Molecular cloning and sequencing of genomic DNA encoding yeast vacuolar carboxypeptidase yscS*

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A *Saccharomyces cerevisiae* genomic DNA encoding vacuolar carboxypeptidase yscS was cloned from a yeast YEp13 library by complementation of the previously characterized mutation *cps1-1* [(1981) J. Bacteriol. 147, 418–426], by means of staining carboxypeptidase activity in yeast colonies. The nucleotide sequence of the cloned gene was determined. The open reading frame of CPS1 consists of 576 codons and therefore encodes a protein of 64961 molecular weight. A stretch of 19 residues near the N-terminus of the deduced polypeptide sequence contains characteristics common to known hydrophobic leader sequences. CPS1 was determined by DNA blot analysis to be a single copy gene located on chromosome X. The cloned fragment was used to identify a 2.1 kb mRNA. A transcriptional activation of CPS1 occurs when cells grow on a substrate of carboxypeptidase yscS as sole nitrogen source.

Carboxypeptidase; Molecular cloning; Vacuole; Yeast

1. INTRODUCTION

The vacuole of the yeast *Saccharomyces cerevisiae* is considered to be the equivalent of the lysosome of mammalian cells (reviewed in [1]). Seven peptidases associated with yeast vacuoles have been characterized, including two endopeptidases, two carboxypeptidases, two aminopeptidases and one dipeptidyl aminopeptidase (see [2,3] for reviews), and their biosynthesis and sorting have been examined (reviewed in [4]). The localization of such proteins to the vacuole requires the early stages of the secretory pathway [5–9]. In addition, localization determinants on carboxypeptidase yscY and proteinase yscA, as well as genes necessary for sorting of these proteins, have been identified [6,7,10–13].

All soluble vacuolar enzymes characterized so far are glycoproteins that are synthesized as higher-molecular weight zymogens (reviewed in [4]) whose proteolytic activation occurs through a cascade triggered by activation of pro-proteinase yscA, the product of the PEP4 gene [14–18].

Interestingly enough, carboxypeptidase yscS is the only soluble vacuolar peptidase whose 'in vitro' enzymatic activity is unaffected by the allelic state of

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* The same gene has been simultaneously cloned and sequenced by D.O. Sporman, J. Heim and D.H. Wolf [Eur. J. Biochem., in press]

PEP4 gene [19]. Its maturation process and intracellular sorting remain unknown. This enzyme was first described by Wolf and Weiser [20] in a mutant strain lacking carboxypeptidase yscY. It is a metal ion-dependent peptidase, located inside the vacuole, which cleaves efficiently Cbz-Gly-Leu and whose activity is regulated by the nitrogen source of the growth medium [20–22].

This paper reports the cloning, sequencing and chromosomal location of the gene encoding carboxypeptidase yscS and provides evidence that at least part of the regulation of the CPS1 expression is at the level of transcription. This cloned gene will facilitate further studies on the biosynthesis, processing, sorting and transport to the vacuole of carboxypeptidase yscS.

2. MATERIALS AND METHODS

2.1. *Materials*

Enzymes used in recombinant DNA manipulation were obtained from Boehringer (FRG) or New England Biolabs (USA). *Saccharomyces* chromodi-hybridizer was from Clontech (USA). Chemicals used in biochemical tests were purchased from either Sigma (USA) or Bachem (Switzerland). Growth media were from Difco (USA).

2.2. *Strains and culture conditions*

S. cerevisiae strain C56 (*MAT a, prc1, cps1, leu2-3, 2-112, ura3-52, trp1-289*) originated from a cross of strain DBY746 (Yeast Genetic Stock Center) and strain D11-1-3B [23]. Yeast growth media were YPD-complete medium and mineral medium. For induction of carboxypeptidase medium without ammonium sulfate containing 5% Cbz-Gly-L-Leu as the sole nitrogen source was used [21]. Bacterial strains HB101 and JM109 used as hosts for plasmids, were grown in LB medium supplemented with ampicillin (100 mg/l) or tetracyclin (12.5 mg/l) [24].

2.3. Enzyme assays

Carboxypeptidase yscS activity in colonies or patches of yeast cells made permeable by chloroform treatment [25] was monitored by pouring a mixture prepared by adding 15 ml of 1.2% melted agar in 0.1 M sodium phosphate buffer pH 7.4 to 6 ml of a solution containing 1.5 mg L-amino acid oxidase, 2.4 mg peroxidase, 39 mg Cbz-glycyl-L-leucine and 0.6 mg o-dianisidine, made in the same buffer. Plates were incubated at 37°C for 60 min. Development of a brown color resulting from the oxidation of the o-dianisidine indicated carboxypeptidase yscS activity. For measurements of peptidase activity in cell extracts the method of Wolf and Weiser [20] was used. Specific activity was expressed as nmol L-leucine liberated min^{-1} mg protein^{-1}.

2.4. Recombinant DNA methodology

All DNA manipulations were performed according to standard procedures [24]. Competent E. coli strains were obtained by using the RbCl method [26]. Yeast transformation was carried out as described [27]. DNA sequencing was performed by the chain termination method [28] using M13 primers or 17-base oligonucleotides synthesized in a soli-phase Applied Biosystems DNA synthesizer model 381A and a Sequenase kit (United States Biochemical Corporation). Yeast RNA was prepared as indicated [29]. Southern and Northern analysis

were performed as described [30,31]. The yeast chromosomal agarose gel (i.e., a 'Saccharomyces chromo-dihybridizer' from Clontech) was probed with the ^{32}P-labeled SalI fragment (0.6 kb) of the yeast carboxypeptidase gene (Fig. 1), according to the supplier's instructions.

3. RESULTS AND DISCUSSION

Plasmids capable of complementing the previously characterized mutation cps1-1 [32] were recovered from the YEp13 genomic library [33]. After transformation [27] of strain C56 approximately 10000 Leu$^+$ transformants were screened for carboxypeptidase yscS activity by overlaying with the substrate-containing mixture described in section 2 and 5 positive clones were identified. Plasmids were purified from these colonies and retransformation of the yeast strain C56 proved that the observed complementation was linked to the genomic DNA insert. The restriction maps revealed that the inserts of all 5 plasmids were identical to that shown in Fig. 1 for plasmid pCB1. Various subclones
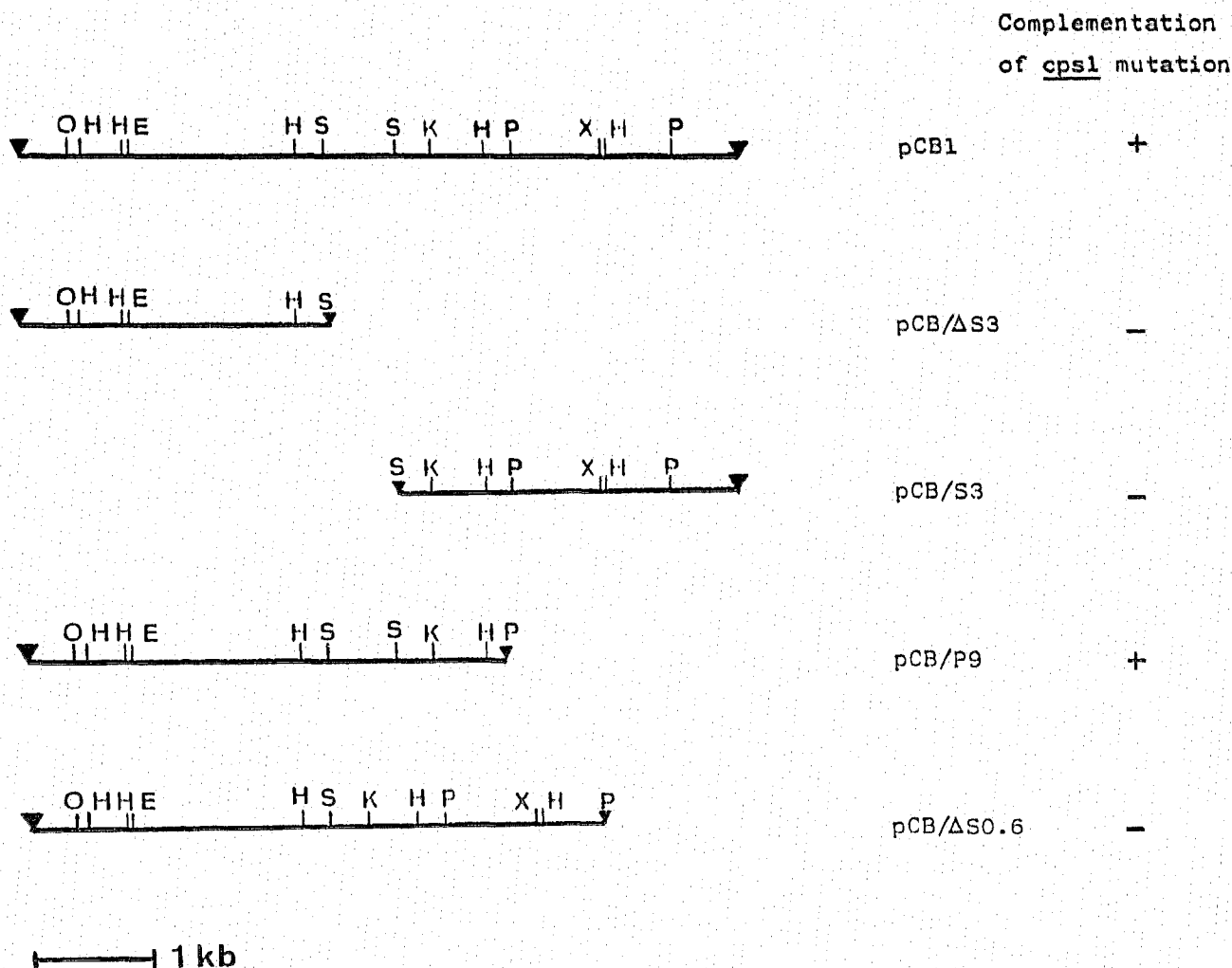

Fig. 1. Restriction maps and complementation results for plasmid pCB1 and its derivatives. Endonuclease cleavage sites are indicated (E, EcoRI; H, HindIII; K, KpnI; P, PvuII; S, SalI; X, XbaI; O, XhoI).

were constructed, and yeast transformants carrying the subcloned plasmids were examined for carboxypeptidase yscS activity (Fig. 1). Deletions that remove the 0.6 kb SalI fragment (pCBS0.6) from the middle of the insert, eliminate complementation. The results of these analyses indicate that the DNA segment which complements cps1 mutation lay in the central SalI fragment and extends both to the right and left of this 0.6 kb fragment.

If the cloned insert described above indeed encodes carboxypeptidase yscS, then multiple copies of the plasmid-borne structural gene should result in overproduction of carboxypeptidase yscS. This has been found to be the case, as cells containing pCB1 exhibit about a 7-fold higher level of intracellular carboxypeptidase yscS than the same strain carrying the parent 2 μm plasmid YEp13 (data not shown), when cells grow on Cbz-Gly-L-Leu as sole nitrogen source. Other vacuolar hydrolases were not overproduced.

Southern blot analysis was carried out to demonstrate that the cloned DNA was present as a single copy gene in the yeast genome. The results presented in Fig. 2 demonstrate that there is a single copy of the carboxypeptidase yscS gene in the yeast genome.

An elegant method for assigning an unmapped gene to a distinct yeast chromosome has recently become available based on the electrophoretic separation of intact chromosomes [34]. When a yeast chromosomal agarose gel was probed with the internal 0.6 kb SalI fragment from pCB1 the hybridization pattern obtained is depicted in Fig. 3 and indicates that CPS1 is located on yeast chromosome X.

Fig. 4 shows the complete DNA sequence and deduced protein sequence for CPS1. An open reading
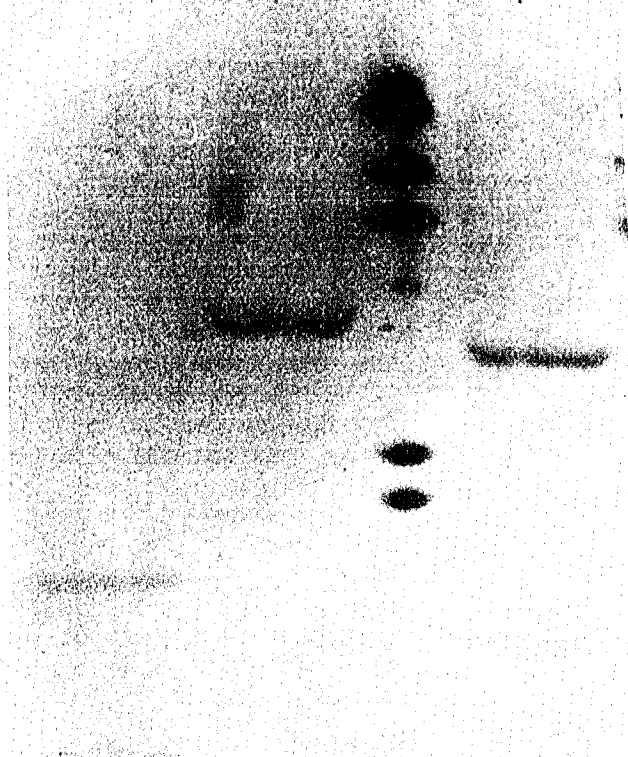

Fig. 2. Southern blot analysis of yeast genomic DNA. Yeast total DNA was digested with either HindIII (lane 1), EcoRI and XbaI (lane 2) or EcoRI and PvuII (lane 4), run on an 0.7% agarose gel, transferred to a nitrocellulose membrane and probed with the 4.1 kb EcoRI-XbaI fragment from Fig. 1. Size standards in lane 3 are E. coli phage DNA digested with HindIII.

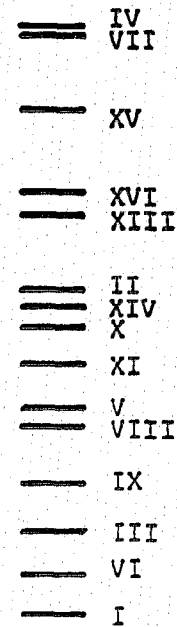

Fig. 3. DNA blot analysis of yeast chromosomal DNA. The yeast chromosomal agarose gel (i.e. a Saccharomyces chromodi-hybridizer from Clontech) was probed with the 32P-labeled SalI restriction fragment (0.6 kb) of the carboxypeptidase yscS gene (Fig. 1).

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-503 AAGCTTTAGCATACACAAACAAAGCGACACCGGGATTTGCATATCGCTTGTTCCTACGACGTTGTCAGTTCCATAACCTCTGATTTCCCATACGGATTTTGAAAATCAGCTACGAATA
-381 GATAGTCTTTTTCTATTTTCAGTAAATGTGTCATTGAAAAAAGTTTCTTTAGAACGACGCTTTTTGCAGGCTACTAGAAACACCCACTTCTAGTAGCTTTACGTGACGAATTAAACCG
-259 CGTGATCTTCTGCACTGTTATCCTAAATGCTCAATGCTCAGAACATCTTCTTGTTATCTCAGCCTCGCCACCCTTATCTTATCATAGCGGCCTTTTATTTTCCCTTTGGGCAAAATAC
-137 GATACAGCCTTCCAAGGTCGGGCAAGAAAGTTTATGACATATAAAAGAAACGGTACCCCAAATACGTCTCTTCCCATTGTAAAACTAATCCTGCATCATCACATTAAGGAATCATTCA
 -15 TCTAACAATTACATT ATG ATC GCC TTA CCA GTA GAG AAG GCC CCT AGA AAG TCC CTA TGG CAA AGG CAC AGA GCC TTT ATT AGT GGA ATA GTT
               MET Ile Ala Leu Pro Val Glu Lys Ala Pro Arg Lys Ser Leu Trp Gln Arg His Arg Ala Phe Ile Ser Gly Ile Val

  79 GCC CTT ATT ATC ATC GGC ACC TTC TTC CTC ACT TCG GGT CTC CAC CCA GCA CCA CCT CAT GAG GCA AAG CGT CCA CAC CAT GGA AAA GGT
  27 Ala Leu Ile Ile Ile Gly Thr Phe Phe Leu Thr Ser Gly Leu His Pro Ala Pro Pro His Glu Ala Lys Arg Pro His His Gly Lys Gly

 169 CCC ATG CAC TCA CCC AAA TGT GAG AAG ATT GAA CCA TTA AGT CCA TCA TTC AAA CAT TCC GTC GAC ACA ATT CTT CAT GAC CCT GCC TTT
  57 Pro MET His Ser Pro Lys Cys Glu Lys Ile Glu Pro Leu Ser Pro Ser Phe Lys His Ser Val Asp Thr Ile Leu His Asp Pro Ala Phe
         *

 259 AGA AAC AGC TCC ATT GAG AAA CTG TCC AAT GCT GTT AGA ATC CCC ACT GTA GTC CAA GAC AAA AAC CCC AAC CCC GCA GAT GAT CCG GAT
  87 Arg Asn Ser Ser Ile Glu Lys Leu Ser Asn Ala Val Arg Ile Pro Thr Val Val Gln Asp Lys Asn Pro Asn Pro Ala Asp Asp Pro Asp

 349 TTC TAT AAG CAT TTT TAT GAA CTA CAC GAC TAT TTT GAG AAG ACT TTC CCT AAT ATT CAC AAG CAT TTG AAA TTG GAG AAA GTC AAT GAG
 117 Phe Tyr Lys His Phe Tyr Glu Leu His Asp Tyr Phe Glu Lys Thr Phe Pro Asn Ile His Lys His Leu Lys Leu Glu Lys Val Asn Glu
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 439 CTG GGT CTT CTA TAC ACA TGG GAA GGT TCT GAT CCT GAT CTA AAA CCA TTA TTG TTA ATG GCC CAT CAA GAT GTT GTA CCT GTA AAC AAC
 147 Leu Gly Leu Leu Tyr Thr Trp Glu Gly Ser Asp Pro Asp Leu Lys Pro Leu Leu Leu MET Ala His Gln Asp Val Val Pro Val Asn Asn

 529 GAA ACT TTA TCA TCC TGG AAG TTC CCT CCA TTT TCT GGT CAC TAT GAT CCA GAA ACA GAT TTT GTT TGG GGG CGT GGT TCT AAC GAT TGT
 177 Glu Thr Leu Ser Ser Trp Lys Phe Pro Pro Phe Ser Gly His Tyr Asp Pro Glu Thr Asp Phe Val Trp Gly Arg Gly Ser Asn Asp Cys
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 619 AAG AAC TTG TTA ATT GCC GAG TTT GAA GCT ATC GAA CAA CTG TTG ATA GAT GGA TTC AAG CCC AAC AGA ACT ATT GTT ATG TCG CTT GGT
 207 Lys Asn Leu Leu Ile Ala Glu Phe Glu Ala Ile Glu Gln Leu Leu Ile Asp Gly Phe Lys Pro Asn Arg Thr Ile Val MET Ser Leu Gly

 709 TTT GAT GAA GAA GCA AGC GGC ACC CTC GGT GCT GCC AGC TTA GCC TCA TTT CTT CAC GAA AGA TAT GGT GAT GAT GGT ATT TAC AGT ATC
 237 Phe Asp Glu Glu Ala Ser Gly Thr Leu Gly Ala Ala Ser Leu Ala Ser Phe Leu His Glu Arg Tyr Gly Asp Asp Gly Ile Tyr Ser Ile

 799 ATT GAC GAG GGT GAA GGT ATC ATG GAA GTC GAC AAG GAT GTC TTT GTT GCC ACT CCA ATC AAC GCT GAA AAA GGC TAT GTC GAC TTC GAA
 267 Ile Asp Glu Gly Glu Gly Ile MET Glu Val Asp Lys Asp Val Phe Val Ala Thr Pro Ile Asn Ala Glu Lys Gly Tyr Val Asp Phe Glu

 889 GTC AGT ATT CTA GGC CAT GGT GGT CAT TCC TCT GTC CCA CCT GAT CAT ACC ACA ATC GGT ATC GCT TCA GAA CTG ATT ACT GAA TTT GAA
 297 Val Ser Ile Leu Gly His Gly Gly His Ser Ser Val Pro Pro Asp His Thr Thr Ile Gly Ile Ala Ser Glu Leu Ile Thr Glu Phe Glu

 979 GCC AAC CCA TTT GAC TAC GAA TTT GAG TTT GAC AAT CCA ATC TAT GGA TTG TTG ACA TGT GCT GCT GAA CAT TCT AAA TCT TTA AGT AAG
 327 Ala Asn Pro Phe Asp Tyr Glu Phe Glu Phe Asp Asn Pro Ile Tyr Gly Leu Leu Thr Cys Ala Ala Glu His Ser Lys Ser Leu Ser Lys
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1069 GAT GTG AAG AAG ACA ATT TTG GGC GCA CCA TTC TGT CCT AGA AGG AAG GAC AAG CTT GTT GAG TAC ATT TCC AAC CAA TCA CAT TTG CGC
 357 Asp Val Lys Lys Thr Ile Leu Gly Ala Pro Phe Cys Pro Arg Arg Lys Asp Lys Leu Val Glu Tyr Ile Ser Asn Gln Ser His Leu Arg

1159 AGC TTA ATA AGA ACA ACA CAA GCT GTT GAT ATC ATC AAT GGT GGT GTT AAA GCT AAT GCT CTG CCC GAA ACT ACC AGA TTC TTG ATC AAT
 387 Ser Leu Ile Arg Thr Thr Gln Ala Val Asp Ile Ile Asn Gly Gly Val Lys Ala Asn Ala Leu Pro Glu Thr Thr Arg Phe Leu Ile Asn

1249 CAC AGA ATT AAT TTA CAT TCT TCT GTG GCT GAA GTC TTT GAA AGA AAC ATA GAA TAT GCG AAA AAG ATT GCT GAG AAG TAT GGC TAT GGT
 417 His Arg Ile Asn Leu His Ser Ser Val Ala Glu Val Phe Glu Arg Asn Ile Glu Tyr Ala Lys Lys Ile Ala Glu Lys Tyr Gly Tyr Gly

1339 TTA TCT AAG AAC GGT GAC GAT TAC ATT ATC CCT GAA ACC GAG TTA GGT CAC ATT GAC ATT ACT CTC TTG AGA GAA TTG GAA CCA GCA CCA
 447 Leu Ser Lys Asn Gly Asp Asp Tyr Ile Ile Pro Glu Thr Glu Leu Gly His Ile Asp Ile Thr Leu Leu Arg Glu Leu Glu Pro Ala Pro

1429 CTT TCG CCA AGT TCA GGC CCT GTT TGG GAC ATT TTG GCA GGT ACT ATT CAA GAT GTT TTT GAA AAC GGT GTT CTA CAA AAC AAC GAA GAG
 477 Leu Ser Pro Ser Ser Gly Pro Val Trp Asp Ile Leu Ala Gly Thr Ile Gln Asp Val Phe Glu Asn Gly Val Leu Gln Asn Asn Glu Glu
                                                                                   *

1519 TTC TAT GTG ACT ACT GGT TTA TTC TCT GGT AAC ACC GAT ACT AAA TAC TAC TGG AAT TTG TCC AAG AAC ATT TAT AGG TTT GTT GGC TCT
 507 Phe Tyr Val Thr Thr Gly Leu Phe Ser Gly Asn Thr Asp Thr Lys Tyr Tyr Trp Asn Leu Ser Lys Asn Ile Tyr Arg Phe Val Gly Ser

1608 ATC ATT GAT ATT GAT TTA CTG AAG ACA TTG CAT TCG GTT AAT GAA CAC GTG GAT GTC CCA GGT CAT TTA TCT GCC ATT GCC TTT GTT TAC
 537 Ile Ile Asp Ile Asp Leu Leu Lys Thr Leu His Ser Val Asn Glu His Val Asp Val Pro Gly His Leu Ser Ala Ile Ala Phe Val Tyr

1698 GAG TAT ATC GTT AAT GTT AAC GAA TAC GCT TAA GCGCAATCATTGAATAGTCAAAGATTTTTTTTTTTTAATTTTTTTTTTTTTAATTTTTTTTTTTTTCATAGACTTTT
 567 Glu Tyr Ile Val Asn Val Asn Glu Tyr Ala TER

1802 ATTTAAATAAATCACGTCTATATATGTATCAGTATATAACGTAAAAAAAAAAACACCGTCAGTTAAACAAAACATAAATAAAAAAAAAAAGAAGTGTTCAAATCAAAGTGTCAAATCAA
1924 AATTTAAATGATGATGATAATATGACCATACAATGACTTAAATCAAATCAAAACCACTTGCGTAAAGGCAGTGAATATGGCCAAAATATAATACCATACACCGTTTTGCATGTTCGTTA
2046 AACGAACACCATTCAGGTGGTCGAAGGCGCGAGTAGCAGAAATGCTCATCAATTGAGAAGAATAGGTTTCGTTTTCTGGAATTGCAGCCAATAAATCGTTAACAGTATCAGCAGAAACA
2168 GTTCGATTGAATGGACTGGCATCAGATATGAAAGAAAACGACATTTCCACTAGAATCGAAAACAACACCTTTCATCGTTCCAGTAGTATTTCTTGTTATGTATCCGTAAGATTGGATAC
2290 CAGTACCCAATCCTCGATACCTTGGAAAGTGTGGAAAGTAGAATAGAGATGGTTTTT
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Fig. 4. Nucleotide and deduced amino acid sequence of carboxypeptidase yscS gene. A TATA-like sequence, TATAAA, is located 97 bases upstream of the initiation codon. A putative hydrophobic leader sequence, starting at residue 25 is underlined and the most probable processing position is marked with a vertical arrow. Asterisks identify potential glycosylation sites. The polyadenylation signals AAAtAAA at the 3'-end are marked.
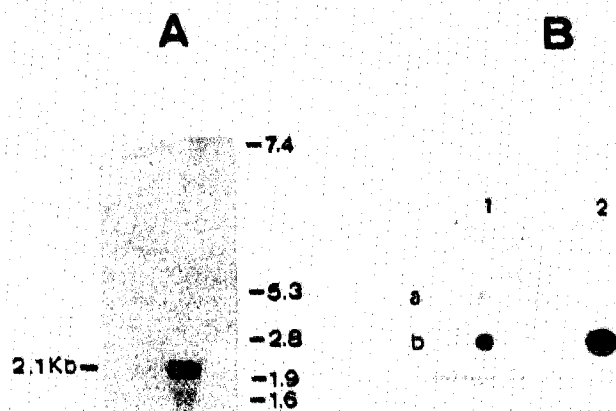
A   **B**

Fig. 5. (A) Northern blot analysis of yeast total RNA. RNA was prepared from a strain transformed with plasmid pCB1 and grown in minimal medium without ammonium sulfate containing 5% Cbz-Gly-L-Leu. (B) Dot blot of yeast total RNA. RNA was prepared from logarithmic growing cells from a CPS1 strain (lane 1) and from a strain transformed with plasmid pCB1 (lane 2). (a) Cells grown in ammonium sulfate minimal medium. (b) Cells grown in Cbz-Gly-L-Leu as sole nitrogen source. Blots were hybridized with the 0.6 kb Sall fragment from pCB1 (Fig. 1).

frame of 1728 nucleotides extends from the most 5' ATG initiation codon to a stop codon TAA (marked TER). Not unexpectedly, since carboxypeptidase yscS is a vacuolar hydrolase and presumably follows the route through the endoplasmic reticulum and Golgi body to the vacuole, there is an hydrophobic leader stretch between residues 25 and 43, which contains characteristics common to known leader sequences [35]. The predicted polypeptide contains 5 potential acceptor sites for Asn-linked glycosylation. Two have the sequence Asn-X-Ser (at Asn-88 and Asn-381) and three have the sequence Asn-X-Thr (at Asn-176, Asn-228 and Asn-321) which are preferred glycosylation sites for vacuolar hydrolases [18]. The calculated molecular weight of the encoded protein is 64961. A computer program was used to compare the derived amino acid sequence of carboxypeptidase yscS with the Swiss Prot (ENBL) protein sequence data bank, and no significant sequence similarity with any other protein was found.

An RNA blot analysis indicated that the CPS1 gene encodes a 2.1 kb RNA (Fig. 5A). Assuming average sized 5' and 3' untranslated regions and a poly(A) tail totaling 400 base pairs one expects about 1.75 kb of coding capacity or 583 amino acids, which fits pretty well with the open reading frame of CPS1 (Fig. 4).

When yeast cells grow on a substrate of carboxypeptidase yscS (Cbz-Gly-L-Leu) as sole nitrogen source, a drastic increase (about 12-fold) in carboxypeptidase yscS activity is observed [21]. We have used RNA blot analysis to determine whether any part of this regulation is transcriptional in nature. The results presented

in Fig. 5B show a substantial increase in mRNA levels during growth on the dipeptide as compared with ammonium sulfate grown cells. The derepression of transcription of CPS1 gene is even more marked in yeast cells transformed with the pCB1 multicopy plasmid (Fig. 5B, lane 2), confirming the suspected transcriptional regulation of the gene.

With the experimental approach used, complementation of cps1 mutation, we cannot exclude completely the possibility of having cloned another gene coding for a product with overlapping specificity. However, we find this possibility very unlikely for several reasons: (i) the nucleotide sequence of the cloned gene does not resemble at all that reported for carboxypeptidase yscY, the only known major activity acting on the same substrate; (ii) the overproduction of activity found with plasmid pCB1 could not be explained if CPS1 coded for a minor carboxypeptidase activity, different from carboxypeptidase yscS not found as far; (iii) the increase in mRNA levels during growth on Cbz-Gly-L-Leu correlates pretty well with the previously described derepression of carboxypeptidase yscS activity.

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