coli* translation of the cloned *SPP2* gene starts not at the predicted AUG beginning at nucleotide 1 but at an unusual UUG beginning at nucleotide 37. We propose that the UUG at nucleotide 37 represented the actual initiation codon of the *SPP2* gene in *E. coli*, because it has been shown that UUG can act as an alternative initiation codon in *E. coli* (15) and a plausible ribosome binding site (37) (GGAG beginning at nucleotide 26; Fig. 1) is also present upstream of the UUG. However, the AUG beginning at nucleotide 1 may be the initiation codon in yeast, because in eukaryotic cells no alternative codon other than AUG is known (23). And the ribosome scans for the first AUG from the 5' cap instead of using a ribosome binding site for initiation (23).

Antisera were prepared from rabbits injected with the gel-purified *SPP2* product made in *E. coli*. As expected, the serum reacted strongly and specifically with the *SPP2* product from *E. coli*. The serum can specifically recognize a polypeptide from yeast cell extracts containing an increased amount of *SPP2* gene product overexpressed from a high-copy plasmid pJDB207-SPP2 or from a single-copy plasmid YCpGal-SPP2 by induction. However, the size of the yeast polypeptide (26 kDa) recognized by the antibody was slightly larger than that of the *SPP2* product made in *E. coli* (22.5 kDa). This discrepancy could be explained by the possibility that the *SPP2* pro-

duct made in *E. coli* is 12 amino acids shorter than that made in yeast, although the expected size difference (1.3 kDa) is somewhat smaller than the observed size difference (3.5 kDa). Alternatively, posttranslational modifications of the Spp2p protein in yeast such as glycosylation, acetylation, phosphorylation, etc. might be responsible for the difference. Glycosylation is not likely to be among those modifications in the Spp2p based on several lines of evidence. First, the Spp2p is probably localized inside the nucleus where no nuclear protein is known to be glycosylated, because it is involved in pre-mRNA splicing. Second, the reading frame of the *SPP2* gene reveals no plausible glycosylation motif. Third, treatment of yeast extracts containing the Spp2p with O-glycosidase or N-glycosidase A or F caused no reduction in size of the Spp2p recognized by the anti-Spp2p serum upon immunoblot analysis (data not shown).

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