PRE5 and *PRE6*, the Last Missing Genes Encoding 20S Proteasome Subunits from Yeast? Indication for a Set of 14 Different Subunits in the Eukaryotic Proteasome Core[†,‡]

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ABSTRACT: The 20S proteasome of eukaryotes is an abundant multicatalytic/multifunctional proteinase complex composed of an array of nonidentical subunits which are encoded by α- or β-type members of the proteasomal gene family. In budding yeast, 14 subunits had been detected and 12 proteasomal genes had been cloned and sequenced so far. Starting from peptide sequences of purified subunits of the yeast 20S proteasome, we cloned two additional proteasomal genes, *PRE5* and *PRE6*, which both encode essential α-type subunits. Sequence comparison of all known eukaryotic proteasomal proteins show the presence of a total of 14 subgroups, which can be divided into seven α- and seven β-type groups. Including the Pre5 and Pre6 proteins, every subgroup contains a single yeast member. We anticipate that the 14 genes encoding subunits of the yeast proteasome represent the complete set of proteasomal genes of this organism. The ancestral archaebacterial proteasome is composed of four stacks of rings, the two outer rings containing seven identical α-subunits and the inner rings containing seven identical β-subunits. We speculate that, in analogy to the archaebacterial proteasome, every eukaryotic proteasome is made of two halves of 14 distinct subunits, each half consisting of seven different α-type and 7 different β-type subunits. In higher eukaryotes, subunit isoforms may contribute to variability in the subunit composition of the 20S proteasome allowing functional modulations.

The 20S proteasome is a high molecular mass (700 kDa) multicatalytic endopeptidase complex found in the cytosol and the nucleus of all eukaryotic cells [for recent reviews, see: Tanaka et al. (1992), Goldberg and Rock (1992), Rechsteiner et al. (1993), Rivett (1993)]. The highly conserved cylinder-shaped particle is made of four stacked rings composed of a multitude of small (20–35 kDa), nonidentical subunits (Tanaka et al., 1988; Kleinschmidt et al., 1988). As the only known noneukaryotic genus, the archaebacterium *Thermoplasma* contains a proteasome, which consists of only two types of subunits called α and β (Zwickl et al., 1992). The two outer rings of this ancestral proteasome are composed of seven identical α-subunits each and the two inner rings of seven identical β-subunits each (Pühler et al., 1992). In contrast, for eukaryotic proteasomes, a large number of nonidentical subunits has been found which varies, depending on the source and investigator, from 12 to 25 [for examples, see Schliephacke et al. (1991) and Haass and Kloetzel (1989)]. All eukaryotic subunits characterized in primary structure represent evolutionary related members of a novel protein class and can be subdivided into α- and β-types by their degree of relationship to either of the two archaebacterial subunits.

The 20S proteasome can assemble with additional components in an ATP-dependent fashion to form a 26S proteolytic complex [for reviews, see Goldberg and Rock (1992) and Rechsteiner et al. (1993)]. In vitro this complex degrades ubiquitinylated proteins (Eytan et al., 1989; Driscoll & Goldberg, 1990; Kanayama et al., 1992) as well as antizyme-complexed, nonubiquitinylated ornithine decarboxylase (Murakami et al., 1992).

Genetic studies with yeast mutants defective in the chymotrypsin-like activity of the proteasome due to alterations in subunits of the 20S core particle (Heinemeyer et al., 1991, 1993; Hilt et al., 1993) revealed functions of the protease complex in a variety of cellular events: It is responsible for removal of abnormal proteins formed after heat treatment or incorporation of amino acid analogues (Heinemeyer et al., 1991, 1993; Hilt & Wolf, 1992; Hilt et al., 1993), as well as for turnover of proteins which are short-lived by nature (Heinemeyer et al., 1991; Richter-Ruoff et al., 1992; Seufert & Jentsch, 1992) or become unstable upon disassembly (Egner et al., 1993). Also metabolically regulated proteins are targets of the proteasome (Schork et al., 1994). Evidence for a participation of the proteasome in the degradation of transcriptional regulators (Richter-Ruoff, Hochstrasser, and Wolf, unpublished results) and in cell-cycle control (Richter-Ruoff & Wolf, 1993; Ghislain et al., 1993; Gordon et al., 1993) is accumulating.

A specialized function of certain proteasomes from mammals seems to reside in antigen processing [for reviews, see: Goldberg and Rock (1992), Driscoll and Finley (1992), DeMars and Spies (1992)]. Degradation of intracellular protein antigens into peptides, which after entry into the endoplasmic reticulum are loaded onto MHC class I molecules and finally presented at the cell surface, is thought to be performed by a certain proteasome species, the low molecular mass protein (LMP)[1] complex, which contains two MHC class II region-encoded, γ-interferon inducible subunits, LMP2 and LMP7 (Gaczynska et al., 1993; Driscoll et al., 1993; Boes et al., 1994; Aki et al., 1994).

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[1] Abbreviations: LMP, low molecular mass proteins; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TFA, trifluoroacetic acid.

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Large gaps exist in our understanding of the mechanisms underlying substrate recognition and degradation by the proteasome, and the functions of 26S proteasome specific subunits are essentially unknown. Furthermore, even though a multitude of sequence information for the 20S proteasome core subunits is available, we know neither the role of these subunits in catalysis nor the exact composition of the core particle. The definition of the composition of the 20S proteasome is complicated by the fact that there exist discrepancies in the reported number of different subunits visible after two-dimensional separation of dissociated, purified 20S proteasomes. This observation may point to variability of the proteasome structure resulting from posttranslational modifications or/and tissue and development specific changes in subunit composition. The existence of the mammalian LMP complex as well as reports on developmental changes in subunit content of the *Drosophila* proteasome (Haass & Kloetzel, 1989) are documented examples for this diversity.

Analysis of the composition of the eukaryotic proteasome should be facilitated by using an unicellular organism such as the yeast *Saccharomyces cerevisiae*. Here, 14 subunits had been found after dissociation of the purified 20S proteasome particle (Heinemeyer et al., 1991). Up to now, sequence information of subunits was available on the basis of 12 cloned proteasomal genes (Heinemeyer et al., 1991, 1993; Hilt et al., 1993; Enenkel et al., 1994; Fujiwara et al., 1990; Emori et al., 1991; Haffter & Fox, 1991; Georgatsou et al., 1992; Lee et al., 1992; Basile et al., 1992), all but one of them being indispensible for cell survival.

Here, we report on the cloning of two additional essential yeast proteasomal genes, *PRE5* and *PRE6*, adding up the known yeast proteasomal genes to the number of 14. Reasons will be discussed which lead to the assumption that the yeast 20S proteasome is composed of precisely 14 different subunits and that composition of 14 subunits of the type found in yeast applies in principle to all eukaryotic proteasomes.

EXPERIMENTAL PROCEDURES

Proteasome Purification, Reversed Phase Chromatography of Subunits and Tryptic Peptides, and Peptide Sequencing. Purification of the yeast 20S proteasome was done essentially as described in Achstetter et al. (1984) and modified by Tröndle (1991). For separation of proteasome subunits by reversed phase chromatography, 250 μg of purified proteasome was applied onto a MN Nucleosil 300 5C4 column (4 × 250 mm; Macherey & Nagel, Düren, Germany) and bound protein was eluted with a linear (38-65%) gradient of acetonitrile in 0.05% aqueous TFA. The 12 resulting peak fractions were lyophilized and dissolved in 1.8 M urea in 20 mM Tris/HCl, pH 8.0. Trypsin was added to a ratio of 1/100 of the subunit protein concentration, and the sample was incubated for 16 h at room temperature. Samples were then applied onto a REP RP HR 5/5 reversed phase column (5 × 50 mm; Pharmacia, Freiburg, Germany) and eluted with a linear (10–35%) gradient of acetonitrile in 0.1% aqueous TFA. Lyophilized peptide fractions were sequenced with an automatic protein sequencer (Applied Biosystems Inc., Foster City, CA).

Manipulations of Microorganisms. *Escherichia coli* strain DH5α (Ausubel et al., 1987), used as host for plasmids, was grown in Luria broth medium in the presence of 50 mg/L ampicillin when required.

S. cerevisiae strains were grown in rich (YPD) or synthetic (SD) media according to Ausubel et al. (1987) and transformed as described in Heinemeyer et al. (1991). Sporulation and tetrad analysis were carried out following standard procedures (Ausubel et al., 1987; Guthrie & Fink, 1991).

Gene Cloning, Deletion, and Mapping. All DNA manipulations were carried out according to standard procedures (Ausubel et al., 1987; Sambrook et al., 1989).

For cloning of *PRE5*, the two peptide sequences "8–9":GA-HLLEFQP and "8–8":TPFTIYDGE from purified yeast proteasome subunit 8 served for the deduction of the two degenerate, inosine-containing oligonucleotide mixtures "8–9-for":5′-CA*TGATCA*(T/C)TI(T/C)TIGA(A/G)TT(T/C)-CA(A/G)CC-3′ and "8–9rev":5′-GC*GAATTC*ICC(A/G)-TC(A/G)TC(A/G/T)ATIGT(A/G)AA-3′, respectively (underlined parts correspond to the underlined stretch in the peptide sequences and restriction enzyme recognition sequences introduced at the 5′ ends for cloning purposes are in italic). Both mixtures (33 pmol each) were added to 10 ng of *S. cerevisiae* genomic DNA from strain WCG4 (Heinemeyer et al., 1993) in 50 mM KCl, 3 mM $MgCl_2$, 10 mM Tris/HCl, pH 8.0, 0.1 mg/mL gelatine, and 0.2 mM each of dGTP, dCTP, dATP, and dTTP. During denaturation at 94 °C, Taq DNA polymerase (2.5 units) was added and the sample was subjected to 29 cycles of each 2 min at 50 °C, 3 min at 72 °C, and 1.5 min at 94 °C followed by a 2 min step at 50 °C and a 5 min step at 72 °C. The resulting 280 bp PCR product was cloned into pUC19 (Yanish-Perron et al., 1985) and sequenced using the chain termination method.

The excised *PRE5* insert was labeled with digoxigenin (Boehringer, Mannheim, Germany) according to the manufacturer's protocol and used as the probe for hybridization with immobilized plasmid DNA from *E. coli* colonies harboring the YCp50 yeast genomic library (Rose et al., 1987). Plasmids from two hybridizing colonies carrying overlapping genomic inserts were subjected to restriction mapping and Southern hybridization analysis in order to determine the localization of *PRE5*. A series of pUC19-based plasmids carrying overlapping restriction fragments from the *PRE5* gene region served to establish the sequence of a continuous stretch of 2.1 kbp comprising *PRE5*.

A *pre5*Δ::*HIS3* deletion allele was constructed in two steps: Plasmid pE5-59ΔAc, carrying the 580 bp *Acc*I/*Hin*dIII fragment from the *PRE5* 3′ region (Figure 1A) in pUC19 was cut in the polylinker besides its *Acc*I insert end with *Eco*RI and *Sma*I, and the insert from plasmid pE5-69ΔAc (850 bp *Eco*RI/*Acc*I fragment from the *PRE5* 5′ region in pUC19; Figure 1A) was inserted as *Eco*RI/filled-in-*Hin*dIII fragment. In the resulting plasmid, a polylinker-derived *Bam*HI site at the 5′–3′ junction was used to insert a 1.77 kbp *Bam*HI fragment carrying the *HIS3* gene, yielding pE5Δ::HIS3. The null allele was excised as a 2.6 kbp *Sna*BI fragment and transformed into the diploid wild type strain WCG4. The expected heterozygous replacement of the *PRE5* gene by the *pre5*Δ::*HIS3* allele was confirmed among several His^+ transformands by PCR using primers annealing in the 5′- and 3′-noncoding regions of *PRE5*. Sporulation of two of these transformands and tetrad dissection resulted in a 2:0 distribution of colony-forming spores (at least 16 tetrads each were examined). All viable spores were auxotrophic for histidine.

Cloning of *PRE6* was done essentially in analogy to that of *PRE5* with the following alterations: PCR primer mixtures were "6for":5′-C*GAATTC*CA(A/G)GTIGA(A/G)-TA(T/C)GCI(T/C/A)TIGA(A/G)GC-3′, deduced from the N-terminal sequence FQVEYA(L/M)EA, which was completely conserved in the expected Pre6p homologues from *Dictyostelium* and *Arabidopsis* and partially conserved in the *Drosophila* PROS28.1 protein (see Figure 2B), and

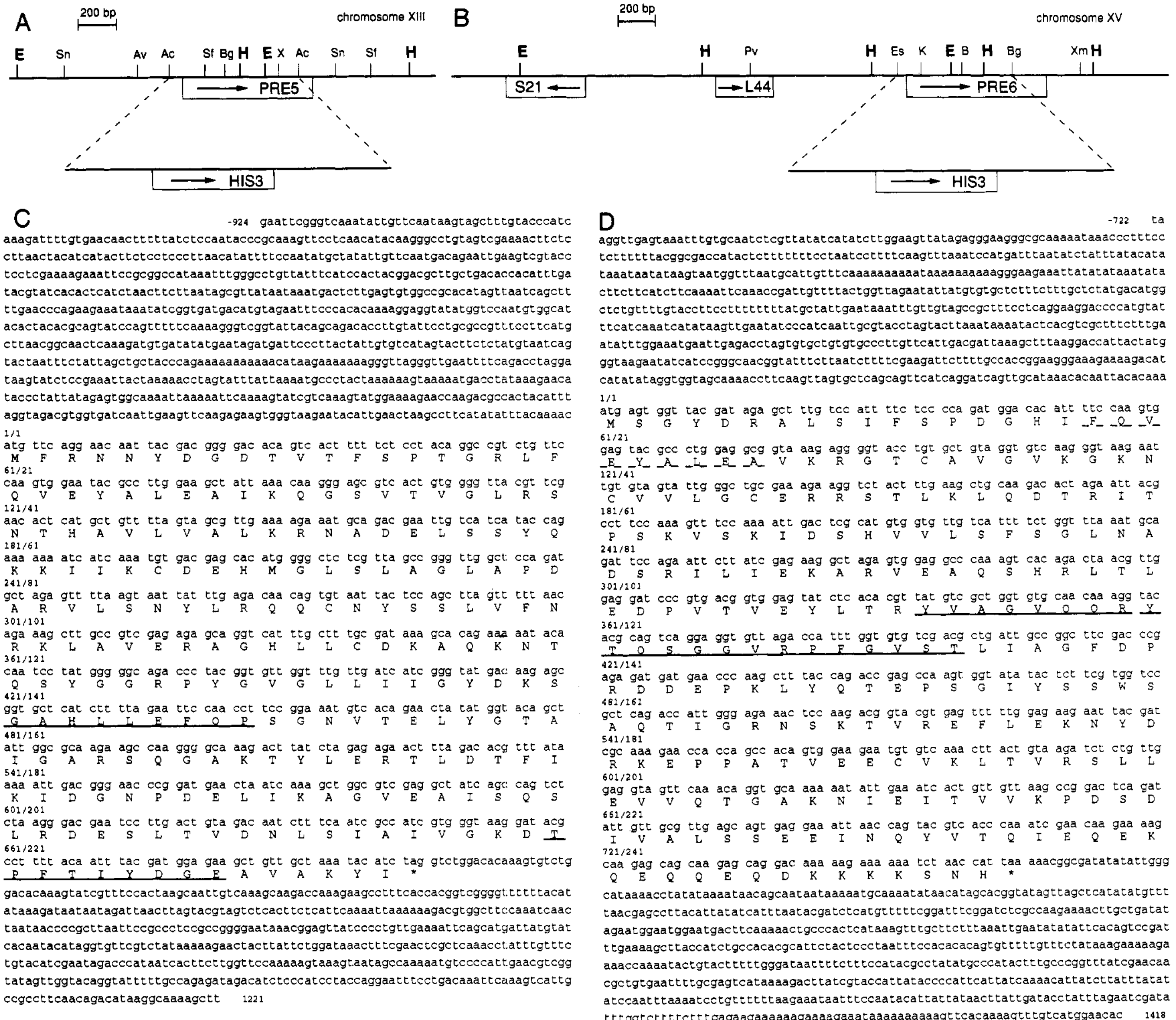

FIGURE 1: *PRE5* and *PRE6* genes. Panels A and B: Organization, restriction maps, and deletion alleles of *PRE5* and *PRE6*, respectively. Coding regions are shown as boxes with arrows indicating the direction of transcription. Dashed lines indicate the regions of *PRE5* and *PRE6* that were replaced with *HIS3* in the respective deletion alleles (see Experimental Procedures). Restriction sites used for subcloning and deletion purposes are Ac, *Acc*I; Av, *Avr*II; B, *Bam*HI; Bg, *Bgl*II; E, *Eco*RI; Es, *Esp*I; H, *Hin*dIII; K, *Kpn*I; P, *Pvu*II; Sf, *Sfu*I; Sn, *Sna*BI; X, *Xba*I; Xm, *Xmn*I. Panels C and D: Nucleotide sequences of the *PRE5* and *PRE6* genes and deduced amino acid sequences of the encoded proteins. Amino acids are shown in single-letter code beneath the encoding nucleotide sequence. Numbering of nucleotides and amino acids, respectively (separated by a slash), begins at the presumed start codon and start methionine. Asterisks indicate the stop codons. The first 220 nucleotides in panel D, starting with the stop codon of the gene encoding ribosomal subunit L44, are taken from the GenBank/EMBL Data Bank nucleotide sequence file with accession number M26503, which overlaps with the *PRE6* 5′-flanking nucleotide sequence determined in our study. Each of the two peptides obtained by amino acid sequencing of purified yeast proteasomal subunits is underlined in the Pre5p and Pre6p amino acid sequences. A N-terminal sequence in Pre6p (panel D), relevant to cloning of the *PRE6* gene (see Results), is indicated by dashed underlining.

"6rev":5′-TC*GGTACC*(T/G)(T/C)TG(T/C)TGIACICC-IGC(A/T/C/G)AC(A/G)TA-3′, deduced from the peptide "5–2":YVAGVQQR, derived from the purified yeast proteasomal subunit 5. PCR was performed with 100 pmol of each oligonucleotide mixture in 30 cycles of each 1.5 min (5 min in the first cycle) at 94 °C, 1 min at 55 °C, and 1.5 min (5 min in the last cycle) at 72 °C. The 310 bp amplification product was cloned into pUC19, sequenced, and employed as the probe for colony hybridization. Five different hybridizing plasmids were detected in the YCp50 library, which after restriction mapping allowed precise localization of the *PRE6* gene. Sequencing of 2.1 kbp comprising the complete *PRE6* coding region was done with plasmids containing overlapping subfragments.

A *pre6*Δ::*HIS3* null allele was constructed by cutting the pUC19-based plasmid pE6-1.7 (carrying a 600 bp *Hin*dIII insert containing *PRE6* C-terminal-coding and 3′-noncoding regions; Figure 1B) with *Bgl*II in the insert and *Eco*RI in the polylinker and ligating to it a 1.77 kbp *HIS3 Bam*HI fragment together with the *Eco*RI/*Bam*HI-ended insert of the pUC19 derivative pE6-6.12ΔEs (carrying 2.1 kbp of *PRE6* 5′ region from *Eco*RI to *Esp*I; Figure 1B). The desired ligation product, plasmid pE6Δ::HIS3, was cut with *Eco*RI and *Xmn*I to liberate the deletion allele, which was transformed into diploid WCG4 wild type yeast cells. His^+ transformands heterozygously carrying the *PRE6* deletion allele (confirmed by PCR) produced two viable histidine auxotrophic and two nonproliferating spores (5 and 25 tetrads, respectively, from

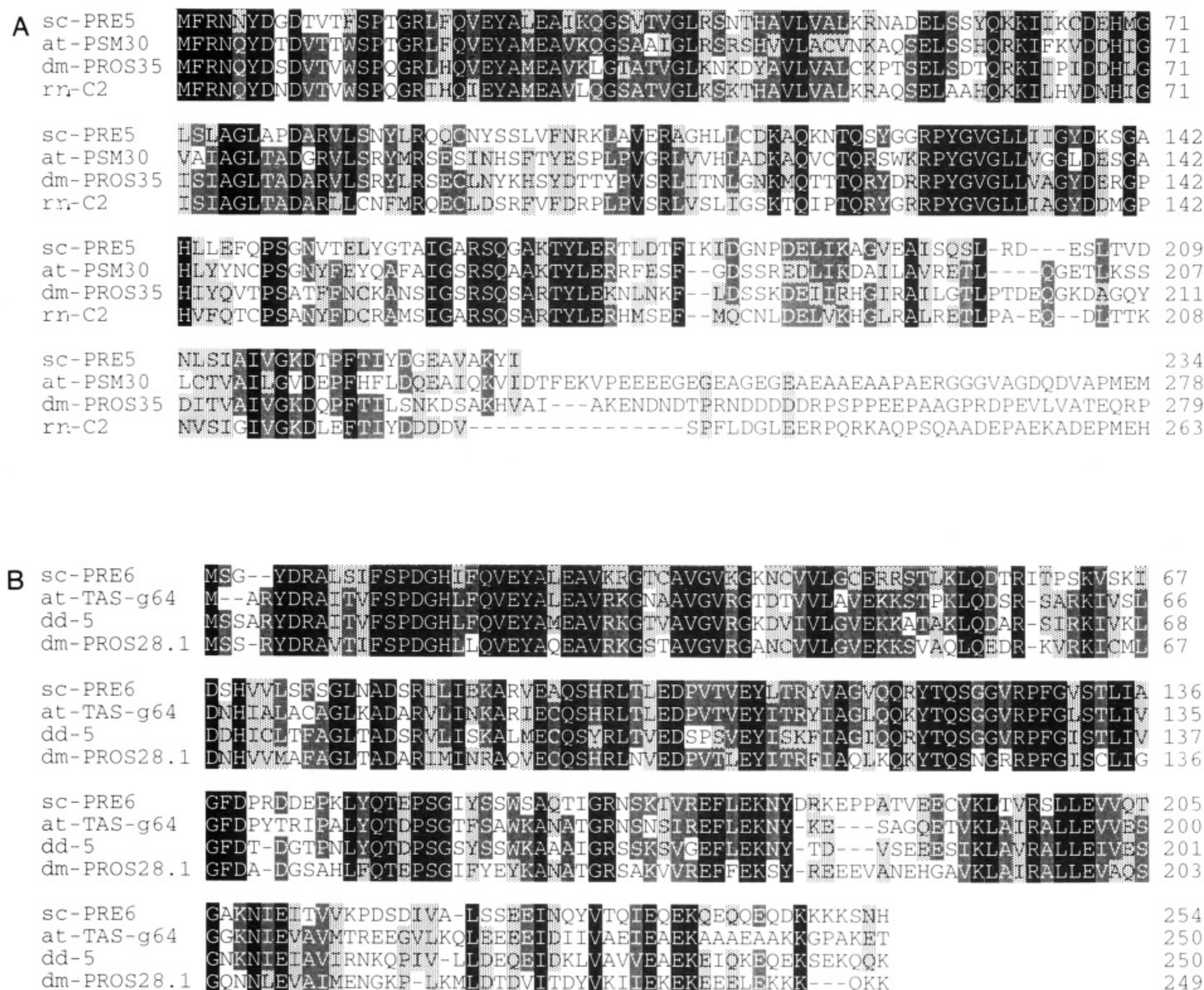

FIGURE 2: Alignments of the Pre5p and Pre6p protein sequences with proteasomal homologues from higher eukaryotes. Panel A: Comparison of Pre5p (sc-PRE5) with the proteasomal subunits PSM30 from *Arabidopsis thaliana* [at-PSM30 (Shirley & Goodman, 1993)] and PROS35 from *Drosophila melanogaster* [dm-PROS35 (Haass et al., 1989; Frentzel et al., 1992b)] and the rat proteasomal C2 component [rn-C2 (Fujiwara et al., 1989)]. Panel B: Comparison of Pre6p (sc-PRE6) with the proteasomal subunits TAS-g64 from *A. thaliana* [at-TAS-g64 (Genschick et al., 1992)], DD5 from *D. discoideum* [dd-5 (Schauer et al., 1993)], and PROS28.1 from *Dr. melanogaster* [dm-PROS28.1 (Haass et al., 1990b; Frentzel et al., 1992b)]. Numbers at the right of each panel are for the last amino acid in the line. Amino acids are shown in the single-letter code. Dashes indicate gaps introduced to maximize alignment. Sequence identities in all four or in three members at a given position are denoted using white letters on black or shaded background, respectively, and similar amino acids (grouped into ACILMV, FHWY, DENQ, ST, RK, and GP) in at least three members are shown as shaded black letters. Alignments were created by the CLUSTAL multiple sequence alignment program (Higgins & Sharp, 1988) using the DNASTAR "Lasergene" software.

two independant transformands were examined).

The chromosomal localizations of the *PRE5* and *PRE6* genes were determined by hybridizing the digoxigenin-labeled 1.35 kbp *Eco*RI fragment from the *PRE5* gene region (Figure 1A) and the digoxigenin-labeled 310 bp PCR amplification product of *PRE6*, respectively, to a set of filters spotted with the yeast lambda prime clone library, which contains an ordered array of DNA representing most of the yeast genome (Riles et al., 1993).

RESULTS

Cloning of PRE5 and PRE6. The 12 so far characterized genes encoding subunits of the *S. cerevisiae* 20S proteasome can be divided into five α-type genes [*PRS1*, *PRS2* (Fujiwara et al., 1990), *PUP2* (Georgatsou et al., 1992), "Y7", and "Y13" (Emori et al., 1991)] and seven β-type genes [*PRE1* (Heinemeyer et al., 1991), *PRE2* (Heinemeyer et al., 1993), *PRE3* (Enenkel et al., 1994), *PRE4* (Hilt et al., 1993), *PRS3* (Lee et al., 1992), *PUP1* (Haffter & Fox, 1991), and the originally unidentified putative proteasomal gene (Basile et al., 1992) here referred to as *PUP3*, coding for a protein with striking homology to the recently characterized rat protesomal subunit RC10II (Nishimura et al., 1993b) and peptide sequences derived from the bovine proteasomal subunit θ (Dick et al., 1992)]. When the primary structures of all known eukaryotic proteasomal proteins were compared with each other, each of the 12 yeast subunits fell into a separate of a total of 14 subgroups. The existence of two α-type subgroups being devoid of a yeast member, as well as knowledge about the architecture of the archaebacterial "urproteasome" (Pühler et al., 1992) consisting of two halves of each seven α- and β-subunits, and the appearance of 14 protein spots after two-dimensional separation of purified, dissociated yeast proteasome (Heinemeyer et al., 1991) led to the postulation that two yet undetected yeast α-type proteasomal genes must exist.

This assumption was confirmed by sequencing of tryptic peptides derived from purified yeast proteasomal subunits (Tröndle, 1991): Among the nine proteins examined, two

gave rise to sequences, which could not be assigned to any of the 12 known yeast proteasomal subunits. However, sequence similarities in these peptides with members of the two α-type subgroups containing no yeast homologue were obvious. Starting from the peptide sequence information, we cloned the corresponding genes by a reversed genetical approach including a PCR step.

For cloning of the *PRE5* gene, sequences of two peptides were available. Their positions in the Pre5 protein were predicted by their similarity to parts of the *Drosophila* PROS35 (Haass et al., 1989; Frentzel et al., 1992b) and the mammalian C2 (Fujiwara et al., 1989; Tamura et al., 1991; Silva Pereira et al., 1992) subunits. A PCR using degenerate primer mixtures deduced from the two yeast peptides and total yeast DNA as template resulted in amplification of a product with the expected length of about 280 bp coding for a protein sequence highly homologous to regions from the C-termini of PROS35 and C2. With this PCR fragment as probe, the full length *PRE5* gene was identified on plasmids from a yeast genomic library (Rose et al., 1987) by colony hybridization. Sequencing the part of the genomic insert containing the hybridizing DNA revealed a continuous open reading frame capable of coding for a 234-amino acid protein with a predicted molecular mass of 25.6 kDa and a p*I* of 7.30. The *PRE5* gene product possesses all the conserved domains characteristic for α-type proteasomal subunits (Haass et al., 1990b) and shows over its entire length striking homology to the *Drosophila* PROS35 (Haass et al., 1989; Frentzel et al., 1992b), the human C2 (Tamura et al., 1991; Silva Pereira et al., 1992), and the rat C2 (Fujiwara et al., 1989) as well as the recently discovered *Arabidopsis* PMS30 (Shirley & Goodmann, 1993) subunits (47.4, 52.6, 53.0, and 50.4% identity, respectively; Figure 2A). Remarkably, the yeast protein lacks a C-terminal extension present in all four of its homologues, in which there is divergence in amino acid sequence but uniformity in the high content of charged amino acids. The C-terminal extension of the rat C2 subunit was recently shown to be partially cleaved off by limited proteolysis leading to a conversion of the proteasome from its latent into its active form (Arribas et al., 1994). This possible regulation mechanism may not be realized in the yeast proteasome and may have been acquired by higher eukaryotes later during evolution.

The *PRE5* gene was localized on chromosome XIII close to the right telomere. In databases, no sequences overlapping with the *PRE5* locus were found.

For cloning of *PRE6*, also two peptide sequences from a purified yeast proteasome subunit (Tröndle, 1991) were available. They exhibited strong homology to parts of the *Drosophila* PROS28.1 (Haass et al., 1990b; Frentzel et al., 1992b), the *Arabidopsis* TAS-g64 (Genschick et al., 1992), and the *Dictyostelium* DD5 (Schauer et al., 1993) subunits. In these proteins, the two regions similar to the yeast peptide sequences were located adjacent to each other. Thus, a PCR was performed with degenerate primer mixtures, of which only one was deduced from one of the yeast peptides, whereas the second mixture was deduced from a strongly conserved N-terminal region of the putative Pre6p homologues. This strategy resulted in amplification of a 310 bp fragment, which after sequence determination indeed turned out to code for a protein sequence very similar to a N-terminal part of each of the three proteasomal proteins predicted to be homologues of Pre6p. The complete *PRE6* gene was again identified by hybridization of the PCR product with a yeast genomic library (Rose et al., 1987). It codes for a 254-amino acid protein of 28.4 kDa with a predicted p*I* of 7.27. Pre6p also represents a typical α-type subunit (Haass et al., 1990b). Its strong relationship to the *Drosophila* PROS28.1 (Haass et al., 1990b; Frentzel et al., 1992b), the *Arabidopsis* TAS-g64 (Genschick et al., 1992), and the *Dictyostelium* DD5 (Schauer et al., 1993) proteasomal subunits (51.6, 56.3, and 57.9% identity, respectively; Figure 2B) is visible over its entire length, which differs only slightly from that of its homologues. Clustering of charged amino acids is found in the C-termini of all four members of the Pre6p subfamily of proteasomal proteins.

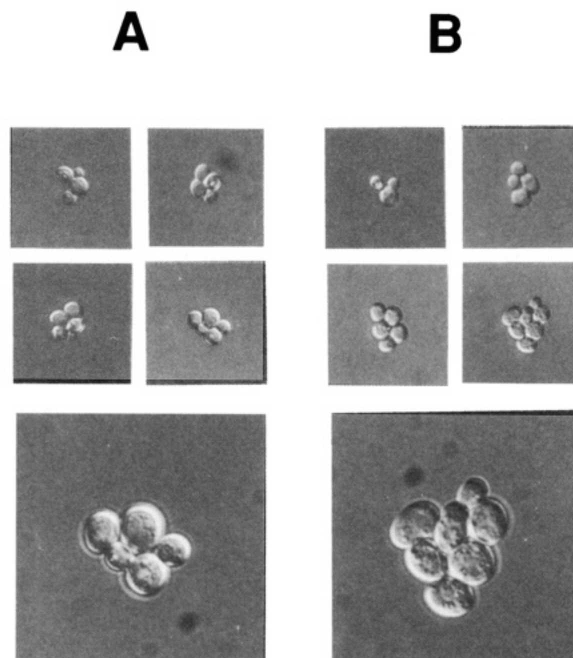

FIGURE 3: Photographs of microcolonies formed from spores bearing the *pre5* or *pre6* deletion alleles. Four examples of colonies formed from *pre5*Δ::*HIS3* spores (panel A) or *pre6*Δ::*HIS3* spores (panel B), respectively, are shown (top). For each case one colony is shown in higher magnification (bottom).

The *PRE6* gene was found to reside on the left arm of chromosome XV. A sequence overlap of the 5′-noncoding region of *PRE6* with two genes coding for ribosomal subunits [S21 (Takasawa et al., 1992) and L44 (Remacha et al., 1988); Figure 1B] was found in a database search.

Chromosomal Deletion of PRE5 and PRE6. With the single exception of the gene encoding the Y13 subunit of the yeast proteasome (Emori et al., 1991), deletions of proteasomal genes are lethal for the yeast cell (Heinemeyer et al., 1991, 1993; Hilt et al., 1993; Enenkel et al., 1994; Fujiwara et al., 1990; Emori et al., 1991; Haffter & Fox, 1991; Georgatsou et al., 1992; Lee et al., 1992; Basile et al., 1992). To investigate the effect of a *pre5* or *pre6* null allele, we heterozygously replaced the respective wild type gene with a deletion construct (marked with the selectable *HIS3* marker gene; Figure 1A,B) each in a diploid wild type strain. Sporulation and tetrad analysis showed that in each case only the histidine auxotrophic spore clones were viable. Thus, like the majority of yeast proteasomal genes, *PRE5* and *PRE6* are essential for cell proliferation.

As observed for *pre1*, *pre2*, *pre3*, and *pre4* null mutant spores (Heinemeyer et al., 1991, 1993; Hilt et al., 1993; Enenkel et al., 1994), spores deleted in *PRE5* and *PRE6* were able to germinate and undergo few cell divisions. Depending on the time of sporulation, between two and four cell divisions were observed. Microcolonies with up to 16 cells were formed after a 2 day sporulation time, whereas no more than eight

Table 1: The 14 Yeast Proteasomal Genes and Properties of Their Encoded Proteins

gene (other names)	type	chromosomal location	predicted protein[a] mol mass (kDa)	predicted protein[a] p*I*	motifs	correlation with proteolytic activity
PRS1 (*YC-1*[b])	α	XV	31.6	5.20	–	–
PRS2 (*SCL1*,[c] *YC-7α*,[b] *Y8*[d])	α	VII	28.0	6.05	NLS1	–
"Y7"	α	nd[g]	27.2	5.54	2 × cAMP-PS	–
"Y13"	α	nd	28.7	4.96	2 × cAMP-PS, Tyr-PS	–
PUP2	α	nd	28.6	4.58	cAMP-PS	–
PRE5	α	XIII	25.6	7.30	Tyr-PS	–
PRE6	α	XV	28.4	7.27	cAMP-PS, Tyr-PS, NLS1	–
PRE1	β	V[f]	22.5	6.01	NLS2	chymotryptic
PRE2 (*PRG1*[e])	β	XVI	31.6 (23.3)	6.02 (6.19)	Tyr-PS	chymotryptic
PRE3	β	X	21.2	5.37	Gln-synthetase	PGPH
PRE4	β	VI	29.4 (25.1)	5.75 (5.84)	Tyr-PS, NLS1	PGPH
PRS3	β	II	27.1 (25.1)	6.11 (6.86)	cAMP-PS	–
PUP1	β	XV	28.3 (25.2)	6.60 (6.53)	–	–
PUP3	β	V	22.6	4.93	–	tryptic?

[a] The molecular mass and p*I* values were calculated for the longest predictable translation products of the respective open reading frames. Values in brackets correspond to putative processed forms predicted from sequences of N-terminal peptides of homologous proteins from other species (Lee et al., 1990; Lilley et al., 1990; Schauer et al., 1993). Except for NLS1, the motifs listed were found via search in the "PROSITE" Database (Bairoch, 1990). Abbreviations: cAMP-PS, cAMP-dependent phosphorylation site; Tyr-PS, tyrosine phosphorylation site; NLS1, nuclear localization signals of the SV 40 large T antigen type; NLS2, bipartite nuclear localization site; Gln-synthetase, glutamine synthetase consensus sequence; PGPH, peptidyl-glutamyl-peptide hydrolyzing. [b] Fujiwara et al. (1990). [c] Balzi et al. (1989). [d] Emori et al. (1991). [e] Friedman et al. (1992). [f] Heinemeyer, unpublished. [g] nd, not determined.

cells were found after longer times of sporulation. Figure 3 shows representative examples for microcolonies of *pre5*Δ and *pre6*Δ spores after a 4 day sporulation period.

DISCUSSION

Identification of *PRE5* and *PRE6* raises the number of known yeast proteasomal proteins to 14, which can clearly be subdivided into seven α- and seven β-type subunits. A summary of relevant properties of the 14 genes and their protein products is given in Table 1. The available mapping data point to a random distribution of the 14 genes over the yeast genome, with an accumulation of three genes on chromosome XV. Nothing is known about mechanisms guaranteeing a coordinate expression of the proteasomal genes. As found for the majority of genes in *S. cerevisiae*, all 14 genes lack introns. The molecular masses of the predicted translation products range from 21.2 to 31.6 kDa, which is in agreement with the estimated sizes of the protein bands after SDS–PAGE of purified yeast proteasome (Tanaka et al., 1988, 1989; Kleinschmidt et al., 1988). Processing of yeast β-type subunits by cleavage at their N-termini has not been documented so far, but this could be expected from data indicating processing of β-type proteasomal subunits of higher eukaryotes (Lee et al., 1990; Lilley et al., 1990; Glynne et al., 1993; Frentzel et al., 1993) as well as of the archaebacterial β-subunit (Zwickl et al., 1992). Putative nuclear localization signals are found in the Pre1, Pre4, Prs2, and Pre6 protein sequences, and potential phosphorylation sites for tyrosine and cAMP-dependent threonine/serine kinases are found in several predominantly α-type subunits. The biological significance of these motifs, however, remains to be established. The primary structure of none of the 14 yeast proteasomal proteins allows an identification of possible proteolytically active sites when searching for homologies of these subunits with any known type of protease. Genetic data point to the participation of certain β-type subunits in the generation of proteolytic activity of the 20S proteasome. Intact Pre1p and Pre2p are necessary for the chymotrypsin-like activity (Heinemeyer et al., 1991, 1993), and intact Pre3p and Pre4p are necessary for the peptidyl-glutamyl-peptide-hydrolyzing activity (Hilt et al., 1993; Enenkel et al., 1994). Pup3p may participate in the trypsin-like activity of the enzyme complex as it contains a cysteine residue, which in the bovine Pup3p homologue named θ was shown to be accessible to the trypsin protease inhibitor leupeptin (Dick et al., 1992). The correlation of five β-type subunits with proteolytic activities favors the hypothesis of the active sites residing within these subunits, which, in analogy to the localization of the β-subunit in the archaebacterial proteasome (Pühler et al., 1992), are most probably located in the inner two rings of the proteasome particle.

Several reasons lead us to assume that the now known 14 subunits represent the complete set of proteasomal proteins in yeast: (i) the number "14" nicely reflects the 2-fold $\alpha_7\beta_7$ symmetry of the archaebacterial "urproteasome" (Pühler et al., 1992), (ii) two-dimensional separation of purified and dissociated yeast proteasome results in exactly 14 protein spots (Heinemeyer et al., 1991), and (iii) the comparison of the 14 yeast subunit sequences with the primary structures of all so far known proteasomal subunits from other species leads to a dendrogram (Figure 4) comprising 14 subgroups, each containing a single yeast member. With one exception, the Y13 subunit (Emori et al., 1991), all known yeast proteasomal proteins are indispensable for life, indicating that at least for 13 subunits there exist no homologues which could substitute for their essential functions. However, the existence of an additional yet undetected 15th subunit, which is able to take over the role of the Y13 component, cannot completely be excluded.

Regarding the dendrogram in Figure 4, we may assume that the composition of 14 subunit types proposed here for the yeast proteasome most probably applies in principle to all proteasomes from higher eukaryotes. The occurrence of more than 14 protein spots on two-dimensional gel separations of plant, insect, or mammalian proteasomes may partly be due to artificial proteolysis of some proteasome components during purification and storage of the complex. However, the following possibilities are more likely to explain the observed high complexity in the subunit patterns: First, in mammals, the MHC class II-encoded LMP2 and LMP7 subunits, both not essential for cellular life, seem to represent unique cases of specialized proteasome subunits, which after overexpression substitute for strongly related, constitutive subunits of the proteasome which serve housekeeping functions (Gaczynska et al., 1993; Driscoll et al., 1993; Boes et al., 1994; Aki et al., 1994). Hence, in the subgroup containing the mammalian

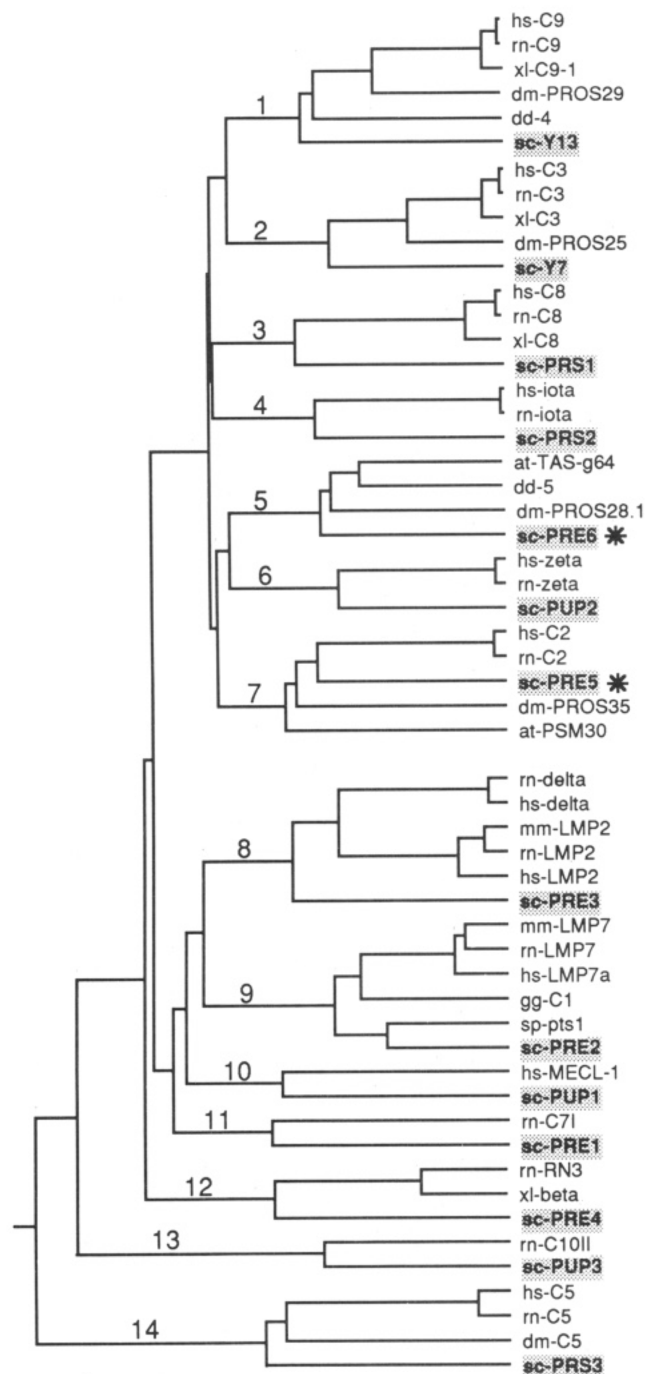

FIGURE 4: Dendrogram showing the relationships among all known eukaryotic 20S proteasome protein sequences. The 14 *S. cerevisiae* proteasome subunits are shown in bold type on shaded background; the Pre5 and Pre6 proteins are highlighted by asterisks. The 14 main branches are numbered. In cases of subunits, for which slightly differing sequences have been reported, only one sequence is considered. Abbreviations for species are at, *A. thaliana*; dd, *D. discoideum*; dm, *Dr. melanogaster*; gg, *Gallus gallus*; hs, *Homo sapiens*; mm, *Mus musculus*; rn, *Rattus norvegicus*; sc, *S. cerevisiae*; sp, *Schizosaccharomyces pombe*; xl, *Xenopus laevis*. Sequences are taken from the following sources: at-PSM30 (Shirley & Goodman, 1993), at-TAS-g64 (Genschick et al., 1992), dd-4 and dd-5 (Schauer et al., 1993), dm-PROS25 (Seelig et al., 1993a), dm-PROS28.1 (Haass et al., 1990b), dm-PROS29 (Haass et al., 1990a), dm-PROS35 (Haass et al., 1989), dm-C5 (Saville & Belote, 1993), gg-C1 (A. Shiratsuchi and S. Sato, unpublished; EMBL Data Base accession number X57210), hs-C2, hs-C3, hs-C5, hs-C8, and hs-C9 (Tamura et al., 1991), hs-ι, hs-ζ and hs-δ (DeMartino et al., 1991), hs-LMP2 (Kelly et al., 1991), hs-LMP7a (Glynne et al., 1993), hs-MECL-1 (Larsen et al., 1993), mm-LMP2 (Martinez & Monaco, 1991), mm-LMP7 (Frentzel et al., 1992), rn-C2 (Fujiwara et al., 1989), rn-C3 (Tanaka et al., 1990a), rn-C5 (Tamura et al., 1990), rn-C8 (Tanaka et al., 1990b), rn-C9 (Kumatori et al., 1990), rn-ι, rn-ζ, and rn-LMP2 (Tamura et al., 1992), rn-LMP7 (Aki et al., 1992), rn-RN3 (Thomson et al., 1993), rn-C7I (Nishimura et al., 1993a), rn-C10II (Nishimura et al., 1993b), sc-Y7 and sc-Y13 (Emori et al., 1991), sc-PRS1 and sc-PRS2 (Fujiwara et al., 1990), sc-PUP2 (Georgatsou et al., 1992), sc-PRE3 (Enenkel et al., 1994), sc-PRE2 (Heinemeyer et al., 1993), sc-PUP1 (Haffter & Fox, 1991), sc-PRE1 (Heinemeyer et al., 1991), sc-PRE4 (Hilt et al., 1993), sc-PUP3 (Basile et al., 1992), sc-PRS3 (Lee et al., 1992), sp-pts1 (Stone, Tanaka, Ichihara, Goebl, and Yanagida, unpublished; EMBL Data Base accession number D13094), xl-C3 (Fujii et al., 1991), xl-C8 and xl-C9-1 (Fujii et al., 1993), and xl-β (Van Riel & Martens, 1991). The dendrogram was created by the CLUSTAL multiple sequence alignment program (Higgins & Sharp, 1988) using the DNASTAR "Lasergene" software.

LMP2 protein, one finds another mammalian subunit, called δ (DeMartino et al., 1991; Tamura et al., 1992). The LMP7-containing group, accordingly, is expected to include another mammalian subunit, which may be subunit ϵ, for which only partial peptide sequences are available up to now (Lee et al., 1990; Lilley et al., 1990). Besides this unique case in mammals, a divergence in proteasome species can be caused, for instance, by duplications of given proteasomal genes or by alternative splicing, resulting in multiple, highly homologous isoforms of a subunit. Several examples for pairs of equivalent subunits differing in only few amino acids exist; two cDNAs each were identified for the human ι/*Hs*PROS27 subunit (DeMartino et al., 1991; Bey et al., 1993), the human C2/*Hs*PROS30 subunit (Tamura et al., 1991; Silva Pereira et al., 1992), and the *Xenopus* C9 protein (Fujii et al., 1993). Additionally, N-terminal amino acid sequences of human and rat β-type proteasome subunits (Lee et al., 1990; Lilley et al., 1990) differ in few positions from the respective parts of corresponding full length sequences derived from cDNA cloning. In agreement with this is the viability of an *Arabidopsis* cell line lacking the gene encoding the Pre5p homologue PSM30 (Shirley & Goodman, 1993): here, evidence exists for a strongly related second gene which may take over the function of PSM30.

Besides gene duplications and alternative splicing, post-translational modifications of proteasomal subunits may account for the observed high number of electrophoretically distinguishable subunits. Reports on glycosylation (Tomek et al., 1988) and phosphorylation (Haass & Kloetzel, 1989; Arrigo & Mehlen, 1993) of subunits favor this idea. Data of Hendil and co-workers (1994) clearly show that the up to 20 spots found in human placenta proteasomes separated by two-dimensional gel electrophoresis can be attributed to 16 different proteins (two of them existing in three different phosphorylation states each). Among the 16 proteins are LMP2 and LMP7. Taking into account the presence of their housekeeping homologues of equivalent function, composition of human placenta proteasomes of 14 different subunits can be calculated.

Individual subunits which were placed in a common subgroup of the dendrogram (Figure 4) are supposed to serve equivalent functions in the assembly and activity of the proteasome from the respective species. This is supported by experiments of Seelig et al. (1993b), which demonstrate the successful substitution of the murine proteasomal C3 component by the homologous *Drosophila* PROS25 subunit.

Taken together, a symmetrical structure containing two sets of 14 subunit types each in defined positions is easily imaginable as the common rule for the 20S proteasome particle. Development or tissue specific variations among proteasome species or even a diversity within one cell is not excluded by this rule. Subunit substitutions and modifications may be a widespread mechanism in higher eukaryotes to modulate the

properties of the 20S proteasome with respect to proteolytic activity and specificity.

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