Mutations in a Peptidylprolyl-cis/trans-isomerase Gene Lead to a Defect in 3'-End Formation of a Pre-mRNA in Saccharomyces cerevisiae*

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In a genetic screen aimed at the identification of trans-acting factors involved in mRNA 3'-end processing of budding yeast, we have previously isolated two temperature-sensitive mutants with an apparent defect in the 3'-end formation of a plasmid-derived pre-mRNA. Surprisingly, both mutants were rescued by the essential gene ESS1/PTF1 that encoded a putative peptidylprolyl-cis/trans-isomerase (PPIase) (Hani, J., Stumpf, G., and Domdey, H. (1995) FEBS Lett. 365, 198–202). Such enzymes, which catalyze the cis/trans-interconversion of peptide bonds N-terminal of prolines, are suggested to play a role in protein folding or trafficking. Here we report that Ptf1p shows PPIase activity in vitro, displaying an unusual substrate specificity for peptides with phosphorylated serine and threonine residues preceding proline. Both mutations were found to result in amino acid substitutions of highly conserved residues within the PPIase domain, causing a marked decrease in PPIase activity of the mutant enzymes. Our results are suggestive of a so far unknown involvement of a PPIase in mRNA 3'-end formation in Saccharomyces cerevisiae.

Despite intensive efforts to unravel the complex process of mRNA 3'-end formation in Saccharomyces cerevisiae, the list of participating factors still awaits its completion.

We have recently isolated a gene complementing the phenotype of two temperature-sensitive yeast mutants that were impaired in mRNA 3'-end formation. This gene, designated PTF1 (processing/termination factor 1; identical with the previously described ESS1 (1)), encodes a protein that, by virtue of sequence similarity, was identified as a peptidylprolyl-cis/trans-isomerase (PPIase) (2). PPIases are ubiquitous enzymes that catalyze the interconversion from cis to trans of peptide bonds preceding a proline and are thought to accelerate this often rate-limiting step in the folding of a number of proteins in vivo (3–6).

PPIases are divided in three families, based on their sensitivities toward two clinically relevant immunosuppressants: the cyclosporin A-binding proteins (cyclophilins), the FK506-binding proteins, and a third family, named after the Escherichia coli protein parvulin, which is not inhibited by either of the two drugs (for review see Refs. 3–6). In addition, the members of each family are characterized by conserved but distinct amino acid domains. By this criterion, PTF1 was predicted to belong to the parvulin family of PPIases (2).

Although disruption of PPIase genes did not generally impair cell growth (7–8), PTF1 was the first PPIase gene shown to be essential for cell viability (1). In fact, PTF1 is the only essential PPIase gene in S. cerevisiae as demonstrated more recently by the viability of a yeast mutant lacking the remaining 12 PPIases, members of the other two immunosuppressant binding families. (8). So far, the only other example of an essential PPIase is the recently discovered PIN1, a human protein, that is structurally and functionally related to Ptf1p (9–10).

In this paper we describe the genetic screen that led to the isolation of PTF1 and the phenotypes of the two temperature-sensitive strains carrying mutations in this gene. Moreover, we demonstrate that Ptf1p displays PPIase activity in vitro and that this activity is drastically reduced in the two mutant PTF1 proteins isolated at nonpermissive temperatures. The intriguing observation that yeast cells harboring mutant PTF1 proteins are defective in the 3'-end formation of a plasmid-encoded pre-mRNA invites speculations on the possible role of a PPIase in mRNA 3'-end processing and/or transcription termination in budding yeast.

EXPERIMENTAL PROCEDURES

Strains—E. coli XL 1 Blue (recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac (F proAB lacZAM15 Tn10 (tet)) (Stratagene) was used for cloning procedures. E. coli BL21 (DE3) (F ompT rpsL mcrA (DE3) (Novagen) was used for expression of Ptf1p.

Yeast DH448 cells (MATa ade2-1 leu2-3 his3-12 can1-100 trp5-48 ura4-1 lys1-1) were used for the production of mutants (11). DBY784 cells (MATa his5-2 (o), leu2-1 (o), can1-100 (o), ura3-52, trp5-48 (o)) were used for mating with the mutant YM23 (12). EJ101 (20B-12-1) (MATa, trp1, prot-126, prb1-112, pep4-3, pro1-126) was used for the production of yeast whole cell extracts (13). W303 (MATaMATa, ade2/ ade2 his3/3his3, leu2/leu2, trp1/1trp1, ura3/ura3) was used as a diploid control (14).

Plasmid Construction—The screening plasmid pJH702CEN was constructed with plasmid pRE200 (15) as basic vector in which the TRP1 gene was replaced by the LEU2 gene. In the single BamHI site of pPF2LEU2 (16) the following fragments were inserted: a 730-bp long BamHI/BglII fragment containing the ACT1 promoter plus flanking sequences, a modified ADH1 terminator without stop codons, and a

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Selective medium to an 60° C. The sporulation analysis, and tetrad analysis were performed as described by Lawrence (21). The production of Parvulin 1 (YPT1) had a length of 143 (pSH101) or 80 nt (pSH102). The 3′-terminal region of ACT1 was inserted into the pLacZ gene (see Fig. 4). For the generation of pGalPTF1, which contains the PTF1 gene under control of the GAL1 promoter, the EcoRI fragment of YIP1-GESS (1) carrying the ess1 gene under GAL1 control was purified and inserted into pBluescript II KS+ (16–17). From the 80 nt-long fragment which still contained a part of the 5′-nontranscribed region was deleted by site-specific mutagenesis according to Kunkel (18). For the overexpression of a mutated parvulin gene, the corresponding DNA was amplified by PCR from pShE538 (19) with the primer MP1 5′-GCA GGA TCC GAT GAC GAT GAC AAA ACA GCA GCA GCA C-3′ which contains an enterokinase cutting site and a BamHI restriction site and the primer MP3 5′-CCG GGC AGC TGG GTA AAG AAG-3′ which is complementary to the 3′-terminal region of ess1. The PCR-amplified construct was modified by site-specific mutagenesis according to Kunkel (18).

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The construction of the plasmids pHDS059s (ACT1 terminator), pHDS11s (CYC1 terminator), pHDS512s (YPT1 terminator), pHSh101 (ADH1 terminator 145 nt), pHSh102 (ADH1 terminator 80 nt) has been described in Heidmann et al. (28). The 3′-terminal fragments of ADH1 had a length of 143 (pSh101) or 80 nt (pSH102). The 3′-terminal region of ADH1 consisted of a 354-nt-long Sau3AI fragment (pHD509s), the CYC1 region consisted of a 241-nt-long Sau3AI fragment (pHD511s), and the YPT1 region of a 494-nt-long Sau3AI fragment (pHD512s).

EMS Mutagenesis and Tetrad Analysis of Yeast Cells—EMS mutagenesis was performed as described by Lawrence (21). The production of diploid yeast cells, the sporation analysis, and tetrad analysis were done according to Haber and Halvorson (22) and Sherman and Hicks (23).

RNA Analysis—Overnight cultures of YPM2 and of DH484 (200 ml), transformed with the selection plasmid pH702CEN, were grown in selective medium to an 60° C. A 100-ml aliquot of medium was removed for RNA preparation, and the remaining 100 ml were mixed with the same amount of medium prewarmed to 50 °C. Incubation was continued at 37 °C for 6 h when another 100 ml were removed for RNA preparation. The last aliquot of 100 ml was taken after another 13 h. Total RNA was prepared from the collected yeast cells by the hot phenol method as described by Köhrer and Domdey (24). Poly(A) RNA was isolated with an mRNA purification kit purchased from Amersham Pharmacia Biotech. Then all RNA aliquots were treated with RNase-free DNase to remove traces of remaining DNA.

For Northern blot analysis, 20 μg of glyoxal-treated total RNA was separated on a 1.5% agarose gel and transferred to a Hybond N nylon membrane (Amersham Pharmacia Biotech). For the quantitation of processed and unprocessed chimeric ACT1-ADH1-locZ transcrip...
the absorbance at 390 nm in a Hewlett-Packard 8452 diode array UV-visible spectrophotometer in 0.5-s intervals for a total of 4 min. Reported data are given as the mean value of three to five measurements.

A disadvantage of the protease-coupled assay is the requirement for high concentrations of helper proteases in order to obtain the two-phase reaction described above. This assay could therefore only be used for proteins that proved to be resistant toward attack from these enzymes, at least for the duration of the experiment. The intactness of each putative PPIase after the reaction was examined on Western blots. In the case of recombinant Ptf1p, thrombin and trypsin cleaved off the oligohistidine tag at the inserted thrombin cleavage site but did not further digest Ptf1p. Subtilisin did not attack the protein under the conditions of PPIase measurements, whereas chymotrypsin digested Ptf1p completely within 2 min of incubation (2). Thus, in the PPIase assay of Ptf1p, trypsin (0.08 and 0.02 mg/ml) was used for cleavage of the Lys-4NA and Arg-4NA bonds, respectively, and subtilisin (0.04 mg/ml) for cleaving the Phe-, Tyr- Leu- and Met-4NA bonds. Substrates were purchased from Bachem (Heidelberg) or synthesized according to Schutkowski et al. (30). Stock solutions of various substrates were prepared in dimethyl sulfoxide.

For inhibition studies, the Suc-Ala-Phe-Pro-Phe-4NA was used as substrate for parvulin and Suc-Ala-Ala-Pro-Arg-4NA as substrate for Ptf1p. FK506 was a gift from Fujisawa Pharmaceutical Co., Osaka. Stock solutions of the inhibitors were prepared in 50% ethanol. The incubation time was 5 min for FK506 and 15 min for cyclosporin A. Three independent experiments were performed.

**Protease-free Assay**—Because of the unexpected sensitivity of the mutant proteins Ptf1-2p and -5p toward proteases, PPIase activity was measured using a modification of the assay described by Kofron et al. (31) and others (32). This assay exploits the difference in the absorption coefficients of cis and trans conformers of the substrates Suc-Ala Ala Pro-(NO$_2$)Tyr-4-fluoranilide at 430 nm.

**RESULTS**

**Identification and Characterization of Yeast Mutants Defective in Pre-mRNA 3’-End Formation**—In an effort to identify trans-acting factors participating in the 3’-end processing reaction of yeast pre-mRNAs, a genetic selection system was established, in which a defect in this reaction could be recognized by the appearance of blue-colored colonies. The system was based on a fusion construct, pJH702CEN, composed of a 3’-end truncated ACT1 gene joined to the lacZ gene by a minimum-sized ADH1 3’-end formation site in a centromere vector (Fig. 1). The ADH1-derived sequences had been modified such that an open reading frame was maintained throughout the originally noncoding 3’-region of the ADH1 gene and that translation of read-through transcripts would result in a lacZ fusion protein. Stable read-through transcripts were expected to occur only in plasmid-carrying cells in which the formation of mRNA 3’-ends was impaired.

Transformation of wild type yeast with pJH702CEN (Fig. 1) yielded, as expected, white colonies on 5-bromo-4-chloro-3-indolyl b-o-galactopyranoside-containing medium, as the plasmid-derived transcripts were processed and polyadenylated at the ADH1 3’-end formation site. In contrast, a positive control construct (pJH712CEN, Fig. 1), in which the 3’-end truncated ACT1 gene was directly connected with the lacZ gene, gave rise to blue colonies on 5-bromo-4-chloro-3-indolyl b-o-galactopyranoside-containing selective medium (data not shown).

After EMS mutagenesis of 1.9 million yeast cells containing the selection plasmid pJH702CEN, three viable mutants (yeast processing mutants YPM2, YPM3, and YPM5) that displayed the expected blue-color phenotype were isolated at 23 °C. Two of them, YPM2 and YPM5, additionally showed temperature-sensitive growth at 30 and 37 °C, respectively. The results of several control experiments demonstrated that the mutations leading to the specific phenotypes were chromosomal mutations (data not shown) as follows. (i) The plasmids isolated from the mutants and retransformed into wild type yeast led to a wild type phenotype, i.e. white colonies on 5-bromo-4-chloro-3-indolyl b-o-galactopyranoside-containing plates. (ii) The DNA
sequence of the \( ADH1 \) 3'-end formation site and the beginning of the \( lacZ \) gene was not altered in the mutants. (iii) Mutants, which had been grown on non-selective medium until they had lost their plasmid, turned blue again after re-transformation with the original selection plasmid.

Shifting the YPM2-mutant cells from 23 \( ^\circ \)C to the non-permissive temperature of 37 \( ^\circ \)C led to a significant decrease of the amount of poly(A)\(^+\) RNA within 2 h after the shift (Fig. 2).

To demonstrate that the blue phenotype of the mutants coincided with the presence of the chimeric \( ACT1-ADH1-lacZ \) read-through transcripts in the mutant cells, RT-PCR was performed with total RNA isolated from the mutant YPM2 and wild type yeast grown at 23 and 37 \( ^\circ \)C, respectively. As a positive control, RNA from wild type yeast DH484, which had been transformed with the test plasmid, was isolated under identical conditions. Two sets of primer pairs were used as follows: the primers JH24 and JH25 amplified all \( ACT1-ADH1-lacZ \)-derived transcripts (Fig. 3A, product I), whereas the primers JH26 and JH27 were designed to amplify only those transcripts that had not been cleaved at the \( ADH1 \)-derived polyadenylation site (Fig. 3A, product II). The radioactively labeled RT-PCR products, separated on agarose gels (Fig. 3B) were quantitated by scanning with a PhosphorImager. In wild type cells, read-through transcripts (Fig. 3A, product II) comprised only 2.5 and 1.5% of the total amount of plasmid-encoded \( ACT1 \) transcripts (Fig. 3A, product I) at 23 \( ^\circ \)C and 37\% respectively. In contrast, 10-fold higher levels of read-through transcripts, i.e. 30\%, were detected in the mutant cells at 37 \( ^\circ \)C, with 13\% already present at the permissive temperature of 23 \( ^\circ \)C.

In order to test whether the observed increase in the amount of read-through transcripts from the selection plasmid in YPM2 was restricted to the special template, the transcripts of three other yeast genes, \( ACT1 \), \( CYC1 \), and \( YPT1 \), were examined on Northern blots. To facilitate the analysis, YPM2 cells were transformed with plasmids containing 3' terminal fragments of these genes inserted between the \( ACT1 \) promoter and the terminator of either \( ACT1 \) or \( ADH1 \) (Fig. 4 (20)). As a control, \( ADH1 \) was included with either a complete or truncated version of its 3'-terminal region (Fig. 4). The latter was very similar to the one used in the mutant screen.

The plasmid constructs and positions of the oligonucleotide probes SH16 and SH18 are shown schematically in Fig. 4. The two probes enabled the distinction between transcripts ending in the inserted fragment and unprocessed products ending within the downstream adjacent \( ADH1 \) or \( ACT1 \) terminal region.

Additionally, both probes could also detect endogenous \( ACT1 \) mRNA. The corresponding signal was used as a standard for the amount of RNA isolated from YPM2 cells that differed only in the DNA constructs with which they had been transformed. Northern blot analysis was carried out with total RNA from cells that had been grown at either the permissive or the non-permissive temperature (Fig. 5). The same blot was successively probed with SH16 (Fig. 5A) and SH18 (Fig. 5B).

Hybridization performed with SH16 and RNA that had been synthesized at 23 \( ^\circ \)C resulted mostly in strong signals, corresponding well in size to transcripts that had initiated at the plasmid-derived \( ACT1 \) promoter and ended at the polyadenyl-
goals. Restriction sites are as follows: BHI, DRA, HII, HindIII, and SI, SpHII.

Fig. 4. Schematic presentation of the constructs, which were examined for their ability to yield mature mRNA 3’-ends. The cloning of these constructs was described in Heidmann et al. (10). Horizontal arrows indicate the orientation of the insertions. SH16 and SH18 indicate regions where the corresponding oligonucleotides hybridize. Restriction sites are as follows: BHI, BamHI; DI, DraI; ERV, EcoRV; HII, HindIII; and SI, SpHII.

To establish whether the mutant phenotype could also morphologically be distinguished from wild type cells, mutant and wild type protoplasts were inspected under the microscope. This analysis revealed that protoplasts of the mutant YPM2, maintained at the non-permissive temperature for 10 h, had about double the diameter of wild type protoplasts (data not shown).

In addition, immunofluorescence microscopy with β-tubulin antibodies showed that the spindles in YPM2 cells were very large, compared with the ones seen in the wild type and occurred also in cells that did not show budding (data not shown). This observation indicates that the mutants were arrested in the late anaphase I.

Consistent with the observed lethal phenotype of PTF1 mutants, tetrad analysis of diploid YPM2/DBY874 yeast reproducibly yielded a 2:2 co-segregation of the expression of β-galactosidase together with the temperature sensitivity (data not shown).

Identification of the PTF1 Locus and Characterization of Wild Type and Mutant Genes—Complementation of the temperature sensitivity of both YPM2 and YPM5 with two different genomic libraries resulted in the cloning and isolation of the same genomic region and the identification of PTF1 (2). Its predicted gene product is characterized by a PPIase domain with homology to E. coli parvulin and a WW domain (W denotes the invariant tryptophans), which is assumed to mediate protein-protein interactions (34).

The mutant PTF1 gene locus that caused the temperature sensitivity was isolated by PCR amplification of genomic DNA from the mutants. The amplified products were either sequenced directly or after cloning in a Bluescript vector. In YPM2, a single point mutation led to a change from the glycine residue at position 127 to aspartic acid. The same was true for YPM5, where again a single nucleotide difference caused an amino acid change, this time from a glycine at position 163 to a serine. On the DNA level, both mutations resulted from

The presence of a disproportional signal at the position of the endogenous ACT1 mRNA in lane 8 of Fig. 5B clearly demonstrated that the YPT1 transcript, present in mutant cells grown at 37 °C, was a genuine read-through transcript. A similarly clear result was obtained for the ADH1 transcript expressed from the DNA construct carrying the truncated version of the ADH1 3’-terminal element; a read-through transcript was visible at 37 °C (lane 9) and at 23 °C (lane 4). Interestingly, the presence of the complete ADH1 3’-end formation site totally suppressed the formation of longer transcripts which seems also to be true for the ACT1 and CYC1 constructs. The shorter RNA species, detected in the overexposed autoradiogram of Fig. 5B, lanes 1 and 3, and 6 and 8, are presumably transcripts initiating at cryptic promoter sites downstream of the ACT1 promoter.
transitions of a guanine to adenine. More importantly, both mutations were located within the highly conserved regions of PPIases (Fig. 6).

Coexpression of wild type Ptf1p in each mutant strain restored cell growth at non-permissive temperatures. Moreover, in contrast to results from a previous publication (1), overexpression of Ptf1p in wild type yeast did not lead to cell death (data not shown).

Western Blot Analysis and Mass Determination of Ptf1p—A polyclonal antiserum raised against denatured Ptf1p was obtained from rabbit by immunization with the recombinant His-tagged protein that was described previously (2). Using this antiserum, one of two signals, corresponding to proteins of 70 (Fig. 7, lanes 4 and 6) and 23 kDa (Fig. 7, lanes 3 and 5), respectively, appeared on Western blots prepared with proteins contained in yeast whole cell extracts or fractions thereof. The appearance of either species depended on the method of extract preparation. In extracts, prepared with glass beads, a single species of 70 kDa was identified, which is in obvious contrast to the predicted molecular mass of Ptf1p (19,404 Da). This species survived even the most stringent denaturation protocol applied to the protein sample prior to gel electrophoresis. However, when the extract was prepared in the same manner as for the in vitro 3'-end processing reactions (35), the only signal visible matched the expected molecular mass of Ptf1p (about 23 kDa). Surprisingly, after fractionation of the same extract with 40% ammonium sulfate, the 23-kDa species was found in the supernatant, whereas the 70-kDa signal appeared in the pellet. So far, we can only hypothesize about the nature of the latter protein species. It might represent Ptf1p in an unusually stable multimeric state or in tight complex with another protein, possibly its target. A third possible explanation that the observed high molecular weight protein resulted from cross-reaction with the Ptf1p antiserum can be largely excluded, since in a computer search we did not detect any Ptf1p-related sequences within the complete yeast genome (36).

The molecular mass of the recombinant proteins was analyzed by electrospray mass spectrometry. The resulting values of 21,438 Da for the oligohistidine-tagged protein and 19,263 Da for the one without tag agree well with the predicted sizes and provide no indication for a post-translational modification in E. coli besides removal of the starting Met residue.

PPIase Activity of Recombinant Ptf1p—A protease-coupled assay (see Refs. 37 and 38 and “Experimental Procedures”) was employed to determine the PPIase activity of wild type Ptf1p isolated from E. coli cells that expressed the recombinant, His-tagged protein. cis/trans-Isomerization of the proline bond in synthetic tetrapeptide substrates was measured spectroscopically, and activity was expressed as $k_{\text{cat}}/K_M$.

The values for PPIase activity of Ptf1p and its substrate specificity are summarized in Table I. Using the standard PPIase substrate Suc-Ala-Ala-Pro-Phe-4NA, only moderate enzymatic activity was detected ($k_{\text{cat}}/K_M = 5.9 \times 10^3 \text{M}^{-1} \text{s}^{-1}$). Using a series of peptides, which differed in the residues flanking a single proline, the highest specificity constant was obtained with Suc-Ala-Glu-Pro-Phe-4NA ($k_{\text{cat}}/K_M = 4.2 \times 10^6 \text{M}^{-1} \text{s}^{-1}$). This almost 1000-fold higher enzymatic activity of Ptf1p toward the latter substrate in which glutamic acid, a phosphorylated serine surrogate, preceded proline, led us to investigate peptides containing phosphorylated Ser, Thr, or Tyr residues in the equivalent position. As depicted in Table I, the enzyme showed the highest increase in activity over the standard value when a phosphorylated serine ($k_{\text{cat}}/K_M = 1.7 \times 10^7 \text{M}^{-1} \text{s}^{-1}$) or, to a lesser extent, a phosphorylated threonine ($k_{\text{cat}}/K_M = 2 \times$...
Activitues are expressed as 10 °C in a protease-coupled assay (see "Experimental Procedures"). Ptf1p expressed in E. coli factor, and more importantly, E. coli the values obtained with the standard substrate.

ever, phosphorylated tyrosine in this position did not enhance crease rather than an increase of their specificity constants.

TABLE I
PPIase activity and substrate specificity of Ptf1p

| Substrate                        | \(k_{\text{cat}}/K_M\) | Activity |
|----------------------------------|-------------------------|----------|
|                                  | \(\text{M}^{-1}\text{s}^{-1}\) | %        |
| Suc-Ala-Glu-Pro-Phe-4NA          | 4,170,000               | 100      |
| Suc-Ala-Pro-Leu-4NA              | 69,400                  | 100      |
| Suc-Ala-Ala-Pro-Met-4NA          | 58,200                  | 100      |
| Suc-Ala-Ala-Pro-Arg-4NA          | 36,800                  | 100      |
| Suc-Ala-Gln-Pro-Phe-4NA          | 12,600                  | 100      |
| Suc-Ala-Pro-Lys-4NA              | 7,300                   | 100      |
| Suc-Ala-Pro-Phe-4NA              | 6,600                   | 100      |
| Suc-Ala-Pro-Phe-4NA              | 5,900                   | 100      |
| Suc-Ala-Leu-Pro-Phe-4NA          | 4,400                   | 100      |
| Suc-Ala-Nle-Pro-Phe-4NA          | 3,400                   | 100      |
| Suc-Ala-His-Pro-Phe-4NA          | 2,000                   | 100      |
| Suc-Ala-Ala-Pro-Phe-4NA          | 1,400                   | 100      |
| Suc-Ala-Phe-Pro-Phe-4NA          | <1,000                  |          |
| Suc-Ala-Ile-Pro-Phe-4NA          | <1,000                  |          |
| Suc-Ala-Trp-Pro-Phe-4NA          | <1,000                  |          |
| Suc-Ala-Phe-Pro-Phe-4NA          | <1,000                  |          |
| Suc-Ala-Lys-Pro-Phe-4NA          | <1,000                  |          |
| Ac-Ala-Ala-(PO_4H_2) Ser-Pro-Tyr-4NA | 17,200,000          | 100      |
| Ac-Ala-Ala-(PO_4H_2) Ser-Pro-Arg-4NA | 12,000,000          |          |
| Ac-Ala-Ala-(PO_4H_2) Ser-Pro-Lys-4NA | 8,740,000            |          |
| Ac-Ala-Ala-(PO_4H_2) Thr-Pro-Arg-4NA | 4,730,000            |          |
| Ac-Ala-Ala-(PO_4H_2) Thr-Pro-Lys-4NA | 2,080,000            |          |
| Ac-Ala-Ala-(PO_4H_2) Thr-Pro-Phe-4NA | 960,000              |          |
| Ac-Ala-Ala-(PO_4H_2) Tyr-Pro-Arg-4NA | 436,000              |          |
| Ac-Ala-Ala-(PO_4H_2) Tyr-Pro-Lys-4NA | 5,200               |          |
| Ac-Ala-Ala-(PO_4H_2) Tyr-Pro-Phe-4NA | 15,500               |          |
| Ac-Ala-Ala-(PO_4H_2) Tyr-Pro-Lys-4NA | 13,400               |          |
| Ac-Ala-Ala-(PO_4H_2) Tyr-Pro-Phe-4NA | 11,300               |          |
| Ac-Ala-Ala-(PO_4H_2) Tyr-Pro-Lys-4NA | 3,800                |          |
| Ac-Ala-Ala-(PO_4H_2) Tyr-Pro-Phe-4NA | 2,200                |          |
| Ac-Ala-Ala-(PO_4H_2) Tyr-Pro-Lys-4NA | 2,900                |          |

10^6 M⁻¹ s⁻¹ occurred N-terminal to the proline moiety. However, phosphorylated tyrosine in this position did not enhance the values obtained with the standard substrate.

Other PPIases like human FKBP12, human CYP18, trigger factor, and more importantly, E. coli parvulin showed a decrease rather than an increase of their specificity constants when tested with the phosphorylated substrates.³

In order to rule out that the His-tag of the recombinant protein might have affected the activity and specificity of the enzyme, an authentic His-tag-free recombinant Ptf1p was expressed in E. coli and purified to homogeneity. The integrity of the protein was confirmed by high performance liquid chromatography and mass spectrometry. No differences were found between the enzymatic activities of oligohistidine-tagged and the authentic His-tag-free recombinant Ptf1p (data not shown).

PPIase Activity of Recombinant Ptf1p from the Yeast Mutants YPM2 and YPM5—As described above, one single, although distinct, amino acid change in Ptf1p led to temperature sensitivity of the yeast mutants YPM2 and YPM5 (Fig. 6). To test the PPIase activity of the mutant forms, the corresponding recombinant proteins were expressed in E. coli and isolated from cells grown at non-permissive temperature (37 °C). The mutant proteins turned out to be very unstable and sensitive toward all proteases used for activity measurements. Therefore, activity was determined in a protease-free assay (see “Experimental Procedures” and Ref. 32).²

The data in Table II demonstrate that the PPIase activity of both mutants was strongly reduced as compared with the wild type protein. Moreover, the degree of temperature sensitivity correlated directly with the relative decrease in enzymatic activities of the corresponding enzymes, i.e. Ptf1p from the mutant YPM2 (Ptf1-2p) which showed temperature sensitivity at 30 °C was about half as active as Ptf1p from YPM5 (Ptf1-5p) which is still viable at 30 °C.

To establish whether the mutations at the conserved Gly residues in Ptf1p affected also the activity of another PPIase within the same family, the equivalent Gly substitutions were introduced into E. coli parvulin, resulting in the parvulin mutants Gly103-Asp, corresponding to Ptf1-2p, and Gly105-Ser, comparable to Ptf1-5p. Like the Ptf1p mutants, both parvulin mutant proteins were found to be very unstable. Most notably, as shown in Table III, the presence of either mutation virtually abolished the enzymatic activity of parvulin (less than 0.1%).

**DISCUSSION**

The most curious aspect of *PTF1*, the first essential PPIase gene identified in *S. cerevisiae*, is the fact that it was discovered in a genetic screen aimed at the identification of factors involved in the mRNA 3′-end processing pathway in bakers’ yeast (2). Moreover, *PTF1* was the only gene found in this screen to complement the two independently isolated, EMS-induced temperature-sensitive mutants, YPM2 and YPM5, with an apparent defect in the 3′-end formation of a plasmid-encoded pre-mRNA. Isolation and sequence determination of the *PTF1* genes from both mutants revealed a single, albeit distinct, point mutation at the DNA level, each resulting in a drastic change of highly conserved amino acid residues within the PPIase domain. These changes were accompanied by a drastic decrease in PPIase activity. Coexpression of wild type Ptf1p in

³ B. Schelbert, A. Bernhardt, G. Fischer, and J.-U. Rahfeld, unpublished results.
either mutant was sufficient to restore growth at non-permissive temperatures.

Our mutant screen was based on the detection of plasmid-derived transcripts arising from lack of cleavage and polyadenylation at a truncated ADH1 terminator region, located between yeast actin sequences and the bacterial lacZ gene (Fig. 1 and text). Still, with the notable exception of YPT1, no such read-through events were observed in the mutant strains, when the truncated ADH1 terminator region was replaced by its complete version, or by equivalent sequences from the ACT1 and CYC1 genes (Fig. 4).

A similar “diffuse” effect on mRNA 3’-end formation was reported by Russnak et al. (39). The ref2-1 mutants, which the authors identified, showed differences in mRNA 3’-end processing only for transcripts encoded by certain, artificially designed plasmids. By this criterion, the authors defined “weak” and “strong” polyadenylation sites, a definition which had been introduced earlier by Inniger et al. (40). Moreover it was suggested that impairment of processing efficiency should not occur without a negative effect on termination frequency in yeast cells, as in this organism, unlike in higher eukaryotes, transcription and polyadenylation are tightly coupled. In fact, it might even have been impossible to distinguish in our screen whether the primary defect had occurred in transcription termination or at the level of endonucleolytic cleavage and polyadenylation because of their expected common consequences.

Any failure in transcription termination should also interfere with the transcription initiation of the gene following immediately downstream, as in yeast there is generally little space between polyadenylation sites and the transcription start site of the adjacent gene (41). Additionally, read-through transcripts should not be able in either scenario, whether they fail to be processed at all or lack the 5’-cap, once they are cleaved at their respective poly(A) sites but transcription continues. The very first consequence would be a rapid decline in mRNA accumulation, which is what we observed in the YPM2 mutant at non-permissive temperatures as early as 2 h after the shift (Fig. 2, lane 6).

The multiple phenotypes of the mutant strains described before could be reversed by coexpression of a gene (PTF1 (23)) encoding a peptidylprolyl-cis/trans-isomerase. Whereas the previous identification of Ptf1p as a putative PPIase resulted from its sequence similarity with the E. coli protein parvulin (29), two eucaryotic counterparts of Ptf1p have been discovered in the meantime: DODO from Drosophila melanogaster (42) and PIN1 (9) from human cells. All three proteins are clearly distinct from the prokaryotic members of the Parvulin family of PPIases and have recently been suggested to be named products of the dodo gene family (43). In addition to their high degree of structural identity, these proteins are also functional homologues as demonstrated by the fact that the human PIN1, as well as the fly DODO, complemented the lethal phenotype of ESS1/PTF1 disrupted yeast cells (9, 42). Moreover, intact PPIase activity of PIN1 was necessary for successful complementation. Yet, whereas PTF1 and PIN1 were shown to be essential for growth of yeast and HeLa cells, respectively, total removal of the fly gene did not impair development of the mutant insects (42). Whether the differences in importance of these related genes in their natural host organisms reflect differences in their functions or in host cell requirements remains to be seen.

Unusual for PPIases, Ptf1p revealed a distinct substrate specificity with respect to the amino acid residues preceding the prolyl-peptide bond. The highest activity was achieved with peptides containing phosphorylated Ser/Thr moieties at this position. Most remarkably, the activity toward the optimal substrate (Ac-Ala-(PO_3H_2)-Ser-Pro-Tyr-4NA) was enhanced up to 3000-fold over the value obtained with the standard substrate. This rather unique substrate specificity is also shared by the human PIN1 (9), very likely reflecting the nature of in vivo targets (discussed below).

In agreement with the putative function of Ptf1p as a PPIase in vivo, its mutated forms in YPM2 and YPM5 displayed significantly reduced activities in vitro compared with the wild type protein.

Taken together, our results clearly correlate a PPIase activity with efficient pre-mRNA 3’-end processing and/or transcription termination in S. cerevisiae. Thus, the most compelling question arising from our studies concerns the nature of the putative involvement of a PPIase in these processes. In the absence of more experimental clues, we speculate that Ptf1p interacts with components of the mRNA transcription complex, if not only at the final stages of RNA transcription. Upon the appropriate trigger (or at the time of entry), Ptf1p might induce a conformational switch in either the accessory proteins or the polymerase itself, ultimately causing the dissociation of RNA polymerase II from the DNA template, i.e. transcription termination.

The discovery of a PPIase of the cyclophilin family, shown to interact with the C-terminal domain of mammalian polymerase II in a yeast two-hybrid system (44), seems to lend support to the postulated interaction of Ptf1p with the transcription machinery in yeast. More importantly, the former interaction depended on the presence of phosphoepitopes on the C-terminal domain, as extensive treatment of the yeast extracts with phosphatases resulted in complete loss of this interaction. As the C-terminal domain, which consists of multiple tandem repeats of a heptapeptide Tyr-Ser-Pro-Thr-Ser-Pro-Ser, is highly conserved between yeast and mammals, this domain represents an ideal candidate for a PPIase, with specificity toward phosphorylated serines N-terminal to prolines.

Moreover, two recent publications (45–46), in which another cyclophilin, USA-CyP (a member of the cyclosporin A-binding PPIase family), was shown to be tightly associated with the spliceosomal (U4/U6.U5) tri-snRNA ribonucleoprotein in HeLa cells, also support the proposed role of Ptf1p in the dissociation of the transcription machinery from the DNA template. In one of the papers (45), the authors discuss the intriguing possibility that the role of USA-CyP in the human spliceosome is not to act as a chaperone in the folding or assembly steps but rather to assist in the disassembly of spliceosomes. This view was spurred by the example of one of the best characterized cyclophilins, cyclophilin-A, for which there is convincing evidence that it exerts its function by binding specifically to the HIV-1 capsid protein (CA), destabilizing interactions between the CA molecules and thus facilitating the disassembly of the CA core (47–49). Nevertheless, as much as it is tempting to draw parallels from the above examples to the physiological function of Ptf1p, its putative role in RNA transcription (and/or termination) has first to await experimental confirmation.

**Table III**

| Phosphatase | Activity |
|------------|----------|
| U^-1 M^-1 | %        |
| Parvulin pSEP H6 | 2.5 x 10^4 | 100 |
| Gly^-2Asp | 5.3 x 10^4 | <1  |
| Gly^-2Ser | 1 x 10^4 | <1  |

**Measurements** were performed at 35 mM HEPES buffer, pH 7.8, at 10 °C in a protease-coupled assay (see “Experimental Procedures”) using Suc-Ala-Phe-Pro-Phe-4NA as substrate.
Finally, we should like to consider again PIN1, the functional and structural human homologue of yeast Pin1p. When underexpressed, both proteins induced mitotic arrest of their respective host cells, suggesting a role in cell cycle regulation. In agreement with this notion, PIN1 has been found to be part of the nuclear speckle (33), a large protein complex, which contains several other mitosis-related proteins and, most notably, some splicing factors. Given the fact that PIN1 substitutes functionally for the putative pre-mRNA 3' end-processing factor ESS1/PTF1 in yeast cells, the presence of Pin1 in the nuclear speckle, the components of which are supposedly involved in cell cycle regulation, is rather intriguing. It may be that factors such as these serve as checkpoints for the integrity of the mRNA maturation process thus acting as a link between pre-mRNA 3' end processing and cell cycle regulation.

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REFERENCES

1. Hanes, S. D., Shank, P. R., and Bostian, K. A. (1989) Yeast 5, 55–72
2. Hani, J., Stumpf, G., and Domdey, H. (1995) FEBS Lett. 365, 198–202
3. Fischer, G. (1994) Angew. Chem. Int. Ed. Engl. 33, 1415–1436
4. Galat, A., and Metcalfe, S. (1995) Prog. Biophys. Mol. Biol. 63, 67–118
5. Kay, J. E. (1996) Biochem. J. 314, 361–385
6. Schmid, F. X., Mayr, L. M., Mucke, M., and Schoenbrunner, E. R. (1993) Adv. Protein Chem. 44, 25–66
7. Manning-Krieg, U. C., Henriquez, R., Cammas, F., Gaff, P., Gaveriaux, S., and Movva, N. R. (1994) FEBS Lett. 352, 98–105
8. Dolinski, K., Muir, S., Cardenas, M., and Heitmann, H. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 13093–13098
9. Lu, K. P., and Hunter, T. (1995) Cell 81, 413–424
10. Lu, K. P., Hanes, S. D., and Hunter, T. (1996) Nature 380, 544–547
11. Domdey, H., Apostol, B., Lin, R.-J., Newman, A., Brody, E., and Abelson, J. (1984) Cell 39, 611–621
12. Johnson, P. F., and Abelson, J. (1983) Nature 302, 381–387
13. Lin, R.-J., Newman, A. J., Cheng, S.-C., and Abelson, J. (1985) J. Biol. Chem. 260, 14780–14792
14. Schutkowski, M., Bernhardt, A., Zhou, X. Z., Shen, M., Reimer, U., Rahfeld, J.-U., Lu, K. P., and Fischer, G. S. (1998) Biochemistry 37, 5566–5575
15. Kofron, J. L., Kuzmic, P., Colon-Bonilla, E., and Rich, D. H. (1991) Methods Enzymol. 185, 60–89
16. Alting-Mees, M. A., and Short, J. M. (1989) Nucleic Acids Res. 17, 9494
17. Alting-Mees, M. A., and Short, J. M. (1989) Prog. Natl. Acad. Sci. U. S. A. 82, 488–492
18. Rahfeld, J.-U., Rucknagel, K. P., Schelbert, B., Ludwig, B., Hacker, J., Mann, K., and Fischer, G. (1994) FEBS Lett. 352, 180–184
19. Heidmann, S., Obermaier, B., Vogel, K., and Domdey, H. (1992) Mol. Cell. Biol. 12, 4215–4229
20. Lawrence, C. W. (1991) Methods Enzymol. 194, 273–281
21. Haher, L. E., and Halvorson, H. O. (1975) Methods Cell Biol. 11, 45–69
22. Sherman, F., and Hicks, J. (1991) Methods Enzymol. 194, 21–37
23. Kohr, K., and Domdey, H. (1991) Methods Enzymol. 194, 398–405
24. Rosenberg, A. H., Lade, B. N., Chui, D., Lin, S., Dunn, J. J., and Studier, F. W. (1987) Gene (Amst.) 56, 125–135
25. Studier, F. W., Rosenberg, H., Dunn, J. J., and Dubendorf, J. W. (1990) Methods Enzymol. 185, 60–89
26. Haid, A., and Suissa, M. (1983) Methods Enzymol. 96, 6921–6927
27. Lu, K. P., Hanes, S. D., and Hunter, T., and Noel, J. P. (1997) Cell 89, 875–886
28. Chen, H. I., and Sudol, M. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 7819–7823
29. Butler, J. S., Sadhale, P. P., and Platt, T. (1990) Mol. Cell. Biol. 10, 2599–2605
30. Ozawa, A., et al. (1997) Nature 387, (suppl.) 1–105
31. Fischer, G., Bang, H., and Mech, C. (1984) Biomed. Biochim. Acta 43, 1101–1111
32. Fischer, G., Wittmann-Liebold, B., Lang, K., Kiefhaber, T., and Schmid, F. X. (1989) Nature 337, 476–478
33. Russnak, R., Nehrke, K. R., and Platt, T. (1995) Mol. Cell. Biol. 15, 1689–1697
34. Rininger, S., Egli, M., and Braus, G. H. (1991) Mol. Cell. Biol. 11, 3060–3069
35. Russn, P. and Sherman, F. (1989) Proc. Natl. Acad. Sci. U. S. A. 86, 8348–8352
36. Maleszka, R., Hanes, S. D., Hackett, R. L., de Couet, H. G., and Miklos, G. L. G. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 447–451
37. Maleszka, R., Lupas, A., Hanes, S. D., and Miklos, G. L. G. (1997) Gene (Amst.) 203, 89–93
38. Bourquin, J. P., Stagljar, I., Meier, P., Moosmann, P., Silke, J., Bacchi, T., Geppert, O., and Schaffner, W. (1997) Nucleic Acids Res. 25, 2055–2061
39. Horowitz, D. S., Kobayashi, R., and Kainer, A. R. (1997) RNA (NY) 3, 1374–1387
40. Teigelkamp, S., Achel, T., Mundt, C., Göthel, S. F., Cronshagen, U., Lane, W. S., Marahiel, M., and Lührmann, R. (1998) RNA (NY) 4, 127–141
41. Braatren, D., Franke, E. K., and Luban, J. (1996) J. Virol. 70, 3551–3560
42. Gamble, T. R., Vajdos, F. F., Yoo, S., Worthylake, D. K., Houseweart, M., Sundquist, W. J., and Hill, C. P. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 2599–2605
43. Braatren, D., Frank, D. K., and Luban, J. (1996) J. Virol. 70, 3551–3560
44. Zhao, Y., Chen, Y., Schutkowski, M., Fischer, G., and Ke, H. (1997) Nucleic Acids Res. 17, 7583–7600
45. Alting-Mees, M. A., and Short, J. M. (1989) Nucleic Acids Res. 17, 9494