The last step of tRNA splicing in the yeast Saccharomyces cerevisiae is catalyzed by an NAD-dependent 2'-phosphotransferase, which transfers the splice junction 2'-phosphate from ligated tRNA to NAD to produce ADP-ribosyl 1'-2' cyclic phosphate. We have purified the phosphotransferase about 28,000-fold from yeast extracts and cloned its structural gene by reverse genetics. Expression of this gene (TPT1) in yeast or in Escherichia coli results in overproduction of 2'-phosphotransferase activity in extracts. Tpt1 protein is essential for vegetative growth in yeast, as demonstrated by gene disruption experiments. No obvious binding motifs are found within the protein. Several candidate homologs in other organisms are identified by searches of the data base, the strongest of which is in Schizosaccharomyces pombe.

tRNA splicing is essential in both the yeast Saccharomyces cerevisiae and humans, since both organisms contain tRNA gene families whose members all contain intervening sequences. Yeast has 10 such intron-containing tRNA gene families (of the approximately 45 total tRNA gene families) (see Ref. 1 for review), and humans have at least one intron-containing tRNA gene family (2). Since all known eukaryotic nuclear-encoded tRNA genes contain introns, it is likely that tRNA splicing is essential in all eukaryotes for processing of tRNA genes.

tRNA introns are invariably located 1 base 3' of the anticodon, and this location is critical for the first step of splicing. In both Xenopus and yeast the endonuclease binds the mature domain of the precursor tRNA, measures the length of the anticodon stem to locate the intron (3, 4), and excises it if the structure at the 3' splice site is correct (5, 6). The products of the reaction are exons bearing 2'-3' cyclic phosphates and 5'-hydroxyl groups at their ends, as shown in Fig. 1 (7, 8).

Joining of the exons involves a ligase that generates a mature sized tRNA bearing a splice junction 2'-phosphate (9). The 2'-phosphate from yeast catalyzes four distinct chemical steps to effect ligation: the 2'-3' cyclic phosphate at the end of the 5' exon is opened to a 2'-phosphate by a cyclic phosphodiesterase activity; the 5'-OH at the beginning of the 3' exon is phosphorylated by a polynucleotide kinase activity in the presence of GTP; the 5'-phosphate is activated by adenylylation from ATP; and then ligation occurs with loss of the adenylate moiety (9–11). The result of ligation is a mature sized tRNA bearing a splice junction 2'-phosphate (see Fig. 1). A ligase present in wheat germ (12, 13), Chlamydomonas (14), and humans (15) also generates splice junctions with a 2'-phosphate, and the wheat germ protein is very similar in catalytic activities to the yeast enzyme (16, 17). Since removal of the 2'-terminal phosphate prevents the yeast ligase from working in vitro (10, 18), the 2'-phosphate is likely formed at the splice junction when this ligase acts in vivo. The yeast ligase is known to be responsible for tRNA splicing in yeast, since conditional ligase mutants accumulate unligated tRNA exons under nonpermissive conditions (19). However, a second ligase, which uses a completely different chemical reaction and does not generate a splice junction 2'-phosphate, has been implicated in tRNA splicing in humans in vitro (20, 21) and in Xenopus oocytes in vivo (22).

Removal of the splice junction 2'-phosphate occurs by a highly unusual reaction: a 2'-phosphotransferase transfers the splice junction phosphate to NAD, forming the novel NAD phosphate 2'-phosphate (see Fig. 1). A ligase present in wheat germ (12), Chlamydomonas (14), and humans (15) also generates splice junctions with a 2'-phosphate, and the wheat germ protein is very similar in catalytic activities to the yeast enzyme (16, 17). Since removal of the 2'-terminal phosphate prevents the yeast ligase from working in vitro (10, 18), the 2'-phosphate is likely formed at the splice junction when this ligase acts in vivo. The yeast ligase is known to be responsible for tRNA splicing in yeast, since conditional ligase mutants accumulate unligated tRNA exons under nonpermissive conditions (19). However, a second ligase, which uses a completely different chemical reaction and does not generate a splice junction 2'-phosphate, has been implicated in tRNA splicing in humans in vitro (20, 21) and in Xenopus oocytes in vivo (22).

Two lines of evidence support the claim that the yeast enzyme catalyzes this step in the cell (24, 25). First, the phosphotransferase is highly specific for substrates bearing an internal 2'-phosphate; an oligonucleotide bearing an internal 2'-phosphate is efficiently dephosphorylated, whereas oligonucleotides terminating with 5'-, 3', 2', or 2'-3' cyclic phosphates are not detectably dephosphorylated. Second, this is the only activity detected in crude extracts that can efficiently remove the 2'-phosphate from ligated tRNA. A similar 2'-phosphotransferase has been described in HeLa cell extracts; like the yeast enzyme the HeLa enzyme is highly specific for substrates with internal 2'-phosphates and is the only activity that can efficiently dephosphorylate 2'-phosphorylated ligated tRNA (25). Moreover, it is likely that the phosphotransferase can act in vivo on tRNA substrates: Xenopus oocytes injected with 2'-phosphorylated ligated tRNA catalyze formation of Appr-p concomitant with dephosphorylation (23).

To begin to study the role of the phosphotransferase in yeast, we have purified the protein and cloned its structural gene (TPT1; tRNA 2'-phosphotransferase). Phosphotransferase was purified ~28,000-fold, the N-terminal amino acid sequence was determined, and the appropriate DNA was isolated by colony

The abbreviations used are: Appr-p, ADP-ribosyl 1'-2' cyclic phosphate; r-p, ribose 1,2-cyclic phosphate; ORF, open reading frame; DTT, dithiothreitol; BSA, bovine serum albumin.
hybridization of a yeast genetic library. The identified ORF was expressed in Escherichia coli and shown to catalyze 2'-phosphotransferase activity, implying that phosphotransferase is a single catalytic polypeptide. 2'-Phosphotransferase is essential for vegetative growth in yeast, as demonstrated by analysis of strains with chromosomal deletions in the TPT1 gene. The sequence of the phosphotransferase does not reveal any obvious binding or catalytic motifs. Several significantly similar ORFs are identified by searches of the data base, including a particularly strong one in Schizosaccharomyces pombe.

**EXPERIMENTAL PROCEDURES**

**Preparation of Ligated tRNA—Ligated tRNA\(^{32P}\) with a 32P-labeled pre-tRNA\(^{32P}\) transcript was derived from T7 RNA polymerase transcription of a plasmid-borne copy of the end-matured pre-tRNA\(^{32P}\) transcript (340 Ci/mol) was derived from T7 RNA polymerase and primers dP-1 (AGAGGAATTCACAGGTG-11) and adding a dIII linker, generating pGMC17 (EcoRI-PstI fragment), the LEU2 2-μm vector yEPlac195 to construct pGMC4 (EcoRI-PstI fragment), the LEU2 2-μm vector yEPlac195 to construct pGMC5 (EcoRI-PstI fragment), and pSP72 vector to construct pGMC7 (EcoRI-HpaI fragment of EMP981 ligated into the EcoRI and PstI vector fragment).

Disruptions of the TPT1 gene were generated by insertion of a LEU2 HindIII fragment (obtained from a polynucleotide-inserted LEU2 SpaI chromosomal fragment, modified to contain HindIII ends) into one of two positions within the TPT1 gene, as illustrated in Fig. 4B: to replace the HindIII fragment of 226 nucleotides around the ATG translation start site of pGMC7, generating pGMC22 (pT1-D1:LEU2), or to replace the 744 base pair HindIII-A/HindIII fragment of pGMC7, after filling in the A/HindIII end and adding a HindIII linker, generating pGMC17 (pT1-D2:LEU2).

pGMC10 contains the TPT1 ORF bounded by engineered EcoRI sites, ligated into pSP72. It was generated by polymerase chain reaction amplification of the TPT1 gene from the ATG of the ORF to a site 240 nucleotides downstream of the TAA termination codon (see Fig. 4B), using primers dP-1 (AGAGGAATTCACAGGTG-11) and adding a dIII linker, generating pGMC17 (EcoRI-PstI fragment), the LEU2 2-μm vector yEPlac195 to construct pGMC4 (EcoRI-PstI fragment), the LEU2 2-μm vector yEPlac195 to construct pGMC5 (EcoRI-PstI fragment), and pSP72 vector to construct pGMC7 (EcoRI-HpaI fragment of EMP981 ligated into the EcoRI and PstI vector fragment).

**Expression of Tpt1 Protein in E. coli**—E. coli strain RZ510 (relevant genotype lac \(^{+}\)) was transformed with pKK223-3 vector or with pGMC9 to express the TPT1 gene. 50-ml cultures were grown at 37 °C in L broth to an \(A\text{OD}_{600}\) of 0.8, induced with 1 mM isopropyl-\(\beta\)-D-thiogalactopyranoside for 1 h, and cells were harvested, resuspended in 2 ml of buffer containing 50 mM Tris 7.5, 1 mM EDTA, 450 mM NaCl, 5 mM DTT, and 10% glycerol, and sonicated on ice 10 bursts for 1 min. Extracts obtained after centrifugation (12.5 mg/ml) were aliquoted, frozen, and thawed to measure phosphotransferase activity.

**Protein Concentration and Visualization**—Protein concentration was determined using Bradford reagent (Bio-Rad). Polypeptides were visualized after SDS-polyacrylamide gel electrophoresis by silver staining, as described (35).

**Purification of 2'-Phosphotransferase**—2.5 kg (wet weight) of frozen yeast were thawed, washed with 5 liters of Wash Buffer (0.1 M Tris-HCl, pH 7.5, and 10 mM DTT), resuspended in 1.25 liters of lysis buffer (20 mM Tris, pH 7.5, 2 mM EDTA, 10 mM DTT, 10% \(<\text{v/v}\) glycerol, 1 mM NaCl, 1.7 mM phenylmethylsulfonyl fluoride, 1.2 μg/ml leupeptin, and 1.2 μg/ml pepstatin A), and diluted with 0.5-1 ml buffer. Centrifugation (at 5,000 x g) was repeated until the supernatant was clear (approximately 3 times). The crude extract was obtained by passing the mixture through a coarse sintered glass funnel, followed by centrifugation at 13,000 x g for 40 min.

**Protein** was precipitated by the addition of solid ammonium sulfate to 80% saturation, followed by centrifugation at 14,700 x g for 45 min. The protein pellet was washed with 2 liters of Buffer A (0.2 M Tris-HCl, pH 7.5, 1 mM EDTA, pH 8.0, 200 mM NaCl, 1 mM DTT, and 10% \(<\text{v/v}\) glycerol).
protein cross-linking reactions were assembled in 15 m methanol.

were dialyzed against Buffer B containing 40 mM NaCl and 20% glyc-phoric acid). Peak fractions from the hydroxylapatite column (8 ml) apparates at room temperature for 4 h at 0.65 mA/square cm or 300 mA.

an intensity of 2 milliwatts/cm². Samples were supplemented with 55 m

cross-linked to RNA for 10 min at room temperature, using a UV source

mixtures were preincubated for 15 min at 30 °C, and proteins were

Gel HTP, Bio-Rad) equilibrated in Buffer C (20 mM Tris, pH 7.5, 0.5 mM

(250 m

dialyzed against Buffer B containing 40–900 mM NaCl. Active fractions (350 ml) from the blue Sepharose column were
dialyzed against Buffer B containing 40–900 mM NaCl. Active fractions (25 ml) were loaded directly onto a 15-ml hydroxylapatite column (DNA grade Bio-Gel HTP, Bio-Rad) equilibrated in the same buffer, washed, and active fractions (700 ml) flowed through the column. These fractions were pooled, diluted 2-fold with Buffer B lacking NaCl, reapplied to the blue Sepharose column equilibrated with Buffer B containing 0.175 M NaCl, and retained protein was eluted with a 2-liter linear gradient of Buffer B from 0.175 to 2 M NaCl. 2'-Phosphotransferase eluted at 450–500 mM NaCl.

Active fractions (350 ml) from the blue Sepharose column were
dialyzed against Buffer B containing 40 mM NaCl, applied to a 250-ml heparin-Sepharose column, and activity was eluted with a gradient of Buffer B containing 40–900 mM NaCl. Active fractions (25 ml) were loaded directly onto a 15-ml hydroxylapatite column (DNA grade Bio-Gel HTP, Bio-Rad) equilibrated in the same buffer, washed, and active fractions (700 ml) flowed through the column. These fractions were pooled, diluted 2-fold with Buffer B lacking NaCl, reapplied to the blue Sepharose column equilibrated with Buffer B containing 0.175 mM NaCl, and retained protein was eluted with a 2-liter linear gradient of Buffer B from 0.175 M to 2 M NaCl. 2'-Phosphotransferase eluted at 450–500 mM NaCl.

A streamlined purification yielded material that was about 4-fold less pure.

Protein Sequencing—100–200 pmol of purified phosphotransferase

was resolved on a 12% SDS-polyacrylamide gel, transferred electrophoretically to polyvinylidene difluoride membranes, stained with Coo- massie Blue R-250, destained, and used for sequencing essentially as described by Matsudaira (36). Transfer was done in a Bio-Rad transblot apparatus at room temperature for 4 h at 0.65 mA/square cm or 300 mA in transfer buffer containing 39 mM glycine, 48 mM Tris base, and 20% methanol.

UV Cross-linking of tRNA to Phosphotransferase Fractions—RNA-protein cross-linking reactions were assembled in 15 μl of phosphotransferase assay buffer containing 200 μg/ml BSA, 180,000 cpm of spliced tRNA, 750 units (approximately 5 ng) of 2'-phosphotransferase from the orange A column peak, 5 mM AMP, and no NAD. Reaction mixes were preincubated for 15 min at 30 °C, and proteins were cross-linked to RNA for 10 min at room temperature, using a UV source at 254 nm (UV-C Blei 155 lamp, Schott Corp., Westbury, NY) with an intensity of 2 milliwatts/cm². Samples were supplemented with 55 μl of 10 mM Tris, pH 7.5, and digested with 7.5 units of ribonuclease T1 at 50 °C for 45 min. Proteins were precipitated at −20 °C by the addition of trichloroacetic acid to 10%, followed by centrifugation to pellet the protein, washing with ice cold acetone, resuspension in SDS-polyacryl-amide gel electrophoresis loading buffer, boiling for 15 min, and electrophoresis on a 12% SDS-polyacrylamide gel.

RESULTS

Purification of 2'-Phosphotransferase Implicates a 30-kDa Polypeptide—The purification of 2'-phosphotransferase yields a prominent polypeptide of 30 kDa which comigrates with activity. This is illustrated in Fig. 2, in which fractions from the final purification step were analyzed for both polypeptides and phosphotransferase activity. The amount of 30-kDa polypeptide closely parallels phosphotransferase activity in different fractions, both across the final orange A column (compare fractions 20–36 in Figs. 2, A and B) and in the peak fractions from the heparin-agarose and hydroxylapatite columns of the purification (Fig. 2A). Three other minor polypeptides are visible in this preparation of 2'-phosphotransferase activity, with apparent molecular masses of 52, 45, and 20 kDa. Neither of the chromatographic profiles of the 52-kDa or the 45-kDa polypeptides corresponds to the observed activity peak from the orange A column (Fig. 2); however, the profile of the 20-kDa polypeptide does appear to correspond to the observed activity peak from this column. The same 30-kDa polypeptide, but not the 20-kDa polypeptide, also copurifies with activity using a streamlined purification procedure (see “Experimental Procedures”), and if the material from the penultimate step of this streamlined procedure is chromatographed on DEAE or on another heparin-agarose column (with isocratic elution) instead of the orange A column. Thus it seemed likely that the 30-kDa polypeptide is the limiting component responsible for 2'-phosphotransferase activity.

The 30-kDa Protein Cross-links to Spliced tRNA—The sug-gestion that the 30-kDa polypeptide is a component of the
phosphotransferase is supported by the observation that it can be cross-linked to its substrate. As shown in Fig. 3, ligated tRNA bearing a 2'-phosphate, prepared from (α-32P)ATP-labeled pre-tRNA transcript, is cross-linked to a polypeptide of 30 kDa (band B1) from the peak orange A fraction, as visualized after RNase T1 digestion by electrophoresis on an SDS-polyacrylamide gel. Cross-linking requires both phosphotransferase and UV illumination. No UV-dependent cross-linking is observed to BSA, which was deliberately present at a 600-fold higher concentration than 2'-phosphotransferase and to the contaminating 52- and 45-kDa polypeptides. A band migrating at around 20 kDa (band B2) was present in all lanes, and thus represents an RNase T1-resistant background. Based on the apparent molecular mass of the cross-linked polypeptide (band B1), we conclude that the 30-kDa polypeptide comigrating with activity in the purification is the cross-linked protein. Separate experiments with less purified fractions demonstrate that the interaction of the 30-kDa polypeptide with tRNA is specific: cross-linking with less purified fractions demonstrate that the interaction of the 30-kDa polypeptide comigrating with activity in the phosphorylation procedure, which contained slightly different contaminants. Furthermore, the yield of amino acids obtained from the initial steps of the sequencing run corresponded roughly to the number of moles of 30-kDa polypeptide subjected to sequencing. Thus, we were likely sequencing the major polypeptide present at 30 kDa and not a minor contaminant.

The 2'-phosphotransferase gene (TPT1) was isolated by reverse genetics. The sequence of 14 amino acids was compared with the information in the yeast database, and a single perfect match was found at the N-terminal end of an ORF of 26.2 kDa, located on chromosome XV. The sequence of this ORF is shown in Fig. 4A. No other near matches were found in the yeast database. An oligonucleotide probe was designed from the database DNA sequence, and this probe was used to isolate the gene from a yeast genomic library by colony hybridization, as described under “Experimental Procedures.” A schematic of a portion of chromosome XV that was isolated is illustrated in Fig. 4B, showing the position of the TPT1 ORF and neighboring ORFs. Plasmids containing the phosphotransferase gene, constructed as described under “Experimental Procedures,” were then used to confirm that the gene encodes 2'-phosphotransferase.

**TPT1 Encodes the 2’-Phosphotransferase—Overproduction of Tpt1 protein in yeast results in overproduction of 2’-phosphotransferase activity.** This was established in two experiments, which are summarized in Table II. First, high gene dosage results in overproduction of activity. Extracts made from a strain bearing a high copy plasmid containing the gene and its regulatory regions (2-μm TPT1) have about 55-fold more phosphotransferase activity than extracts from the same strain bearing the plasmid vector alone (Table II). Second, placing the open reading frame under control of a regulatable promoter in yeast results in regulated overproduction of the phosphotransferase activity. To this end, a plasmid was constructed with the TPT1 open reading frame immediately downstream of the GAL10 promoter and transcription start site, as described under “Experimental Procedures.” A strain bearing this plasmid (pGAL10-TPT1) overproduces phosphotransferase about 20-fold when grown in galactose, which induces transcription, compared with the phosphotransferase produced when cells are grown in glucose, which represses transcription (Table II). These two experiments demonstrate that expression of the TPT1 gene is the limiting factor determining the observed 2'-phosphotransferase activity in yeast extracts.

To establish that TPT1 encodes the 2’-phosphotransferase, the gene was expressed in E. coli. As shown in Table III, extracts from an E. coli strain bearing the expression vector pKK223-3 have little, if any, detectable 2’-phosphotransferase activity. However, extracts from an E. coli strain containing the TPT1 ORF fused immediately downstream of the hybrid trp-lac promoter of pKK223-3 have 100-fold more 2’-phosphotransferase activity than the control extracts when expression is induced in the presence of isopropyl-β-D-thiogalactopyranoside. Since the TPT1 gene encodes a protein of 26.2 kDa, which has 2’-phosphotransferase activity, and its size is nearly the same as that identified in the purification (Fig. 2), and in cross-linking experiments (Fig. 3), it is highly likely that Tpt1 protein is the phosphotransferase protein.

**Isolation of the Phosphotransferase Gene**—To clone the structural gene for the phosphotransferase, we sequenced the N-terminal end of the 30-kDa protein in the final orange A column, after transfer of the polypeptides to polyvinylidene difluoride paper and excision of the appropriate region. We obtained 14 amino acids of N-terminal sequence. The same sequence was also obtained from two other experiments with equivalent, but less pure, material obtained from the stream.
comigration of the product (in several different thin layer chromatography systems) with Appr-p made by the purified yeast protein, as well as by phosphatase resistance of the product (data not shown). Similarly, Tpt1 protein produced in E. coli, like the yeast enzyme, prefers substrates with tRNA structure: for each protein 2'-phosphorylated tRNA is about 50-fold more efficient a substrate than a synthetic oligonucleotide (pUp-UpU), which contains a 2'-phosphate. Assuming that E. coli extracts do not fortuitously supply missing factors that can act in concert with Tpt1 protein, it is likely that 2'-phosphotransferase is a single polypeptide that can recognize its substrates and catalyze the complete phosphotransfer reaction.

**Table I**

| Fraction          | Volume (ml) | Activity (units) | Protein (mg) | Specific activity (units/mg) | Fold purification, step (overall) | Yield, step (overall) |
|-------------------|-------------|------------------|--------------|-----------------------------|----------------------------------|----------------------|
| Extract           | 1520        | 6.7 x 10⁴        | 136,000      | 4.9 x 10²                   | 4.1 (4.1)                       | 52                   |
| 46–59% (NH₄)SO₄   | 1040        | 3.5 x 10⁴        | 17,300       | 2.0 x 10⁴                   | 1.2 (4.9)                       | 69 (36)              |
| Blue Sepharose #1 | 1400        | 2.4 x 10⁵        | 9,500        | 2.4 x 10⁴                   | 14 (69)                         | 17 (6.3)             |
| Blue Sepharose #2 | 35          | 4.2 x 10⁵        | 123          | 3.4 x 10⁴                   | 8.9 (10)                        | 160 (9.9)            |
| Heparin Agarose   | 25          | 6.6 x 10⁵        | 21.8         | 3.0 x 10⁴                   |                                  |                      |
| Bio-Gel-HPA       | 8.2         | 2.6 x 10⁵        | 1.17         | 2.2 x 10⁴                   | 7.3 (4500)                      | 39 (3.9)             |
| Orange A Sepharose| 1.1         | 1.1 x 10⁵        | 0.080        | 1.4 x 10⁸                   | 6.2 (28,000)                    | 42 (1.6)             |

**2'-Phosphotransferase Gene Implicated in tRNA Splicing**

**Tpt1 Protein Is Essential for Vegetative Growth**—Removal of the 2'-phosphate from ligated tRNA is likely to be critical for tRNA function. Since the splice junction 2'-phosphate is 1 base 3' of the anticodon, its bulk and charge would be expected to interfere with anticodon recognition and thus impair growth. If Tpt1 protein is the enzyme that catalyzes removal of the splice junction 2'-phosphate from ligated tRNA in vivo, then cells lacking this protein would likely be dead (or very sick).

Gene disruption experiments demonstrate that the TPT1 gene is essential. To show this, we did a standard one-step gene disruption experiment, in which we replaced one allele of the TPT1 gene in a diploid with a copy of the LEU2 gene, as described under “Experimental Procedures,” and sporulated the resulting diploid. Two separate gene disruptions were made by replacement of a fragment spanning the ATG of the ORF with the LEU2 gene: in tpt1-1::LEU2, a 226-nucleotide HindIII fragment extending from −174 in the promoter to +53 in the coding region was replaced; and in tpt1-2::LEU2 a 744 nucleotide HindIII-AfII fragment from −174 in the promoter to +570 in the coding region was replaced (see Fig. 4B). Southern analysis confirmed in both cases that the chromosomal DNA from the transformant diploids contained one normal sized copy of the TPT1 gene and one copy of the TPT1 gene that was altered by the presence of the LEU2 gene (data not shown). In SC804 (relevant genotype: TPT1+/tpt1-1::LEU2) 6 tetrads were examined; all segregated two live:two dead spores, and all the live spores lacked the LEU2 marker. Similarly in SC805 (relevant genotype: TPT1+/tpt1-2) seven tetrads were examined and all segregated two live Leu` spores and two dead spores. These are the expected results if disruption of the TPT1 gene is lethal. Since, in addition, microscopic examination of the nonsurviving spores demonstrated that they germinated and grew into microcolonies, these results suggest that the TPT1 gene is essential.

To confirm that the lethality of the disruptions was caused by lack of the TPT1 gene itself, we demonstrated that the TPT1 gene on a plasmid could complement the deletion and suffice for viability. As shown in Table IV, SC804 (tpt1-1::LEU2/TPT1+) carrying a plasmid bearing the TPT1 gene on either a single copy plasmid (CEN URA3) or a multi-copy plasmid (2-µm URA3) could readily segregate Leu+ (tpt1+) spores as long as the spores also contained a URA3 TPT1+ plasmid; by contrast, SC804 carrying just a URA3 plasmid segregated only Leu- spores. Furthermore, only the TPT1 gene itself was required to complement the tpt1-1::LEU2 disruption, since Leu` Ura+ spores could be readily recovered if the sporulated diploid carried a URA3/CEN plasmid containing only the TPT1 ORF (fused to the GAL1 promoter) and 236 nucleotides of downstream DNA (to allow for termination of transcription). Since the TPT1 ORF is the only ORF on this plasmid, and it still complements the lethality of the tpt1 disruption, it is highly likely that the TPT1 gene is essential. Furthermore, as expected if the TPT1 gene is essential for vegetative growth, cells bearing the TPT1 deletion require the plasmid-borne TPT1 gene for continued viability. Whereas wild type cells bearing a URA3 plasmid can easily lose such plasmids when the URA3 gene is selected against on media containing 5-fluoroorotic acid, tpt1-1::LEU2 haploid strains bearing the TPT1 gene on a URA3 plasmid cannot lose the plasmid and therefore die on media containing 5-fluoroorotic acid (see Table IV). This is the expected result if expression of the TPT1 gene is necessary for vegetative growth.

**Analysis of the TPT1 Gene**—The phosphotransferase is a 230-amino acid protein, with a calculated molecular weight of 26,196 and an isoelectric point of 9.30. It is a highly charged protein; fully 30% of the residues are potentially ionized at neutral pH, and there is a substantial excess of basic residues (12 Arg, 20 Lys, 12 His) over acidic residues (11 Asp, 14 Gln).

Examination of the amino acid sequence with either GCG or Swiss-Prot, or by visual inspection, reveals little about possible domains of the protein. No well characterized RNA binding motifs are found in the Tpt1 protein, including the RNP1, arginine-rich (ARM), RGG, K homology (KH), and double-stranded RNA binding motifs (see Ref. 38 for review). Similarly, there are no sequences identical to the RNA binding motifs of the class I aminoacyl tRNA synthetases, which are involved in binding the amino acid acceptor stem of the tRNA (39, 40), and no marked similarities with the less well characterized class II aminoacyl tRNA synthetase sequences (41). Among the tRNA synthetases there is a lack of a consensus site for binding the tRNA anticodon (42, 43). The best match to an NAD binding domain is to that of diphtheria toxin (44). The residues of diphtheria toxin (and by analogy the related exotoxin A from Pseudomonas aeruginosa) that contact NAD are characterized by the sequence beginning at amino acid 117 (HGTNLQSVIKIIES-P/R. A reasonable match is found in the Tpt1 protein, including the RNP1, arginine-rich (ARM), RGG, K homology (KH), and double-stranded RNA binding motifs (see Ref. 38 for review). Similarly, there are no sequences identical to the RNA binding motifs of the class I aminoacyl tRNA synthetases, which are involved in binding the amino acid acceptor stem of the tRNA (39, 40), and no marked similarities with the less well characterized class II aminoacyl tRNA synthetase sequences (41).

The TPT1 gene itself, as demonstrated by the presence of the LEU2 gene (data not shown). In SC804 (relevant genotype): TPT1+/tpt1-1::LEU2) 6 tetrads were examined; all segregated two live:two dead spores, and all the live spores lacked the LEU2 marker. Similarly in SC805 (relevant genotype: TPT1+/tpt1-2) seven tetrads were examined and all segregated two live Leu` spores and two dead spores. These are the expected results if disruption of the TPT1 gene is lethal. Since, in addition, microscopic examination of the nonsurviving spores demonstrated that they germinated and grew into microcolonies, these results suggest that the TPT1 gene is essential.

2 S. Spinelli and E. M. Phizicky, unpublished results.
2'-Phosphotransferase Gene Implicated in tRNA Splicing

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**FIG. 4.** A, predicted amino acid sequence of the Tpt1 protein. The sequence was obtained from the *Saccharomyces cerevisiae* genome data base (HRE230 accession number SS1897) and independently confirmed. B, restriction map of the *TPT1* gene and surrounding region on chromosome XV. The *TPT1* ORF and neighboring ORFs are represented by heavy arrows in the direction in which they are transcribed. Restriction sites within and around the *TPT1* gene, which were used for plasmid construction, are represented by light vertical lines.

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**TABLE II**

| Strain | Growth Specific activity units/mg |
|--------|----------------------------------|
| EMP177 + yEpLac181 (2-μm LEU2 vector) | -Leucine 9 × 10^3 |
| EMP177 + pGMC5 (2-μm LEU2 TPT1) | -Leucine 500 × 10^3 |
| SC466 + pBM150 (P*gal16* vector) | YP + glucose 6.1 × 10^3 |
| SC466 + pBM150 (P*gal16* TPT1) | YP + galactose 6.8 × 10^3 |
| SC466 + pGMC21 (P*gal16*TPT1) | YP + glucose 8.6 × 10^3 |
| SC466 + pGMC21 (P*gal16*TPT1) | YP + galactose 170 × 10^3 |

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**DISCUSSION**

We have cloned the *S. cerevisiae* TPT1 gene, which encodes the 2'-phosphotransferase activity implicated in the last step of tRNA splicing: removal of the splice junction 2'-phosphate from ligated tRNA. This gene was cloned from the purified protein by reverse genetics and demonstrated to be authentic by overproduction of the activity in both yeast and *E. coli* under regulated promoter control. 2'-Phosphotransferase appears to be a single catalytic polypeptide (Fig. 1, Table III). The purified protein is the predominant silver-staining band in highly purified preparations, and the bacterially expressed protein catalyzes the same NAD-dependent 2'-phosphotransferase reaction in extracts from *E. coli*. Since formation of Appr-p is at least a two-step chemical reaction, this single polypeptide likely carries out both steps, if both steps are enzyme-catalyzed.

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Sanger Center in the United Kingdom (http://www.sanger.ac.uk/~yeastpub/swpombe.html.). The predicted amino acid sequence of this gene is shown in Fig. 5. The *S. pombe* ORF is 34% identical and 57% similar to that of *S. cerevisiae*, with three distinct blocks of 10 or more amino acids where the identity approaches or exceeds 80%. The conservation of sequence extends over the entire length of the *S. cerevisiae* protein, suggesting that the *S. pombe* ORF might be a functional homolog of the Tpt1 protein. Tpt1 protein also shares significant conserved amino acid sequence with an ORF in *E. coli* and several ESTs from higher eukaryotes, as illustrated in Fig. 5. The amount of conserved sequence, and the fact that the conservation is largely in the same regions among all the potential proteins, suggest that these ORFs form a family.
sequences of a lack of the protein. The cellular role of Appr diesterase that can convert Appr

We have previously identified a highly specific cyclic phospho-

would most easily be addressed by altering its levels in vivo.

sequences in the data base.

The

metabolic products has a cellular role, then the failure to make

this product might also impair growth. We have recently iso-

lated conditional tpt1 mutants to begin to ascertain the con-

sequences of a lack of the protein. The cellular role of Appr would most easily be addressed by altering its levels in vivo. We have previously identified a highly specific cyclic phosphodiesterase that can convert Appr or r to the correspond-

ing ribose-1-P derivative in yeast extracts (50), an activity that appears to be related to a similar activity in wheat germ (50, 51). A cyclic phosphodiesterase from Arabidopsis with very similar properties has recently been cloned (52); analysis of its function in Arabidopsis or of the function of the corresponding gene in yeast may directly address the question of the role of Appr in cells.

A crude calculation based on the purification (Table I) indicates that there is on the order of 10 times more phosphotrans-

ferase protein in a yeast cell than there is of the other splicing enzymes: tRNA ligase protein and endonuclease (34, 53). En-

endonuclease and ligase may form a complex in vivo, based on their similarities within the cell, their localization in similar subdomains of the nucleus (7, 54), and the concerted splicing reaction observed in vitro with tRNA precursors (55). If such a complex includes the phosphotransferase, there is likely an excess of uncomplexed phosphotransferase. This is consistent with the observation that there is at least 12 times as much 2'-phosphotransferase activity in cells as is necessary for normal growth. Since the GAL10 promoter is tightly repressed by glucose-containing medium, it might be expected that a tpt1-D::LEU2 strain with a GAL10- TPT1 plasmid would die on glucose. Unfortunately this is not the case; cells with only a galactose-regulated TPT1 gene have wild type growth rates after prolonged growth in glucose. Under these conditions, phosphotransferase activity is down 12-fold from that observed in wild type cells; thus there is an excess of phosphotransferase. The high degree of conservation of the S. cerevisiae Tpt1 protein is striking. Since the enzymes: tRNA ligase protein and endonuclease (34, 53). En-

endonuclease and ligase may form a complex in vivo, based on their similarities within the cell, their localization in similar subdomains of the nucleus (7, 54), and the concerted splicing reaction observed in vitro with tRNA precursors (55). If such a complex includes the phosphotransferase, there is likely an excess of uncomplexed phosphotransferase. This is consistent with the observation that there is at least 12 times as much 2'-phosphotransferase activity in cells as is necessary for normal growth. Since the GAL10 promoter is tightly repressed by glucose-containing medium, it might be expected that a tpt1-D::LEU2 strain with a GAL10- TPT1 plasmid would die on glucose. Unfortunately this is not the case; cells with only a galactose-regulated TPT1 gene have wild type growth rates after prolonged growth in glucose. Under these conditions, phosphotransferase activity is down 12-fold from that observed in wild type cells; thus there is an excess of phosphotransferase in the cell.

The high degree of conservation of the S. cerevisiae Tpt1 protein sequence with sequences from E. coli suggests strongly that these proteins constitute a family. Given the similarities of sequence, it seems reasonably likely that the S. pombe ORF, and perhaps the mouse and rice ORFs, encode functional Tpt1 proteins; these ORFs all share the same ORFs, translation termination signal, gaps in the alignment.

metabolic products has a cellular role, then the failure to make this product might also impair growth. We have recently iso-

lated conditional tpt1 mutants to begin to ascertain the con-

sequences of a lack of the protein. The cellular role of Appr would most easily be addressed by altering its levels in vivo. We have previously identified a highly specific cyclic phosphodiesterase that can convert Appr or r to the correspond-

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ates splice junctions with a 2'-phosphate, it seems unlikely that this protein encodes a tRNA 2'-phosphotransferase. However, the E. coli ORF might encode a related catalytic or binding activity. Understanding the function and/or the biochemical activity of the E. coli protein might therefore lead to an understanding of the origin of the unusual activity catalyzed by the yeast 2'-phosphotransferase.

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