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Molecular cloning of an essential yeast gene encoding a proteasomal subunit

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We present the cloning and sequence of a *Saccharomyces cerevisiae gene, PUP2*, which encodes for a proteasomal subunit. The PUP2 protein is similar to other proteasomal components from yeast, as well as from *Drosophila* and rat. Although not-properly-folded proteins have been implicated to constitute substrates of proteasomes, we show that the accumulation of such proteins does not induce expression of the *PUP2* gene. Finally, gene disruption experiments demonstrate that PUP2 belongs to the class of yeast proteasomal subunits that are essential for cell viability.

PUP2; Proteasome; Yeast; *Saccharomyces cerevisiae*

1. INTRODUCTION

Proteasomes are large, multicatalytic proteinase complexes which are found in all eukaryotes examined ranging from man to yeast [1,2]. They are composed of multiple subunits (10–20) which are different in size and charge and which are organised into cylindrical 20 S particles [3–6]. The proteasome catalyses the cleavage of peptide bonds on the carboxyl side of basic, neutral and acidic residues [7–9] but its exact function in the cell is still unknown. It has been implicated in playing a role in the degradation of proteins via the ATP-dependent ubiquitin-mediated proteolysis [10,11] and it was reported recently that *pre1-1* yeast cells (mutations in the PRE1 gene encoding a proteasomal subunit affecting the proteolytic activity of the proteasome) exhibit decreased protein degradation and accumulate ubiquitin–protein conjugates [28]. The 20 S particles have been proposed to be also involved in tRNA processing and mRNA translation [12,13]. The expression of proteasomal subunits has been shown to be elevated in malignant cells [14] and tissue-specific expression has been reported in *D. melanogaster* [15]. Finally, proteasomes are localised both in the nucleus and the cytoplasm [2,6,15] and developmentally specific accumulation into the nucleus has been reported [16,17]. All these results suggest an important role for proteasomes in cellular proliferation and differentiation.

Cloning of a number of proteasomal subunits from rat [18–23], *D. melanogaster* [15,24,25] and yeast [26–29] has revealed another interesting property of these proteins. They belong to a unique family of proteins which share extensive inter- and intra-species similarities. This suggests that the proteasome is organised from multiple similar subunits which are evolutionarily related. Very recently the molecular cloning of six yeast proteasomal subunits revealed that these similar subunits might serve different functions since only five of them are essential for cell viability [26–29]. In this report we present the sequence of a yeast gene encoding a proteasomal protein which is also essential for cellular survival.

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2. MATERIALS AND METHODS

Saccharomyces cerevisiae strains used were W303-1B (Mata/α, his3/his3, ade2/ade2, leu2/leu2, trp1/trp1, ura3/ura3) for gene disruption and S288C (Mata) for PUP2 expression analyses. Plasmids used for disruption and sequencing were pUC18 recombinants. DNA sequencing was carried out by the dideoxy termination method with a United States Biochemical Corporation 70770 sequenase version 2.0 sequencing kit.

Sequence alignment was prepared using the CLUSTAL algorithm [30] and gaps were introduced for optimisation.

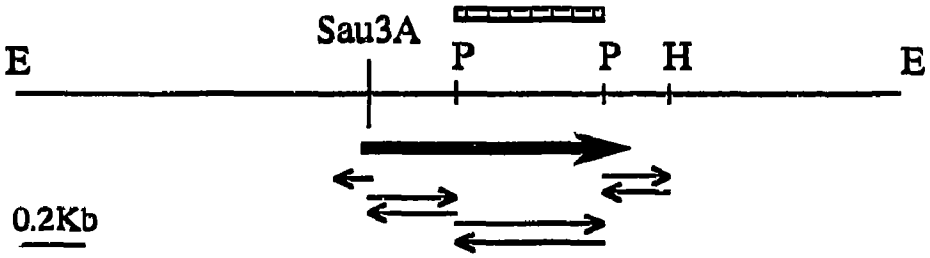
Yeast DNA was prepared according to Struhl et al. [31] and total RNA as described previously [32]. Yeast transformation was performed by the LiCl procedure [33] and sporulation and random tetrad analysis as described in [34].

3. RESULTS AND DISCUSSION

3.1. *Cloning and sequencing of a putative gene*

Part of a putative gene was first identified in a cloned yeast genomic DNA fragment which complemented a *gcn5* strain [32]. Nucleotide sequence determination of this DNA fragment defined in close proximity to the

A



B

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   1 TTTTATTAAGCGTGCAAACATGAATGTAGCCCAAGTCCGTAAGGATAATTTAGTGCTTAA
  61 ATTAAATAAATCACTATTCACGAAAATATTTTTGAAATTTCATTATACAGGGTTATCATA
 121 AACCAAGACCGGTGGCAAAAGAGTAACCTAGGAAATTGGACAAGAGAGCGCTATAACTAT
                                                          M  F     2
 181 AGAATACAAGTCAATAAGAACATAAATTCCAATTGTCTAATATAACTAGAAAGGATGTTC
     L  T  R  S  E  Y  D  R  G  V  S  T  F  S  P  E  G  R  L  F    22
 241 TTAACTAGAAGTGAATATGATCGTGGTGTAAGCACATTTTCCCCAGAAGGGAGGTTATTC
     Q  V  E  Y  S  L  E  A  I  K  L  G  S  T  A  I  G  I  A  T    42
 301 CAAGTTGAGTATTCTTTGGAGGCCATCAAATTAGGATCTACAGCAATTGGTATTGCCACG
     K  E  G  V  V  L  G  V  E  K  R  A  T  S  P  L  L  E  S  D    62
 361 AAAGAAGGTGTTGTACTAGGTGTGGAGAAACGTGCCACCTCACCACTATTAGAATCTGAT
     S  I  E  K  I  V  E  I  D  R  H  I  G  C  A  M  S  G  L  T    82
 421 TCTATTGAGAAAATCGTGGAGATTGATCGTCATATTGGTTGTGCAATGAGTGGGCTTACA
     A  D  A  R  S  M  I  E  H  A  R  T  A  A  V  T  H  N  L  Y   102
 481 GCAGATGCTCGTTCCATGATTGAACATGCGCGTACTGCTGCAGTAACGCATAATCTATAC
     Y  D  E  D  I  N  V  E  S  L  T  Q  S  V  C  D  L  A  L  R   122
 541 TACGATGAAGACATCAATGTTGAGTCCTTAACGCAATCGGTGTGTGATCTTGCTCTAAGG
     F  G  E  G  A  S  G  E  E  R  L  M  S  R  P  F  G  V  A  L   142
 601 TTCGGTGAAGGTGCCTCTGGTGAAGAAAGACTAATGTCTCGACCATTCGGGGTTGCCTTG
     L  I  A  G  H  D  A  D  D  G  Y  Q  L  F  H  A  E  P  S  G   162
 661 TTAATTGCTGGCCATGATGCGGATGATGGTTATCAACTTTTCCATGCAGAACCATCTGGG
     T  F  Y  R  Y  N  A  K  A  I  G  S  G  S  E  G  A  Q  A  E   182
 721 ACATTTTACCGTTATAATGCCAAAGCCATTGGTTCAGGCTCTGAAGGAGCACAAGCAGAA
     L  L  N  E  W  H  S  S  L  S  L  K  E  A  E  L  L  V  L  K   202
 781 TTATTGAATGAATGGCATTCTTCTTTAAGTTTGAAAGAAGCTGAGCTTCTTGTTCTAAAA
     I  L  K  Q  V  M  E  E  K  L  D  E  N  N  A  Q  L  S  C  I   222
 841 ATCCTAAAGCAAGTGATGGAAGAGAAACTTGATGAAAATAACGCGCAATTGAGTTGTATA
     T  K  Q  D  G  F  K  I  Y  D  N  E  K  T  A  E  L  I  K  E   242
 901 ACAAAGCAAGACGGATTCAAAATATATGATAACGAAAAAACTGCTGAATTGATAAAAGAG
     L  K  E  K  E  A  A  E  S  P  E  E  A  D  V  E  M  S  *      260
 961 TTGAAAGAGAAAGAAGCTGCAGAAAGTCCAGAAGAAGCGGATGTTGAAATGTCATAAATA
1021 TAGTTACATAAAGGTAAATACCAACGTCACACTAACAAAGAAAGAAAACCTTACAAAAAT
1081 GAAAGGAAAAGTAGTAAAATAACCTCAATTGATCACATCGTCTCGCCGTACTAAACATTT
1141 ATTTCTTCTTCGAAAGGAATAGTAGCGGAAAAGC
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Fig. 1. (A) Schematic representation of the *Eco*RI (E) yeast genomic fragment containing the coding region (black arrow indicating the 5'-to-3' direction of the coding strand) of the *PUP2* gene. The *Sau*3A site indicates the border of the original clone in which the *PUP2* gene was detected. P, *Pst*I; H, *Hin*dIII. The arrows show the sequencing strategy. The hatched bar indicates the *Pst*I fragment replaced by the *HIS3* gene in the disruption procedure. (B) Nucleotide and deduced amino acid sequence of the *PUP2* gene.

GCN5 gene, which is involved in the general control of amino acid biosynthesis in yeast [35,36], a second open reading frame with the coding capacity of 252 carboxy-terminal amino acids located at the very end of the cloned piece (Fig. 1A). By using an overlapping clone and a synthetic primer homologous to the end of the

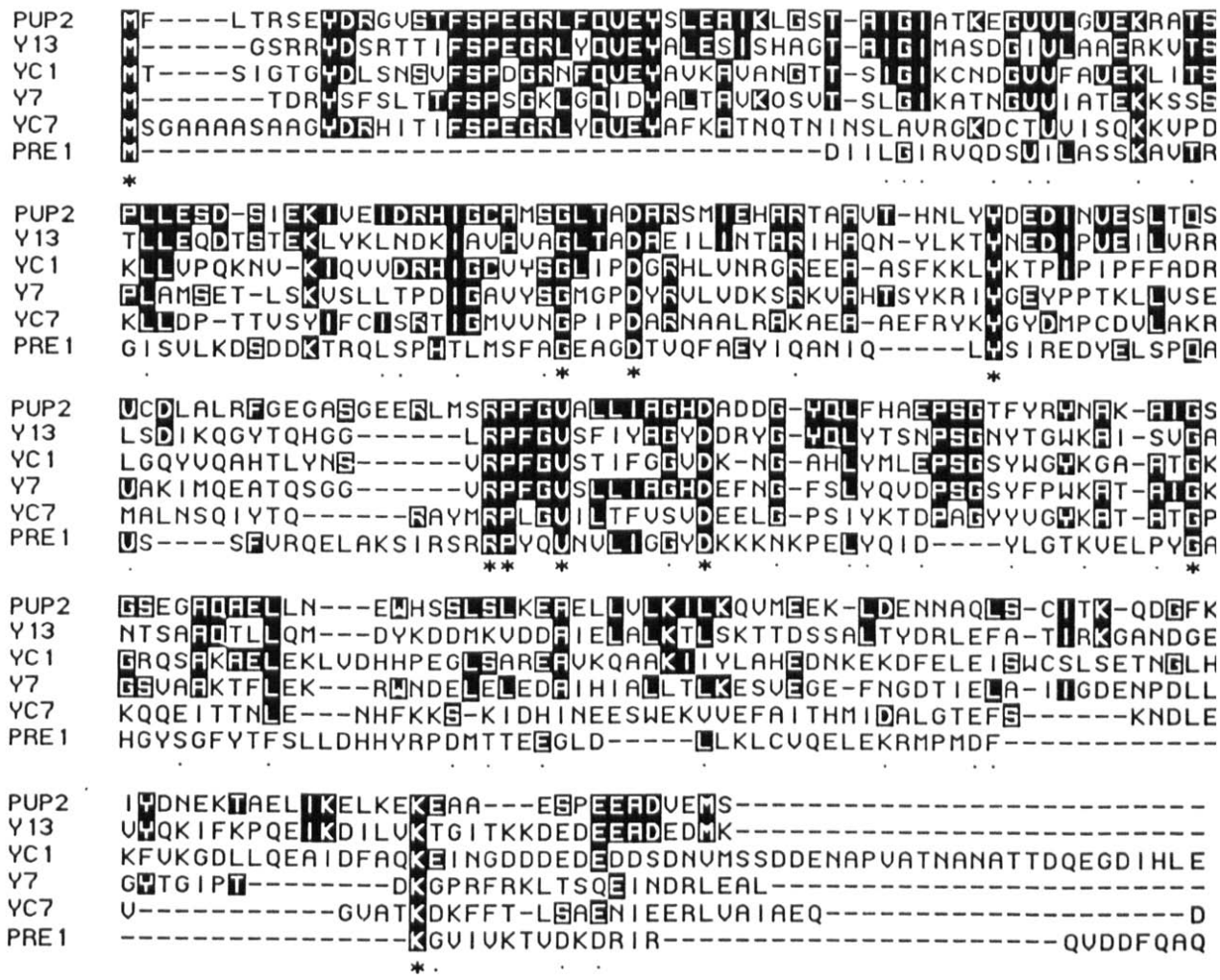

Fig. 2. Multiple sequence alignments of the *PUP2* amino acid sequence and other cloned yeast proteasome subunits. Identical residues between *PUP2* and the other subunits are blackened. Stars and dots denote identities and conserved substitutions, respectively.

starting clone, we extended the sequence towards the amino terminus of this open reading frame. The complete sequence of this region is shown in Fig. 1B. The putative encoded protein has 260 amino acids with a predicted molecular weight of 28.6 kDa.

3.2. *The putative protein is similar to proteasome subunits*

Computer comparison of the sequence of the putative protein with the SWISS protein data base revealed that this protein was similar to protein subunits of the proteasome of *D. melanogaster* and rat. Recently the sequence of additional proteasome subunits has been determined from both these species [20–25] as well as from yeast [26–29], and we have found, by doing multiple alignments, that they all share a high degree of similarity. This family of proteins is unique and no other known protein is related to it. Since the putative protein exhibits the same extent of similarities with this unique family of proteins sharing the same highly conserved regions, we have concluded that this gene encodes for a subunit of the yeast proteasome which we named *PUP2* (PUtative Proteasome subunit). The overall similarities among other already cloned subunits of the yeast proteasome and the PUP2 protein are shown in Fig. 2. As is the case for all proteasomal subunits sequenced from rat, *Drosophila* and yeast there is approximately 30% identity among these subunits and the most conserved region is at the amino-terminus of these proteins. The carboxy-terminus of the PUP2 protein also has conserved characteristics of proteasomal subunits in that it contains clusters of charged amino acids which have been implicated in the regulation of their nuclear translocation [37].

3.3. *Regulation of expression of the* PUP2 *gene*

Proteasomes are thought to be involved in the nonlysosomal degradation of proteins, and biochemical [10,11,38,39] and genetic [28] studies have indicated that their function is coupled to the ATP-dependent ubiq-

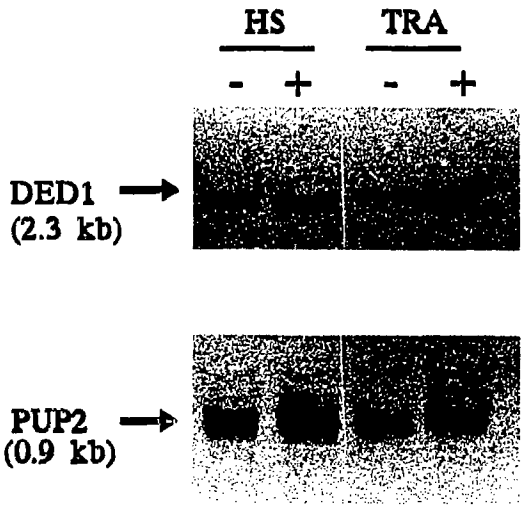

Fig. 3. The expression of *PUP2* mRNA under conditions that result in the accumulation of improperly folded proteins. Total yeast RNA was prepared from cells grown in minimal media (−), in minimal media following a 30 min heat-shock treatment at 37°C (HS+), or in minimal media following a 4 h incubation in the presence of 0.25 mM triazolalanine (TRA+). 2 μg each of these RNAs was electrophoresed and transferred onto a nylon filter. The filter was first hybridised with a ^{32}P *PUP2*-containing DNA probe, and following dehybridisation, with a ^{32}P *DED1*-containing DNA probe [42] used to control for the amount of RNA in each lane.

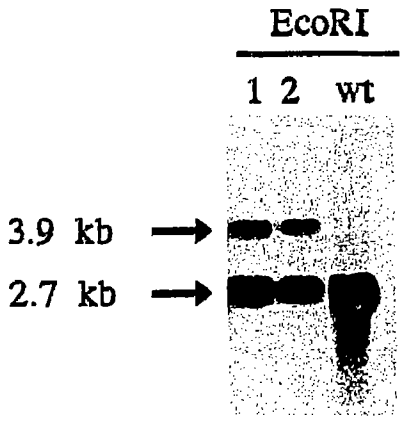

Fig. 4. Verification of the *PUP2* gene disruption. Genomic DNA from two independent diploid transformants (lanes 1, 2) as well as from a non-transformed strain (wt) was isolated, digested with *Eco*RI and subjected to electrophoresis–DNA transfer hybridisation analysis. A ^{32}P *Sau*3A–*Hin*dIII DNA fragment (Fig. 1) was used as probe.

uitin-mediated pathway of proteolysis. The ubiquitin-mediated pathway serves an essential function for cell viability and it is induced during heat-shock conditions or other stresses that result in the accumulation of not-properly-folded proteins [40,41]. In order to investigate whether the expression of the *PUP2* gene is regulated by those signals we have analysed the steady-state accumulation of *PUP2* mRNA under stress conditions. Fig. 3 shows that the level of *PUP2* mRNA remains unaffected following treatment of the cells either with a 30 min heat shock or by including in the media triazolalanine, an analogue of histidine that is incorporated into proteins. Thus, we have concluded that the expression of the *PUP2* gene, unlike the ubiquitin genes, is not induced under conditions that result in the accumulation of not-properly-folded proteins. The proposed role for proteasomes in cellular proliferation and differentiation (cf. Introduction) prompted us to examine a possible cell cycle specificity in their expression. By analysing the accumulation of *PUP2* mRNA in synchronised cells and over a period of two cycles we were able to conclude that the *PUP2* gene is not regulated, at least at the mRNA level, by cell cycle-specific factors (data not shown).

3.4. *The PUP2 protein is essential for cell viability*

Among the six already cloned yeast proteasomal genes, five of them, *YC1*, *YC7α*, *Y7*, *PRE1* and *PUP1* are essential, whereas the *Y13* gene is not essential but is required for normal growth rates. Thus, despite their overall structural similarities, different functions are served by the different subunits of the proteasome. In order to determine the phenotype of a *pup2*-disrupted strain we substituted the 461 bp *Pst*I DNA fragment that is within the coding region of *PUP2* (Fig. 1) with the *HIS3* gene. The 461 bp *Pst*I DNA fragment of a pUC18 *Sau*3A–*Eco*RI subclone (Fig. 1) was replaced with the 1.7 kb *Bam*HI DNA fragment containing the *HIS3* gene [42]. The insert was excised from the resulting clone and used to transform a *his3* diploid strain to histidine prototrophy. Confirmation that the disrupted gene had replaced, by homologous recombination, one of the wild-type alleles of the *PUP2* gene was achieved by isolating genomic DNA from transformed strains, digesting it with appropriate restriction endonucleases, followed by DNA-transfer and hybridisation analysis (Fig. 4). In such strains the PUP2 DNA probe hybridises to two fragments, one corresponding to the non-disrupted gene on one chromosome and the larger one corresponding to the disrupted allele. These strains were sporulated and among the randomly collected haploids (>100) all were unable to grow on minimal media without histidine. Thus the *HIS3*-containing chromosome could not segregate to a viable haploid demonstrating that the *PUP2* gene encoded for an essential cell viability function.

The function of the proteasome in eukaryotic cells is still unknown but at least in yeast six out of seven cloned genes are essential for cell viability. Since disrupted strains are non-viable we cannot conclude whether null mutations of these proteasomal subunits result in the failure of the proteasome to be organised or whether each one of these subunits serves for an essential function. The acquirement of conditional mutations on these genes will allow the elucidation of this issue.

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REFERENCES

[1] Orlowski, M. (1990) Biochemistry 29, 10290–10297.
[2] Tanaka, K., Yoshimoura, T., Kumatori, A., Ichihara, A., Ikai, A., Nishigai, M., Kameyama, K. and Takagi, T. (1988) J. Biol. Chem. 263, 16209–16217.
[3] Falkenburg, P.-E., Haass, C., Kloetzel, P.-M., Neidel, B., Kopp, F., Kuehn, L. and Dahlmann, B. (1988) Nature 331, 190–192.
[4] Arrigo, A.-P., Tanaka, K., Goldberg, A. and Welch, W.J. (1988) Nature 331, 192–194.
[5] Tanaka, K., Yoshimura, T., Ichihara, A., Ikai, A., Nishigai, M., Morimoto, Y., Sato, M., Tanaka, N., Katsube, Y., Kameyama, K. and Takagi, T. (1988) J. Mol. Biol. 203, 985–996.
[6] Martins de Sa, C., Grossi de Sa, M.-F., Akhayat, O., Broders, F., Scherrer, K., Horch, A. and Schmid, H.-P. (1986) J. Mol. Biol. 187, 479–493.
[7] Orlowski, M. and Wilk, S. (1981) Biochem. Biophys. Res. Commun. 101, 814–822.
[8] Orlowski, M. and Michaud, C. (1989) Biochemistry 28, 9270–9278.
[9] Driscoll, J. and Goldberg, A.L. (1989) Proc. Natl. Acad. Sci. USA 86, 787–791.
[10] Hough, R., Pratt, G. and Rechsteiner, M. (1988) in: Ubiquitin (M. Rechsteiner ed.) pp. 101–134, Plenum Press, New York.
[11] Ganoth, D., Leshinsky, E., Eytan, E. and Hershko, A. (1988) J. Biol. Chem. 263, 12412–12419.
[12] Castano, J.G., Ornberg, R., Koster, J.G., Tobian, J.A. and Zasloff, M. (1986) Cell 46, 377–387.
[13] Schmid, H.P., Akhayat, O., Martins de Sa, C., Puvion, F., Koehler, K. and Scherrer, K. (1984) EMBO J. 3, 29–34.
[14] Kumatori, A., Tanaka, K., Inamura, N., Sone, S., Ogura, T., Matsumoto, T., Tachikawa, T., Shin, S. and Ichihara, A. (1990) Proc. Natl. Acad. Sci. USA 87, 7071–7075.
[15] Haass, C., Pesold-Hurt, B., Multhaup, G., Beyreuther, K. and Kloetzel, P.-M. (1989) EMBO J. 8, 2373–2379.
[16] Gautier, J., Pal, J.K., Grossi de Sa, M.-F., Beetschen, J.C. and Scherrer, K. (1988) J. Cell Sci. 90, 543–553.
[17] Pal, J.K., Gounon, P., Grossi de Sa, M.-F. and Scherrer, K. (1988) J. Cell Sci. 90, 555–567.
[18] Fujiwara, T., Tanaka, K., Kumatori, A., Shin, S., Yoshimura, T., Ichihara, A., Tokunaga, F., Aruga, R., Iwanagi, S., Kakizuka, A. and Nakanishi, S. (1989) Biochemistry 28, 7332–7340.
[19] Tanaka, K., Fujiwara, T., Kumatori, A., Shin, S., Yoshimura, T., Ichihara, A., Tokunaga, F., Aruga, R., Iwanaga, S., Kakizuka, A. and Nakanishi, S. (1990) Biochemistry 29, 3777–3785.
[20] Tamura, T., Tanaka, K., Kumatori, A., Yamada, F., Tsurumi, C., Fujiwara, T., Ichihara, A., Tokunaga, F., Aruga, R. and Iwanaga, S. (1990) FEBS Lett. 264, 91–94.
[21] Kumatori, A., Tanaka, K., Tamura, T., Fujiwara, T., Ichihara, A., Tokunaga, F., Onikura, A. and Iwanaga, S. (1990) FEBS Lett. 264, 279–282.
[22] Tanaka, K., Kanayama, H., Tamura, T., Lee, D.H., Kumatori, A., Fujiwara, T., Ichihara, A., Tokunaga, F., Aruga, R. and Iwanaga, S. (1990) Biochem. Biophys. Res. Commun. 171, 676–683.
[23] Sorimachi, H., Tsukahara, T., Kawasaki, H., Ishiura, S., Emori, Y., Sugita, H. and Suzuki, K. (1990) Eur. J. Biochem. 193, 775–781.
[24] Haass, C., Pesold-Hurt, B., Multhaup, G., Beyreuther, K. and Kloetzel, P.-M. (1990) Gene 90, 235–241.
[25] Haass, C., Pesold-Hurt, B. and Kloetzel, P.-M. (1990) Nucleic Acids Res. 18, 4018.
[26] Fujiwara, T., Tanaka, K., Orino, E., Yoshimura, T., Kumatori, A., Tamura, T., Chung, C.H., Nakai, T., Yamaguchi, K., Shin, S., Kakizuka, A., Nakanishi, S. and Ichihara, A. (1990) J. Biol. Chem. 265, 16604–16613.
[27] Emori, Y., Tsukahara, T., Kawasaki, H., Ishiura, S., Sugita, H. and Suzuki, K. (1991) Mol. Cell Biol. 11, 344–353.
[28] Heinemeyer, W., Kleinschmidt, J.A., Saidowski, J., Escher, C. and Wolf, D.H. (1991) EMBO J. 10, 555–562.
[29] Haffter, P. and Fox, T.D. (1991) Nucleic Acids Res. 19, 5075.
[30] Higgins, D.G. and Sharp, P.M. (1989) CABIOS 5, 151–153.
[31] Struhl, K., Stinchcomb, D.T., Sherer, S. and Davis, R.W. (1979) Proc. Natl. Acad. Sci. USA 76, 1035–1039.
[32] Penn, M.D., Galgoci, B. and Greer, H. (1983) Proc. Natl. Acad. Sci. USA 80, 2704–2708.
[33] Ito, H., Fukuda, Y., Murata, K. and Kimura, A. (1983) J. Bacteriol. 153, 163–168.
[34] Spenser, J.F.T. and Spenser, D.M. (1988) in: Yeast. A Practical Approach (I. Campbell and J.H. Duffus eds.) Yeast genetics, pp. 78–83.
[35] Driscoll-Penn, M., Thireos, G. and Greer, H. (1984) Mol. Cell Biol. 4, 520–528.
[36] Hinnebush, A.G. (1988) Microbiol. Rev. 52, 248–273.
[37] Tanaka, K., Yoshimura, T., Tamura, T., Fujiwara, T., Kumatori, A. and Ichihara, A. (1990) FEBS Lett. 271, 41–46.
[38] McGuire, M.J., Reckelhoff, J.F., Croall, D.E. and DeMartino, G.N. (1988) Biochem. Biophys. Acta 967, 195–203.
[39] Matthiews, W., Tanaka, K., Driscoll, J., Ichihara, A. and Goldberg, A.L. (1989) Proc. Natl. Acad. Sci. USA 86, 2597–2601.
[40] Rechsteiner, M. (1987) Annu. Rev. Cell Biol. 3, 1–30.
[41] Finley, D., Ozkaynak, E. and Varshavsky, A. (1987) Cell 48, 1035–1046.
[42] Struhl, K. (1985) Nucleic Acids Res. 13, 8587–8601.