Rpn4p acts as a transcription factor by binding to PACE, a nonamer box found upstream of 26S proteasomal and other genes in yeast

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Abstract We identified a new, unique upstream activating sequence (5′-GGTGGCAAA-3′) in the promoters of 26 out of the 32 proteasomal yeast genes characterized to date, which we propose to call proteasome-associated control element. By using the one-hybrid method, we show that the factor binding to the proteasome-associated control element is Rpn4p, a protein containing a C2H2-type finger motif and two acidic domains. Electrophoretic mobility shift assays using proteasome-associated control element sequences from two regulatory proteasomal genes confirmed specific binding of purified Rpn4p to these sequences. The role of Rpn4p to function as a transregulator in yeast is corroborated by its ability of stimulating proteasome-associated control element-driven *lacZ* expression and by experiments using the *RPT4* and *RPT6* gene promoters coupled to the bacterial *cat* gene as a reporter. Additionally, we found the proteasome-associated control element to occur in a number of promoters to genes which are related to the ubiquitin-proteasome pathway in yeast.

Key words: 26S proteasome; Rpn4p; Transcription factor; Upstream activating sequences element; Yeast

1. Introduction

The ubiquitin-proteasome system plays key regulatory roles in many aspects of cellular regulation, such as metabolic adaptation, cell differentiation, cell cycle control and removal of abnormal proteins [1,2]. The 26S proteasome, the multisubunit enzyme complex responsible for the destruction of ubiquitin-protein conjugates and other proteins, appears to be present in all eukaryotic cells, its subunit composition being highly conserved among species [2]. To date, cDNAs or genes encoding almost all subunits of human and the budding yeast proteasomes have been isolated and characterized by molecular biological techniques. The proteasosmal subunits are distributed between two sub-assemblies, the core particle (CP or 20S particle) and the regulatory particle (RP or 19S particle). The core particle houses the proteolytic activities of the proteasome while the regulatory particle confers ATP-dependence and specificity for ubiquitin-protein conjugates.

17 RP subunits have recently been characterized from purified yeast proteasomes [3], six of which are putative ATPases of the AAA family [4]. Together with their counterparts in other eukaryotes, these yeast Rpt proteins constitute a well-defined AAA subfamily. The primary structures of these subunits show a high inter-subunit homology and high evolutional conservation among species. The remainder of the RP subunits, 12 of which have been characterized in yeast to date, form a heterogeneous group, now designated the Rpn proteins [5]. The important role of the 26S proteolysis system is underlined by the fact that in yeast, many of the genes for its constituents have been shown to be essential genes. With one exception, individual chromosomal deletions of each of the 14 known yeast 20S proteasome genes are lethal as is chromosomal deletion of 19S cap genes [6]. Conceptions as to the structural and functional organization of the yeast 19S particle [7,8], the molecular interplay between its constituting subunits and the molecular interactions underlying the recognition of ubiquitinylated substrates to be attacked by the 26S proteasome are evolving rapidly [9,10].

A problem that has received little attention thus far is how transcription of the proteasomal genes is regulated in yeast. A first indication for the possibility that the expression of these genes might be subject to coordinate control is our finding that the promoter regions of the regulatory proteasomal genes (*RPT*) and most of the other proteasomal genes share a conserved nonamer sequence motif suggesting that this might function as a common *cis*-regulatory signal through interaction with specific DNA-binding factor(s). Here, we demonstrate that Rpn4p, a protein previously found to be associated with the 26S proteasome [11], binds to the conserved sequence motif in vitro and in vivo. The role of Rpn4p to function as a transregulator in yeast is corroborated by its ability of stimulating proteasome-associated control element (PACE)-driven *lacZ* expression and by experiments using the *RPT4* and *RPT6* gene promoters coupled to the bacterial *cat* gene as a reporter. Interestingly, the conserved sequence motif is found in the promoters of additional genes that can be supposed to be involved in the ubiquitin-proteasome pathway.

2. Materials and methods

2.1. Strains, plasmids, growth media and general methods

Yeast strain YM4271 and plasmids pACT2, pGAD-GH, pHISi-1 and pLacZi were from Clontech YEp*lac181* and YEp*lac195* were as described in [12]. All other yeast strains and plasmids are listed in Table 1. *Escherichia coli* strain XL1-blue (Stratagen) and pUC18 (Pharmacia) were used for all DNA manipulations. The library of yeast genomic DNA fragments cloned in pACT2 was a gift from J.-C. Jauniaux (DKFZ Heidelberg). Basic yeast methods and growth media were as described [14]. Yeast strains were transformed by the lithium acetate method [15]. Standard protocols were used in recombinant DNA methodology [16]. Oligonucleotides were purchased from Eurogentec (Belgium). DNA sequencing was performed by the dideoxy chain termination protocol [17]. The β-galactosidase filter test was as described in [18] and the liquid β-galactosidase assay according to [19].

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2.2. Cloning of PACE binding sites into reporter vectors and isolation of clones from the one-hybrid assay

The MATCHMAKER One-Hybrid System (Clontech) was used and procedures were according to the manufacturer's protocols. A fragment obtained from annealing and amplifying 4PACEw and 4PACEc (Table 2) was transferred into reporter plasmid pLacZi via the *Eco*RI/*Sal*I sites and into pHISi-1 through blunt end ligation. pLacZi-PACE was integrated into the *URA3* locus of yeast host strain YM4271 yielding reporter strain YM4271-PACE-Lac. pHISi-1-PACE was integrated into the *HIS3* locus of the yeast host to yield reporter strain YM4271-PACE-HIS. Correct integrations were verified by PCR. Reporter strain YM4271-PACE-HIS was transformed with a library generated from random genomic yeast fragments cloned into vector pACT2. Transformants were plated on selective medium without histidine and leucine but containing 15 mM 3-amino-1,2,4-triazole to suppress background *HIS3* expression. Plasmid DNA isolated from the resulting clones was transformed into reporter strain YM4271-PACE-Lac and plated on selective medium without leucine. Cells from single colonies were streaked on a matrix plate and subjected to the β-galactosidase filter assay [18] in order to exclude false positive clones from the first round of selection.

YEp*lac181*-RPN4 was constructed by introducing a *Hin*dIII/*Bam*HI fragment containing the *TDH3* promoter into vector YEp*lac181* and finally fusing a PCR-generated segment encoding full-length Rpn4p in-frame to the promoter. pGADGH-RPN4 was constructed by ligating a *RPN4* fragment, $RPN4_{(92-531)}$, in-frame into the vector pGAD-GH. YEp*lac181*-RPN4 and pGADGH-RPN4 were transformed into strain YM4271-PACE-Lac (Table 1).

2.3. Analysis of RPT4 and RPT6 promoters fused to the bacterial cat gene in yeast

*Eco*RI/*Pst*I fragments from the promoter regions of *RPT6* (−2–−265) and *RPT4* (−1–−275) were amplified by PCR (Table 2). The PACE-less mutants of the *RPT6* and *RPT4* promoter fragments were constructed according to [20] using the specific mutagenic primers which exclude the nonamer PACE box. A 2 bp deletion (ΔGC) within the *RPT6* PACE box (RPT6ΔGC) was created using primer Δ2-RPT6. The wild-type (wt) and mutant promoter fragments were cloned into YEp*lac195*. Subsequently, the bacterial *cat* gene (*Hin*dIII fragment from pCM7, Pharmacia) was introduced into the pRPT6 and RPT4 derivatives, respectively. The YEp*lac195* constructs were transformed into yeast strain CEN.PK2. Correctness of mutations and insertions were verified by sequencing. To generate an *rpn4*$^-$ background, the *RPN4* gene in CEN.PK2 was disrupted by the one step replacement method using a PCR-generated *RPN4::URA3* disruption cassette [21] constructed with the appropriate primer pairs and the *URA3* gene as DNA template. Correct replacement of *RPN4* was verified by PCR using appropriate primer pairs. The resulting *rpn4*$^-$ strain was then transformed with plasmids pRPT6wt-181 and pRPT6ΔPACE-181, respectively.

2.4. RPT promoter swap mutants

Plasmids expressing *RPT5* under control of wt or mutated *RPT6* promoters were constructed by substituting the *cat* gene in each of the three YEp*lac195* derivatives by a PCR-generated copy of the encoding moiety of *RPT5* (Table 1). These plasmids were introduced into yeast strain RGSY20 [13] by transformation.

2.5. Computational methods

The web site of MIPS (http://www.mips.biochem.mpg.de/yeast/), the *Saccharomyces* Genome Database (SGD) at Stanford (http://genome-www.stanford.edu/Saccharomyces/), SWISS PROT (http://www.expasy.ch/sprot/) and the Yeast Protein Database (YPD) (http://quest7.proteome.com/YPDhome.html) were used for information. Searches for protein signatures were carried out by the routines offered at these sites. DNA pattern searches were performed by using the PATMATCH routine of SGD and YEAST TOOLS (http://alize.ulb.ac.be/∽YRT/). The Wisconsin GCG Package programs PEPTIDE-STRUCTURE and PEPPLOT were employed for protein secondary structure prediction.

3. Results

3.1. PACE, a novel DNA sequence element in yeast promoters

Analysis of the promoter regions of the *RPT* genes revealed that in addition to known upstream activating sequences (UAS) elements, all of them encompass a conserved nonamer box, 5′-GGTGGCAAA-3′ or its complement, between 83 and 230 bp upstream from the translational start site. By a systematic search of the complete yeast genome sequence [22], we found this element at 69 different chromosomal locations. Table 3 presents a brief overview of particular yeast genes, the promoter regions of which contain this element. The full catalogue of the chromosomal locations of PACE can be obtained via http://www.med.uni-muenchen.de/biochemie/feldmann/. The majority of the elements are located upstream

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  1 M A S T E L S L K R T L T D I L E D E L Y H T N P G H S Q F
 31 T S H Y Q N Y H P N A S I T P Y K L V N K N K E N N T F T W
 61 N H S L Q H Q N E S S A A S I P P Q Q T Y H F P I F N K Y A
 91 D P T L T T T T S F T T S E A T A N D R Q I N N V H L I P N
121 E I K G A S E T P L Q K T V N L K N I M K V S D P Y V P T R
151 N T F N Y D V K I S N D F F D N G D N L Y G N D E E V L F Y
181 E D N Y N P K M Q W S L Q D N S A A I N N E D A R A I F N N
211 E F D S D D D D I S D*D E E D E I E E N C L Q Q E Q H Q E E
241 P L L S L D V T P I S M F G S D Q K T G R A K S S S H L F N
271 E Y S Y V D S N M D S I S S V V S E D L L D E R G H E K I E
301 D E D E D N D L D E D D I Y D I S L L K N R R K Q S F V L N
331 K N T I D F E R F P S P S T S A N V P S T A T T G K R K P A
361 K S S S N R S C V S N S N E N G T L E R I K K P T S A V V S
391 S N A S R R K L I N Y T K K H L S S H S S T N S N S K P S T
421 A S P S A H T S S S D G N N E I F T C Q I M N L I T N E P C
451 G A Q F S R S Y D L T R H Q N T I H A K R K I V F R C S E C
                                hhhhhhhhhhh     ßßßßßßßßß     ß
481 I K I L G S E G Y Q K T F S R L D A L T R H I K S K H E D L
    ßßßßßßßß   ßßßßß       ßßßßßßß hhhhhhhhhhhhhhhhh(hhhh)hhhhh
511 S L E Q R Q E V T K F A K A N I G Y V M G
    hhhhhhhhhhhhhhhhhhhhhhhhhh
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Fig. 1. Sequence characteristics of Rpn4p. Bold, acidic domains (positions 211–229 and 300–315); inverse and bold, putative C2H2 finger (position 477–507); bold italics, bipartite nuclear target signal (position 381–399); underlined, sequence deleted in [11]; doubly underlined and bold characters, amino acid residues confirmed by micro-sequencing of purified Rpn4p. The asterisk indicates the beginning of the segment in clone pACT2-23. β and h underneath the sequence indicate predicted β-sheets and α-helices, respectively.

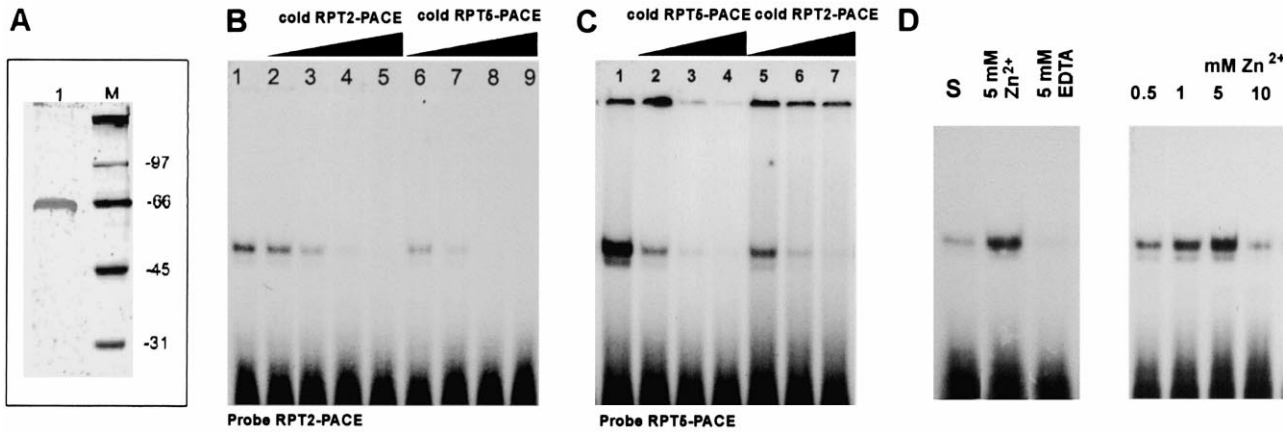

Fig. 2. PACE is bound by purified Rpn4p. (A) Electrophoresis of purified Rpn4p on a 10% SDS gel. M, SDS-PAGE molecular weight standard, broad range (Sigma). Purification of Rpn4p from crude yeast cell extracts was achieved by three consecutive column chromatographies described elsewhere (Karpov et al., in preparation). In brief, heparin agarose (Sigma), DNA cellulose (Pharmacia) and RPT5-PACE oligonucleotide coupled to Sepharose 4B, 6-aminohexanoic acid *N*-hydroxysuccinimide ester (Sigma) were used. The purification procedure was monitored by gel shift experiments similar to those in B and C. Protein from the final preparation was subjected to micro-sequencing (Karpov et al., in preparation). The following amino acids from the N-terminus were identified: 5, Glu; 6, Leu; 7, Ser; 9, Lys; 10, Arg; 12, Leu. (B) and (C): Gel shift competition experiments. Probes of the PACE box(es) from *RPT5* and *RPT2*, respectively, ^{32}P-labelled by nick-translation were used. Binding reactions were carried out in 20 μl 20 mM Tris-HCl, pH 7.5, 0.1 mM EDTA, 10% glycerol, 100 mM KCl, 1 mM DTT, 1 mM PMSF, 1% PIC, 0.1% NP-40, 1 mM spermidine. 0.02% BSA, 1 μg poly(dI-dC), 1 ng of labelled oligonucleotide (200–400 Bq) and 15 ng purified Rpn4 protein (see A) were added (standard conditions, S). In the competition experiments, unlabelled oligonucleotide was added as indicated. Samples were incubated for 15 min at 30°C and applied to a 6% polyacrylamide gel. B, lane 1: RPT5-PACE without competitor; lanes 2–5: addition of a 5-, 25-, 125- and 625-fold excess, respectively, of unlabelled RPT5-PACE; lanes 6–9: competition with excess unlabelled RPT2-PACE in the same order. C, lane 1: RPT2-PACE without competitor; lanes 2–4: addition of a 25-, 125- and 625-fold excess, respectively, of unlabelled RPT2-PACE; lanes 5–7: competition with excess unlabelled RPT5-PACE in the same order. (D) Metal-dependence of Rpn4p binding to RPT2-PACE. Binding reactions were carried out as described above but without adding spermidine. $ZnSO_4$ or EDTA were added to the binding reactions as indicated.

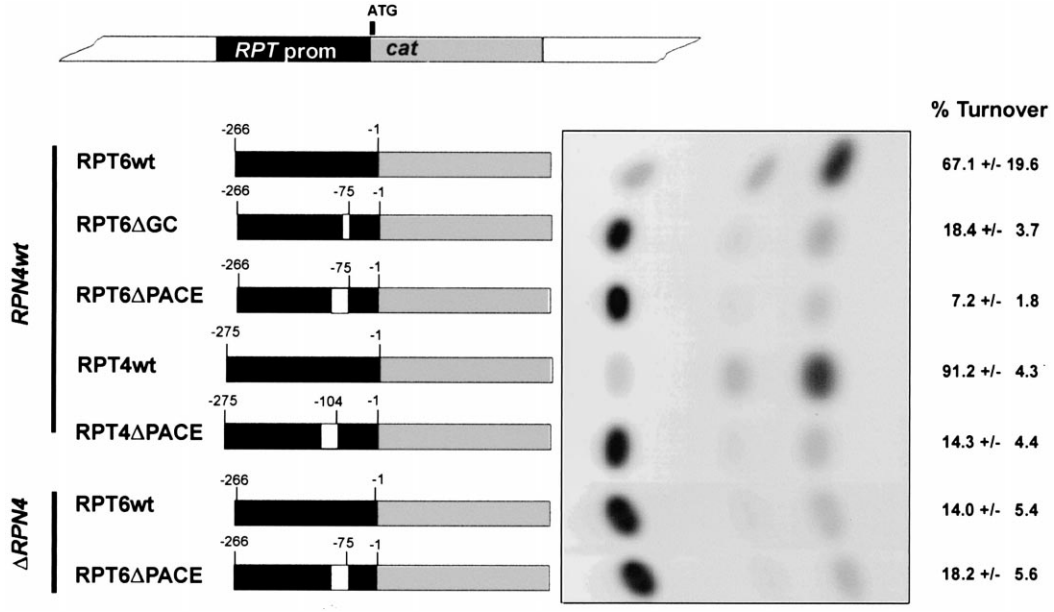

Fig. 3. CAT activity from constructs of *RPT* wt and mutant promoters fused to the bacterial *cat* gene. Procedures for the CAT assays were similar to those described in [26]. Cell-free extracts from liquid cultures grown in selective medium to a cell density of approximately 1.0 A_{600} were prepared by disrupting the cells with glass-beads (0.45 mm) for 2 min in a Brown homogenizer and centrifugation for 30 min at 4°C. Centrifugation of the supernatant was repeated for 60 min. The protein content of cell-free extracts was determined as described in [28]. The chloramphenicol acetyl transferase reaction was carried out according to [27]. Per assay, 0.65 μg protein and 10 μl D-threo-[dichloroacetyl-1-^{14}C]chloramphenicol (Amersham, 925 KBq/ml; 2.04 GBq/mmole) was used in 180 μl incubation mix. Incubation was 1 h at 37°C. The samples were applied to TLC pre-coated aluminium sheets silica gel 60 (Merck) developed in chloroform/methanol (95:5) for 3 h. The sheets were dried, autoradiographed and radioactivity from material of the single spots was measured by scintillation counting. The turnover rate is the percentage radioactivity of reactants, total radioactivity in a reaction set to 100%. Mean values from three independent transformants each are listed. Copy numbers of the reporting plasmids were nearly identical in the different transformants (data not shown).

Table 1
Yeast strains and plasmids generated in this study

Name	Genotype/construction
Yeast strains	
YM4271-PACE-HIS	*MATa ura3-52 ade2-101 lys2-801 leu2-3,112 trp1-903 tyr1-501 gal4-Δ512 gal80-Δ 538 ade5::hisG HIS3::pHISi-1-PACE*
YM4271-PACE-Lac	*MATa ade2-101 lys2-801 leu2-3,112 his3-200 trp1-903 tyr1-501 gal4-Δ512 gal80-Δ 538 ade5::hisG URA3::pLacZi-PACE*
YM4271-PACE-Lac [pACT2-23]	YM4271-PACE-Lac transformed with pACT2-23
YM4271-PACE-Lac [pGADGH-RPN4]	YM4271-PACE-Lac transformed with pGADGH-RPN4
YM4271-PACE-Lac [YEplac181-RPN4]	YM4271-PACE-Lac transformed with pRPN4
YRPT4wt	*MATa ura3-52 trp1-289 leu2-3,112 his3-Δ1* pRPT4wt
YRPT4ΔPACE	*MATa ura3-52 trp1-289 leu2-3,112 his3-Δ1* pRPT4ΔPACE
YRPT6wt	*MATa ura3-52 trp1-289 leu2-3,112 his3-Δ1* pRPT6wt
YRPT6ΔPACE	*MATa ura3-52 trp1-289 leu2-3,112 his3-Δ1* pRPT6ΔPACE
YRPT6ΔGC	*MATa ura3-52 trp1-289 leu2-3,112 his3-Δ1* pRPT6ΔGC
YRPT6wt/ΔRPN4	*MATa ura3-52 trp1-289 leu2-3,112 his3-Δ1 RPN4::URA3* pRPT6wt-181
YRPT6ΔPACE/ ΔRPN4	*MATa ura3-52 trp1-289 leu2-3,112 his3-Δ1 RPN4::URA3* pRPT6ΔPACE-181
RGSY20 [13]	*MATa ade2-1 his3-11,15 leu2-3,112 trp1 ura3-52 kan1-100 YTA1/yta1::HIS3*
RGSY201	As RGSY20, containing YEp*lac195*-RPT6-RPT5
RGSY202	As RGSY20, containing YEp*lac195*-RPT6ΔPACE-RPT5
RGSY203	As RGSY20, containing YEp*lac195*-RPT6ΔGC-RPT5
Plasmids	
pACT2-23	pACT2 containing $RPN4_{(222-531)}$ fused to *GAL4* AD
pGADGH-RPN4	pGAD-GH containing $RPN4_{(92-531)}$ fused to *GAL4* AD
pHISi-1-PACE	pHISi-1 containing multiple PACE as target
pLacZi-PACE	pLacZi containing multiple PACE as target
pRPN4	YEp*lac181*containing *RPN4* under control of *TDH3* promoter
pRPT6wt-181	YEp*lac181* containing *cat* under control of the *RPT6* promoter
pRPT6ΔPACE-181	YEp*lac181* containing *cat* under control of the *RPT6*ΔPACE promoter
pRPT4wt	YEp*lac195* containing *cat* under control of the *RPT4* wt promoter
pRPT4ΔPACE	YEp*lac195* containing *cat* under control of the *RPT4*ΔPACE promoter
pRPT6wt	YEp*lac195* containing *cat* under control of the *RPT6* promoter
pRPT6ΔPACE	YEp*lac195* containing *cat* under control of the *RPT6*ΔPACE promoter
pRPT6ΔGC	YEp*lac195* containing *cat* under control of the *RPT6*ΔGC promoter
pRPT6-RPT5	YEp*lac195* containing *RPT5* under control of the *RPT6* promoter
pRPT6ΔPACE-RPT5	YEp*lac195* containing *RPT5* under control of the *RPT6*ΔPACE promoter
pRPT6ΔGC-RPT5	YEp*lac195* containing *RPT5* under control of the *RPT6*ΔGC promoter

of genes at distances similar to the ones observed for the *RPT* genes. Interestingly, 26 of the 32 proteasomal subunit genes, namely the six *RPT* genes, nine of the 12 *RPN* genes and 12 of the genes for the 20S core subunits, are preceded by the element. This and our findings detailed below prompted us to name this element PACE.

3.2. Rpn4p is a PACE-binding factor

To search for yeast proteins that specifically recognize the PACE sequence, we took advantage of the yeast one-hybrid method [23] using a libary of random yeast DNA segments fused to the Gal4p activating domain in plasmid pACT2 and the appropriate yeast reporter strains. Synthetic oligonucleotides composed of eight repeats of the PACE sequence were placed upstream of the minimal promoters of pHISi-1 or pLacZi, respectively. Transformation of reporter strain YM4271-PACE-HIS with the above pACT2 library yielded approximately 2×10^5 independent colonies. 36 positive clones were obtained in a first screen resulting from *HIS3* activation. Plasmid DNA from each single clone was then transferred into reporter strain YM4271-PACE-Lac and assayed for β-galactosidase activity. In this second round, pACT2-23 was selected as the only clone giving a distinctly positive signal.

The sequence of the insert in pACT2-23 revealed that the activating domain of Gal4p was fused in-frame to the 310 C-terminal amino acids of the nuclear protein Son1p, now renamed Rpn4p [5]. Originally, *SON1* was identified as a suppressor of the *sec63-101* mutation, which showed temperature sensitive growth and mis-localized nuclear proteins [24]. Another *son1* allele, *ufd5*, was found among the ubiquitin fusion degradation (*ufd*) mutants [25]. Recently, Son1p/Rpn4p has been reported to be associated with the yeast 26S proteasome [11]. However, it has not been found among the constituents of purified proteasomes [3]. Although the new quality of Rpn4p (Fig. 1) came as a surprise, we noticed that the presence of a potential C2H2-type zinc finger domain would meet the requirements of Rpn4p to act as a DNA-binding factor. Next, the result from the one-hybrid approach was corroborated by gel retardation assays. 30-mer oligonucleotides including the PACE sequences from *RPT5* or *RPT2* (Table 2) were generated and used in initial experiments together with protein from a crude yeast cell extract, resulting in a band shift (not shown). Subsequently, this assay was used to monitor the protein fractions that were obtained in three consecutive column chromatographies for band shifting capability (Fig. 2). In a SDS gel, the final preparation revealed a singular band (molecular mass 64 kDa) (Fig. 2A) and micro-sequencing confirmed six out of the first 12 N-terminal amino acid residues corresponding to the ones in Rpn4p. A detailed procedure for Rpn4p purification will be presented elsewhere (Karpov et al., in preparation). Fig. 2B and C demonstrate that Rpn4p is capable of specifical binding to the *RPT5*-PACE or *RPT2*-PACE sequences as shown by competition experiments using an excess unlabelled oligonucleotides. Further, Fig. 2D demonstrates that binding of Rpn4p is dependent on Zn^{2+} (optimal stimulation at 5 mM). Binding is also increased by the presence of Mn^{2+}, a quality which is sometimes found in metal-binding proteins (not shown).

To show that Rpn4p by itself is capable of binding to PACE, we introduced YEp*lac*181-RPN4, a low-copy number plasmid expressing full-length Rpn4p, into strain YM4271-PACE-Lac. Two transformants containing pACT2-23 or pGADGH-RPN4, the latter of which expresses an essential part of Rpn4p (encompassing the two acidic domains plus the Zn finger domain) fused to the transactivating domain of Gal4p, were included for comparison. The results given in

Table 2
Particular oligonucleotides used in this study

Name	Sequence (listed 5′ to 3′)	Purpose
4PACEw	aattc-GGTGGCAAA-GGTGGCAAA-GGTGGCAAA-TTTGCCACC	Generation of multiple PACE sites
4PACEc	tcgac-GGTGGCAAA-TTTGCCACC-TTTGCCACC-TTTGCCAC	
RPN4-Δ-start	TTGTATCTTTTCAAAAGTTTTCTAGAATTTTCAAGCAATCGTGAGTTTAGTATACATGCA	*RPN4* disruption
RPN4-Δ-stop	GGTTTTCTTCTTTTATCTCCTATATAATTTGTAACCTTAAGATCTGGCTTTTCAATTCAA	
Eco-RPT6	GGGGAATTCTGTTTGAAAGAGCTGGCTT	Generation of mutant *RPT* promoters
Pst-RPT6	GGGCTGCAGGTCGTGATAGTATCCCACT	
Δ9-RPT6	TGATATTACAACTTCGCTC	
Δ2-RPT6	TGATATTACAACTTTTCACCTC	
Eco-RPT4	GGGGAATTCGACTACGACAATAGGGGG	
Pst-RPT4	GGGCTGCAGTCTTAGTTATTAATATCTTTGCTT	
Δ9-RPT4	GGGGAATTCTGTTTGAAAGAGCTGGCTTCT	
RPT2-PACEw	AGCTTTTTTCTCC**GGTGGCAAA**TGTCTCTTTTG	Generation of PACE oligonucleotide probes for gel shifts
RPT2-PACEc	CTAGGTTTTCTCTGT**AAACGGTGG**CCTCTTTTT	
RPT5-PACEw	AGCTTGCAAATATGT**GGTGGCAAA**AATGAATTG	
RPT5-PACEc	CTAGGTTAAGTAA**AAACGGTGG**TGTATAAACGT	

Table 4 clearly demonstrate that Rpn4p is capable of binding to PACE and that *lacZ* expression is stimulated 3.8-fold over the background (due to endogenous Rpn4p activity). The somewhat higher stimulatory capacity of pACT2-23 and pGADGH-RPN4 transformed into YM4271-PACE-Lac may be due to the additional Gal4p activating domains present in these plasmids. Further, the experiments comparing the activity of a genuine PACE-containing promoter (*RPT6*) in wt and *rpn4*$^-$ strains (see below) are in full support of Rpn4p behaving as a transcriptional activator.

3.3. PACE is a cis-acting element in yeast and Rpn4p is a trans-activating factor

To demonstrate that PACE indeed acts as a *cis*-regulatory element, we decided to use the bacterial *cat* gene as a reporter in combination with promoter segments from *RPT4(SUG2)* and *RPT6(SUG1)*, two of the genes encoding regulatory proteasomal subunits that contain the promoter PACE sequence. We have previously shown that the bacterial *cat* gene fulfils the requirement to serve as a suitable reporter in yeast and that, for example, the expression of the highly-regulated *PHO5* promoter fused to the *cat* gene is correctly monitored when assayed under varying growth conditions [26]. The CAT activity determined in cell extracts from transformants with each of the five promoter-reporter constructs (pRPT4wt, pRPT4ΔPACE, pRPT6wt, pRPT6ΔPACE, pRPT6ΔGC) is reduced in the constructs carrying promoter mutations compared with the corresponding ones carrying wt promoter sequences (Fig. 3). The reduction on average is about 5-fold for the PACE-less *RPT4* promoter and about 4-fold for the PACE-less *RPT6* promoter, while the 2 bp deletion within the PACE box of the *RPT6* promoter has an intermediate effect (2.3-fold). Additionally, we compared the expression of pRPT6wt and pRPT6ΔPACE in an *rpn4*$^-$ background. The CAT activity from these two constructs is similar and at a level comparable to the one of *RPT6*ΔPACE in the wt background.

3.4. Wt and mutated RPT6 promoter segments can substitute for the genuine promoter of RPT5 in vivo

Our experiments indicate that Rpn4p acts as a positive transcriptional factor for the expression of *RPT4* and *RPT6* but that a residual level of transcription is maintained in the absence of Rpn4p. This is in agreement with the notion [11,24]

Table 3
Yeast genes with upstream PACE sequences[a]

Genes[b]	Functions
RPT1 (*CIM5*/*YTA3*); *RPT2* (*YTA5*); *RPT3* (*YTA2*); *RPT4* (*SUG2*); *RPT5* (*YTA1*); *RPT6* (*SUG1*/*CIM3*)	AAA proteins of the regulatory complex of the 26S proteasome
RPN1 (*HDR2*/*NAS1*); *RPN2* (*SEN3*); *RPN3* (*SUN2*); *RPN5* (*NAS5*); *RPN6* (*NAS4*); *RPN9; RPN11* (*MPR1*); *RPN12* (*NIN1*)	"Non-ATPase" proteins of the regulatory complex of the 26S proteasome
SCL1, PUP, PRE6, PRE8, PRE9, PRE10	α-Subunits of the proteolytic 20S core complex of the proteasome
PUP3, PRE1, PRE2, PRE3, PRE4, PRE7	β-Subunits of the proteolytic 20S core complex of the proteasome
UBA1, UBI4, BUL1	Ubiquitin pathway
CDC48 (AAA protein)	Cell division, membrane fusion
CTC6 (chaperonin), *PIM1*(m-AAA protease)	Chaperone/protease
TFP1	Vacuolar endonuclease
ECM12, ECM29, GSF2	Cell wall synthesis
CEG1 (mRNA capping), *FIR1* (mRNA processing), *NAB2* (nuclear mRNA export)	mRNA stability and processing
REB1, BDF2, BMH1, YAP1	Transcription factors
GLK1 (glucokinase), *KIN2* (kinase), *PDI1*(protein disulfid isomerase), *NCA3* (ATP synthase), *YTA7* (AAA protein)	Miscellaneous functions

[a]Only genes with PACE at a canonical distance upstream from the translational start site are included.
[b]The nomenclature for the 26S proteasomal regulatory subunits and references can be found in [5].

Table 4
LacZ expression in PACE-Lac reporter strains

Reporter strain	β-Galactosidase activity (U)[a]	Relative activity (%)
YM4271-PACE-Lac	58.3 ± 12.5	13.6
YM4271-PACE-Lac [pACT2-23]	416.7 ± 13.9	96.8
YM4271-PACE-Lac [pGADGH-RPN4]	430.3 ± 34.5	100.0
YM4271-PACE-Lac [YEp*lac181*-RPN4]	223.4 ± 21.5	51.8

[a]Miller units; mean values of three independent measurements from two independent transformants each.

that knock-out of the *RPN4* gene appears to be not critical for cell survival at an ordinary temperature. Likewise, under normal conditions, PACE-less promoters should not be critical to the cell viability. To address this problem, we performed complementation assays, using yeast strain RGSY20 and promoter 'swap' mutations of the essential *RPT5* gene introduced into YEp*lac195* as the complementing plasmid. We have earlier shown that the segregants from RGSY20, a diploid in which heterozygous deletion of one of the *RPT5* alleles confers a recessive lethal phenotype, can be rescued by complementation with a copy of the corresponding wt allele. Without complementation, the two *rpt5*$^-$ segregants derived from RGSY20 sporulation are growth-arrested after two cell divisions [13]. In contrast, all spores derived from RGSY201, RGSY202 and RGSY203 formed colonies of wt size after outgrowth. Analysis by diagnostic hybridizations and replica plating on selective media confirmed that in all instances, segregation had occurred 2:2, whereby two of the segregants were *RPT5*, whereas the two other segregants were *rpt5*$^-$ but had retained the complementing swap promoter plasmid. From these experiments, we conclude that (i) substitution of the *RPT5* promoter (which contains the PACE sequence) by the *RPT6* promoter is tolerated, (ii) the PACE-less promoter is not critical for the expression of *RPT5* and (iii) upregulation of *RPT5* expression through PACE/Rpn4p is not required for cell viability under normal growth conditions.

4. Discussion

Although protein-protein interactions between the various subunits and the participation of regulatory factors are known to play a pivotal role in the proteasome assembly [2], it is conceivable that the expression of the subunits may already be subject to coordinate control. In this study, we have demonstrated that a unique nonamer box, GGTGGCAAA or its complement (PACE), is located in a similar distance upstream from the translational start site of 26 of the 32 proteasomal subunit genes. By two parallel approaches, we showed that, in fact, PACE is capable of interacting with a particular DNA-binding protein, which turned out to be Rpn4p, a protein that was characterized in some detail before [11,24,25]. Our results demonstrate that in the reporter system used here, an intact PACE sequence is required for optimal expression of the *RPT4* and *RPT6* genes under normal conditions. In an *rpn4* null background, as expected, both the *RPT6*wt and *RPT6*ΔPACE promoter constructs are expressed at a comparably low level. This implies that in the absence of Rpn4p, a basic level of transcription from the *RPT* promoters is retained, which may be sufficient to maintain certain functions of the 26S proteasomes at an ordinary temperature [11,24]. Similarly, many of the moderately *trans*-acting factors in yeast have been found to be dispensible for cell viability. Thus, although not essential for the cell viability at an ordinary temperature, Rpn4p may be needed for specialized functions of the 26S proteasome, such as an involvement in degrading particular ubiquitinated substrates [25] or components of the mating pathway [29] or becoming important for growth under non-favorable conditions [24]. As a *trans*-acting factor, Rpn4p could rather plausibly be regarded to be involved in the modulation of various facets of proteasome activity, but more extended studies on this issue are needed.

Our finding that Rpn4p is a DNA-binding factor and reveals *trans*-activating capacity is well in agreement with its sequence characteristics. Interestingly, the C2H2 finger motif is unusual in that its hydrophobic core encompasses 21 instead of the 12 amino acid residues normally found in classical C2H2 zinc fingers [30]. However, DNA-binding factors with shorter or extended C2H2 fingers similar to that in Rpn4p have been found, for example, in yeast (*ZMS1*, *YML081w*), *Caenorhabditis elegans* (Lin-26) or *Drosophila* (TTK alpha). In further support that the Rpn4p sequence represents a 'true' finger is its requirement of Zn for optimal binding and its predicted arrangement into β-sheets and α-helices which is compatible with those of conventional zinc-binding motifs [31]. Further, the two separate acidic domains [11,24,25] can be considered *trans*-acting domains. This is supported by the earlier finding [11] that Rpn4 with an internal *Eco*RV-mediated deletion of 97 amino acids (Fig. 1) is still functional. This in-frame deletion completely eliminates the second acidic domain but leaves several acidic amino acid residues from the first domain plus the zinc finger motif intact. By contrast, replacement of the 97 amino acid deletion by the *HIS3* marker interrupting the open reading frame rendered the protein non-functional [11]. Also compatible with Rpn4p to function as a transcription factor is a putative bipartite nuclear targeting sequence and localization of the protein to the nucleus as well as the cytoplasm ([24] and our unpublished results). Clearly, further experiments will be necessary to dissect the functions of the various domains in Rpn4p and to investigate whether *RPN4* expression is subject to superior control mechanisms.

Rpn4p acting as a DNA-binding factor and being a potential constituent of the 19S proteasomal sub-complex [11] are not incompatible features in view of the fact that other regulatory subunits of the proteasome are able to fulfil dual functions. For example, Sug1p and its homologues in other organisms are constituents of the 26S proteasome but can also act as mediators in transcription activated by nuclear receptors [32,33]. They have been suggested to be associated with distinct protein complexes and therefore to play multiple roles. The effetcs of Rpn4p on Sec63p [11,24] can be explained by both its presumed functions: (i) retrograde transport mediated by ER translocons is required to transfer abnormal proteins from the ER lumen to the cytosolic face for final proteolysis thus involving the ubiquitin-proteasome machinery in ER degradation (e.g. [34]), (ii) this process may be dependent on

Rpn4p-mediated modulation of the proteasome activity. Synthetic lethality of *rpn4* mutants with mutations of *RPN2*, [29] and *RPN12* [11] have been observed. Rpn2p, the largest subunit of the 26S proteasome, has been shown to function in protein transport to the nucleus [35], while Rpn12p, the smallest subunit, has a function in the nuclear stability [36]. As both the *RPN2* and *RPN12* genes contain PACE in their upstream regions, synthetic lethality may be a consequence of the absence of fully functional Rpn4p affecting the expression of *rpn2* and *rpn12* and/or it may be due to disturbed interactions among these proteins, if Rpn4p is regarded to be a constituent of the 26S proteasome. By contrast, synthetic lethality was not observed for *rpn4* mutants with mutations of *RPN10* [11]. As the *RPN10* promoter is devoid of the PACE sequence, the expression of *RPN10* should not be affected by the loss of Rpn4p.

Fujimoro et al. [11] reasoned that the ability of *rpn4* null strains to grow at normal or higher temperatures might be compensated by (an)other factor(s) with overlapping functions. However, if so, this cannot be a homologue of Rpn4p because no gene with sufficient similarity to *RPN4* is present in the yeast genome. Interestingly, a counterpart of Rpn4p in other organisms is not known. It may well be that such orthologues are found in the future. Otherwise, one could argue that such a factor is either unique to the yeast system or that factor(s) structurally unrelated to Rpn4p may take its role in other organisms.

Altogether, PACE is found at 69 different chromosomal locations, all but a few of these locations fall exclusively into non-encoding regions. In 24 of these, PACE is located in the promoter regions shared by two divergently-transcribed genes. Since only little is known about the transcriptional regulation of divergently-transcribed genes in yeast, the functional significance of any 'intergenic' UAS in the expression of these genes remains completely open. On the other hand, PACE occurs in the upstream regions of 33 genes of known function that are transcribed from a 'unidirectional' promoter. In most of the cases, PACE is located at a distance upstream from the translational start site of these genes which is comparable to those found in the *RPT* genes described here and in accordance with the canonical placement of UASs in yeast. Notwithstanding the need that in each single case the functional significance of PACE has to be addressed by experimentation, it seems worthwile to briefly consider the types of genes potentially concerned. From our list (Table 3), it is obvious that, in addition to most of the genes for proteasomal subunits, these are preferably genes for functions known to be linked to the ubiquitin-proteasome system. Most intriguing in this respect are the genes for the polyubiquitin precursor (Ubi4p), the ubiquitin activating enzyme Uba1p, the ubiquitin ligase-binding protein Bul1p and Cdc48p, a member of the AAA family which has multiple functions in cell division, homotypic membrane fusion, ubiquitin-mediated proteolysis [37] and apoptosis [38]. Another group of genes involved in programmed proteolysis, like *PIM1* (mitochondrial Lon protease) and *CCT6* (member of the TRiC complex) or *TFP1* (vacuolar homing endonuclease) possess the PACE box, but remarkably, none of the promoters of the mitochondrial AAA proteases [13]. It is tempting to speculate that also several genes for factors involved in the cell wall synthesis, mRNA stability, protein folding and several transcription factors may be subject to control by PACE. In this context, it has to be mentioned that we obtained preliminary results for two genes from the above list, *RPN5* and *CDC48*. When promoter segments from these genes were coupled to the *cat* gene as a reporter, expression from the PACE-less versus the intact promoters was found to be reduced 5–7-fold (Karpov et al., in preparation), similar to the values obtained for *RPT4* and *RPT6*.

Taken together, the presence of PACE in most of the promoters for proteasomal and many other yeast genes together with the finding that Rpn4p is a PACE-binding and behaves as a *trans*-acting factor suggests to us that we have touched a novel regulatory network involved in the control of the ubiquitin-proteasome pathway in yeast.

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