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cDNA Cloning of p112, the Largest Regulatory Subunit of the Human 26S Proteasome, and Functional Analysis of Its Yeast Homologue, Sen3p

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The 26S proteasome is a large multisubunit protease complex, the largest regulatory subunit of which is a component named p112. Molecular cloning of cDNA encoding human p112 revealed a polypeptide predicted to have 953 amino acid residues and a molecular mass of 105,865. The human p112 gene was mapped to the q37.1–q37.2 region of chromosome 2. Computer analysis showed that p112 has strong similarity to the *Saccharomyces cerevisiae* Sen3p, which has been listed in a gene bank as a factor affecting tRNA splicing endonuclease. The *SEN3* also was identified in a synthetic lethal screen with the *nin1–1* mutant, a temperature-sensitive mutant of *NIN1*. *NIN1* encodes p31, another regulatory subunit of the 26S proteasome, which is necessary for activation of Cdc28p kinase. Disruption of the *SEN3* did not affect cell viability, but led to temperature-sensitive growth. The human p112 cDNA suppressed the growth defect at high temperature in a *SEN3* disruptant, indicating that p112 is a functional homologue of the yeast Sen3p. Maintenance of *SEN3* disruptant cells at the restrictive temperature resulted in a variety of cellular dysfunctions, including defects in proteolysis mediated by the ubiquitin pathway, in the N-end rule system, in the stress response upon cadmium exposure, and in nuclear protein transportation. The functional abnormality induced by *SEN3* disruption differs considerably from various phenotypes shown by the *nin1–1* mutation, suggesting that these two regulatory subunits of the 26S proteasome play distinct roles in the various processes mediated by the 26S proteasome.

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Note: The nucleotide sequence data reported in this paper will appear in the GSDB, DDBJ, EMBL, and NCBI Nucleotide Sequence Databases with the following accession number: D44466.

INTRODUCTION

The 26S proteasome is a major ATP-dependent intracellular protease involved in extra-lysosomal proteolytic pathways responsible for selective elimination of various types of cellular proteins, including naturally occurring short-lived proteins related to cell cycle progression and metabolic regulation (Ciechanover, 1994; Hochstrasser, 1995). It also rapidly degrades potentially harmful proteins with aberrant structures, such as misfolded and unfolded proteins generated by genetic mutations or induced by various stresses (Goldberg, 1992; Hershko and Ciechanover, 1992). The 26S proteasome catalyzes the ATP-dependent degradation of proteins marked for degradation by the addition of multiubiquitin chains (Hershko and Ciechanover, 1992; Rechsteiner *et al.*, 1993; Peters *et al.*, 1994), as well as some nonubiquitinated proteins such as ornithine decarboxylase (Murakami *et al.*, 1992) and c-Jun (Jariel-Encontre *et al.*, 1995). c-Jun, however, has also been reported to be degraded by the ubiquitin pathway (Treier *et al.*, 1994). The proteasome-ubiquitin system is also responsible for generating antigenic peptides for presentation on the cell surface by class I proteins of the major histocompatibility complex (Michalek *et al.*, 1993; Rock *et al.*, 1994; Heemels and Ploegh, 1995), and for the conversion of p110 precursor of NF-κB to its mature form, p50 (Palombella *et al.*, 1994). Thus the 26S proteasome is involved in a number of important cellular processes.

The 26S proteasome is a large multisubunit complex with a molecular weight of 2,000,000 (Yoshimura *et al.*, 1993; Peters, 1994). It is composed of two large subcomplexes with distinct sedimentation coefficients of approximately 20S and 19S/22S, respectively (Lupas *et al.*, 1993; Tanahashi *et al.*, 1993). The former subcomplex, called the 20S proteasome, has proteolytic functions. It is composed of 28 subunits with molecular masses of 21–32 kDa arranged in a cylinder-shaped particle. The latter sub-complex, which has variously been termed "ball" (Hoffman *et al.*, 1992), μ particle (Udvardy, 1993), PA700 (DeMartino *et al.*, 1994), or 19S cap (Peters *et al.*, 1994), has regulatory functions and is comprised of approximately 20 subunits with masses of 25–112 kDa. The organization of the 26S proteasome consists of a central 20S proteasome and two PA700 sub-complexes attached to each of the terminal rings of the proteasome (Lupas *et al.*, 1993; Tanahashi *et al.*, 1993). The primary structures of all the subunits of the 20S proteasome have been determined for several eukaryotic species and in each of these species the subunits comprise a family of 14 or more homologous gene products that have been conserved during evolution (Hilt and Wolf, 1995; Tanaka, 1995). In contrast, much less is known about the structure and functions of the PA700 complex of the 26S proteasome. Recent work from several laboratories has established that at least six subunits of PA700 regulatory complex are members of a highly conserved protein family with a consensus sequence for ATP-binding (Dubiel *et al.*, 1995; Tanaka, 1995; DeMartino *et al.*, 1996). These subunits may catalyze the ATP hydrolysis required for assembly and/or function of the 26S proteasome. These proteins belong to an expanding family of putative ATPases that are known to be involved in diverse processes, including vesicle-mediated transport, peroxisome assembly, cell cycle control, and gene expression. The ATPase family has been designated the AAA-family (ATPases Associated with a variety of cellular Activities) (for review, see Confalonieri and Duguet, 1995). The PA700 regulatory complex probably has multiple functions and enzymatic activities. For example, a de-ubiquitinating activity, which is required to release the ubiquitin moiety from ubiquitin-ligated proteins for re-utilization in the proteolytic process, appears to be an integral part of the 26S proteasome (Eytan *et al.*, 1993), Moreover, *DOA4*, a gene required for degradation of yeast *MATα2* repressor, has been shown to be a member of a family of de-ubiquitinating enzymes and seems to be associated with the 26S proteasome (Para and Hochstrasser, 1993). Recently Deveraux *et al.* (1994) found a 50-kDa subunit, named S5a, that can bind specifically to proteins conjugated to a multiubiquitin chain. This subunit probably functions as a ubiquitin receptor used by the 26S proteasome to trap ubiquitinated proteins for degradation (Deveraux *et al.*, 1995). In addition, PA700 has also been postulated to have a protein-unfoldase activity that disrupts the three-dimensional structure of protein substrates such that they can gain access to the cavity in the proteasomal complex (Löwe *et al.*, 1995) where protease catalytic sites reside (Lupas *et al.*, 1993; Rubin and Finley, 1995). However, the structural bases of these possible functions have not yet been clarified.

To date over 10 proteins unrelated to members of the ATPase family have been reported to be subunits of the PA700 complex (DeMartino *et al.*, 1994; Dubiel *et al.*, 1995), but little is yet known about their primary structures. Recently we reported cDNA cloning of p40, one regulatory subunit, of human 26S proteasome (Tsurumi *et al.*, 1995), which is equivalent to the S12 subunit designated by Dubiel *et al.* (1995). Interestingly, human p40 is a homologue of previously described products of the *Mov-34* gene from mouse and *Drosophila*. Disruption of this gene by proviral integration induces recessive embryonic lethality in mice (Grindley *et al.*, 1990). In addition, an *Arabidopsis* ubiquitin recognition component of the 26S proteasome, named MBP1 (van Nocker *et al.*, 1996), and its *Drosophila* homologue (Haracska and Udvardy, 1995) have been cloned. Very recently, we have cloned another cDNA encoding p31, another regulatory subunit of the human 26S proteasome (Kominami *et al.*, 1995).

p31 was found to be a homologue of yeast Nin1p, which is necessary for activation of Cdc28p kinase of *Saccharomyces cerevisiae* and is involved in the G_1/S and G_2/M transitions of the cell cycle (Kominami *et al.*, 1995). We are now determining the structures of the remaining non-ATPase components by cDNA cloning. We report here the molecular cloning of cDNA encoding p112, the largest regulatory subunit of the human 26S proteasome, and functional analysis of its homologue Sen3p, in budding yeast.

During the preparation of this manuscript, DeMarini *et al.* (1995) reported the primary structure of Sen3p and a functional analysis of the *SEN3* gene in yeast. Their results are in excellent accord with those described here. Combined with the additional data on the human p112, these results provide a basis for the role of p112/sen3p in protein degradation.

MATERIALS AND METHODS

Biochemical Analysis

The purification of the PA700 regulatory subunit complex from rat liver will be described elsewhere (Tanahashi *et al.*, 1993). Subunit p112 was isolated from purified bovine PA700 and the amino acid sequences of its fragments were described previously (DeMartino *et al.*, 1994; Ma *et al.*, 1994). Preparation of extracts was performed essentially as described (Kominami *et al.*, 1995). The assays of peptidase activities were described previously (Tanaka *et al.*, 1988).

Preparation of Total RNA and Poly(A)$^+$ RNA

Total RNA was extracted by the guanidinium thiocyanate method as described (Chirgwin *et al.*, 1979), and poly(A)$^+$RNA was isolated by oligo(dT)$_{30}$-latex (Oligotex-dT$_{30}$, Takara Shuzu).

Isolation of a cDNA Clone and DNA Sequencing

A cDNA library was constructed from the poly(A)$^+$RNA of human hepatoblastoma HepG2 cells. cDNA was synthesized with a cDNA synthesis kit (Pharmacia LKB Biotechnology, Uppsala, Sweden) using oligo(dT)$_{15}$ as a primer for synthesis of the first strand of cDNA. The cDNA were ligated with an *Eco*RI adapter containing the *Not*I site and then inserted at the *Eco*RI site of a ZAPII vector (Stratagene, La Jolla, CA) for the construction of the phage library. On the basis of partial amino acid sequences of p112, including the N-terminal amino acid sequence, the sequences of 10 tryptic peptides of purified bovine p112 (DeMartino *et al.* 1994), and four sequences produced by lysylendopeptidase digestion of purified rat p112 (see Figure 1), we designed oligonucleotides for amplification by polymerase chain reaction (PCR) with the first strand cDNA complementary to mRNA from rat liver. The largest PCR product, approximately 720 bp in length, was obtained using the following primers: forward primer, 5'AAT(T/C)T(T/G)TATCAGGATGATGCTGT(T/G)AC(T/G)TGAGG-3'; and reverse primer, 5'-CCAGCATC(A/C)AAAATACCCTGAGC(A/C)A(A/G)AAT(A/C)GC-3'. This DNA fragment was used as a probe to screen a cDNA library derived from human hepatoblastoma cell HepG2. cDNA clones were sequenced by a double-strand strategy in an automatic DNA sequencer (Pharmacia LKB Biotechnology). 5' and 3' deletion mutants were constructed to analyze internal sequences.

Northern Blot and Reverse Transcription (RT)-PCR Analyses

For RNA blot hybridization analysis, a Northern blot filter containing 2 μg of poly(A)$^+$ RNAs from various human tissues (Clontech, Cambridge, UK) was hybridized with ^{32}P-labeled probes as described previously (Tanaka *et al.*, 1990). cDNAs for p112, X and Y of the human 20S proteasome (Akiyama *et al.*, 1994), EF1α (a gift from Dr. S. Nagata, Osaka Bioscience Institute), β-actin (Oncor), and glycerol-3-phosphate dehydrogenase (G3PDH), were synthesized by PCR. For RT-PCR analysis, single-stranded cDNAs were synthesized with poly(A)$^+$ RNA from human kidney and HepG2 cells, and p112 cDNA fragments were amplified by the PCR technique. Primers were designed at 74 to 93 bp upstream and 82 to 101 bp downstream from the position of the 170 bp insertion/deletion described in the text.

Chromosomal Localization by Direct R-Band FISH

We used a cDNA clone of the cDNA encoding the human p112 described in this article as a probe. This clone contains a 3.0-kb insert in pBluescript SK$^-$. A direct R-banding fluorescence in situ hybridization (FISH), which is based on FISH combined with replicated prometaphase R-bands, was applied (Takahashi *et al.*, 1991b). For the amplification of the signals of this clone, we used the method reported by Viegas-Pequignot *et al.* (1989) with slight modifications (Takahashi *et al.*, 1991a). The procedures of labeling, hybridization, rinsing, and detection were carried out in a routine manner. Prvia100 film (Fuji, ISO100) was used for the microphotography.

Strains, Plasmids, and Media

Principal yeast strains and plasmids used in this study are listed in Table 1. Other strains and plasmids derived from those listed in Table 1 were also used as described in the text. TM1–4B [pDL120] cells were mutagenized with 3% ethylmethanesulfonate at room temperature for 60 min as described (Fink, 1970). Survival was approximately 30% under these conditions. *URA3* of TM1–4B[pDL120] was disrupted, when necessary, to obtain a host (TM1–4B[pDL120-*ura3*]) that could be selected for Ura$^+$ transformants with YCUp4-based gene library (Fujita, unpublished data). TM1–4B[pHN19] is a strain that was constructed by replacing pDL120 of TM1–4B[pDL120] by pHN19, whose *NIN1* gene had been placed under the control of the *GAL1* promoter (Nisogi *et al.*, 1992). Growth of TM1–4B[pHN19] is dependent on galactose. YPD, SD, and sporulation medium were prepared as described by Sherman (1991). SC is SD supplemented with auxotrophic requirements. YPGal was prepared by replacing glucose in YPD with 5% galactose and 0.3% sucrose. Fluoro orotic acid medium was described previously (Boeke *et al.*, 1984) and used for scoring the Ura3 phenotype; Ura3$^+$ strains cannot grow on this medium.

Plasmid TOp59 possesses the *TDH3* promoter, *URA3*, and 2 μm ori. A unique *Pvu*II site is situated at the 3' side of the *TDH3* promoter, which can be used for ligation of a foreign gene to be expressed in yeast. TOp59 was linearized by cutting with *Pvu*II. A unique *Hin*dIII site was inserted at the 5' side close to the initiation codon, ^{1}ATG^3, of the human p112-L cDNA by PCR. A *Hin*dIII fragment of p112-L cDNA was blunt-ended by the Klenow fragment of *Escherichia coli* DNA polymerase I and four dNTPs. The p112 fragment thus obtained was ligated with TOp59 linearized by *Pvu*II. Plasmid containing the *TDH3* promoter joined with p112 cDNA in the right orientation was screened, confirmed by restriction analysis, and designated pAT540.

Genetic Methods in S. cerevisiae

Standard methods for yeast genetics were described previously (Sherman, 1991). Transformation experiments were carried out as described by Ito *et al.* (1983).

Immunological Analysis

Proteins separated by SDS-PAGE were transferred to nitrocellulose filter (Advantec Toyo, Tokyo) by Sartoblot (Sartorius). Then the

Table 1. Yeast strains and plasmids

Strain	Relevant genotype	Source or reference
W303D	*MATa/MATα Leu2/- his3/- trp1/- Ura3/- ade2/-*	Sutton *et al.* (1991)
YK109	*MATa nin1-1 leu2 his3 trp1 ura3 ade1*	Kominami and Toh-e (1994)
W1710-1B	*MATα nin1-1 leu2 his3 ura3 trp1 ade*	Our stock
TM1-4B	*MATa nin1-1 ade2 ade3 leu2 trp1 ura3*	Our stock
W1646-1C	*MATa leu2 his3 trp1 ura3 ade2 sen3::URA3*	A meiotic segregant of W303D sen3::URA3
W1646-1D	*MATα leu2 his3 trp1 ura3 ade2 sen3::URA3*	A meiotic sister of W1646-1C
YAT1886	*MATa leu2 his3 trp1 ura3 ade2 sen3::LEU2*	URA3 of W1646-1C was replaced by LEU2
YAT2011	*MATα leu2 his3 trp1 ura3 ade sen3::URA3 nin1-1*	A segregant from a cross between W1646-1D and YK109

Plasmid	Type of vector	Relevant character	Source or reference
YEp24	YEp	*URA3 2μ ori Ap^r*	Carlson and Botstein (1982)
pHN4	YCp	*NIN1 URA3 TRP1 ARS1 CEN4 Ap^r*	Nisogi *et al.* (1992)
pHN19	YEp	*P_{GAL1}-NIN1 LEU2 2μ ori Ap^r*	Nisogi *et al.* (1992)
pDL120	YCp	*NIN1 ADE3 URA3 ARS1 CEN4 Ap^r*	This study
pAT540	YEp	*P_{TDH3}-p112-L URA3 2μ ori Ap^r*	This study
TOp59	YEp	*P_{TDH3} URA3 2μ ori Ap^r*	This study
YCUp4	YCp	*URA3 ARS1 CEN4 Ap^r*	Fujita (MITI)
YCUp-SEN3	YCp	*SEN3 URA3 ARS1 CEN4 Ap^r*	This study
Ub-X-lacZ#	YEp	*P_{GAL1}-Ub-X-lacZ URA3 2μ ori Ap^r*	Bachmair *et al.* (1986)
pTG102*	YEp	*P_{GAL1}-NLS-lacZ URA3 2μ ori Ap^r*	This study

#, X is Arg, Leu, Tyr, or Ala.

*, NLS indicates the oligonucleotide encoding the nuclear localization signal of SV40 large T antigen.

filter was processed for Western blotting as recommended by the manufacturer.

Pulse-Chase Experiments

Wild-type and mutant strains carrying the respective ubiquitin-X-β-galactosidase–expressing plasmids, which were kindly supplied by Dr. A. Bachmair, were grown in SC-URA medium containing 2% galactose instead of glucose for 48 h at 25°C. Cells were labeled for 10 min with [^{35}S]methionine. After washing three times, the cells were chased for various times in the same medium supplemented with unlabeled methionine and cycloheximide. Preparation of cell extracts, immunoprecipitation of anti-β-galactosidase antibodies, SDS-PAGE, and fluorography were essentially carried out as described by Bachmair *et al.* (1986).

The Localization of a Nuclear-targeting Protein in Yeast Cells

A gene for MAKIPPKKKRKVLED-β-galactosidase (the underlined sequence corresponds to the nuclear localization signal of SV-40 large T antigen (Kalderson *et al.*, 1984) was constructed by inserting the oligonucleotide encoding this amino acid sequence into the *Bam*HI site of pLGSD5, a plasmid encoding *E. coli* β-galactosidase under *GAL1-GAL10* promoter (Guarente *et al.*, 1982). The resultant plasmid was designated pTG10. Yeast strains were transformed with the plasmid as described (Ito *et al.*, 1983). The transformants were cultured overnight at 30°C in a raffinose-medium containing 0.67% nitrogen base (Difco, Detroit, MI), 2% raffinose, 2% glycerol, 2% lactate, and 20 μg/ml of adenine sulfate, histidine, and tryptophan. The cells were then inoculated into raffinose-medium supplemented with 2% galactose, to a final cell density of about 0.5 OD600, and further cultured for 8 h at 30°C (since the expression of the fusion protein was poorly induced at 37°C in the mutant cells, the experiment was carried out at the semi-permissive temperature for cell growth). After being fixed with 4% formaldehyde in the culture medium for 1.5 h at 30°C, the cells were permeabilized and stained with anti-β-galactosidase antibody (Cappel, West Chester, PA) and fluorescein isothiocyanate-conjugated anti-IgG antibody, successively, according to the method of Kilmartin and Adams (1984). The subcellular localization of the fusion protein was monitored under immunofluorescence microscopy.

RESULTS

cDNA Cloning of p112, the Largest Regulatory Subunit of the Human 26S Proteasome

To isolate a cDNA clone for p112 of the 26S proteasome, about 5×10^5 plaques of a cDNA library derived from human hepatoblastoma cell HepG2 were screened by hybridization with a cDNA fragment that had been synthesized by PCR (for details, see MATERIALS AND METHODS). Eleven positive clones were obtained (frequency of 0.002%). During restriction enzyme mapping and partial sequencing of the cDNA clones, we found that they could be separated into two groups with almost identical nucleotide sequences except for the presence or absence of a 170-nucleotide insert in the coding region close to the N-terminus (see the arrow line in the cDNA structure shown in Figure 1A). The longest inserts representing the two types of cDNAs (about 3.0 kb in length including a poly(A) tail) were subjected to nucleotide sequencing. We named the larger cDNA p112-L and the smaller

p112-S. The structures of these two cDNAs are schematically shown in Figure 1A.

The p112-L clone contains a cDNA insert of 3176 nucleotides, including the entire coding region and 5′- and 3′-noncoding regions. The nucleotide sequence and the deduced amino acid sequence of this clone are shown in Figure 1B. On the basis of the previously identified N-terminal sequence of the p112 protein, we concluded that ATG, located at nucleotides 1 to 3, is the initiation codon. Subunit p112-L corresponds to a protein of 953 amino acids with a calculated molecular weight of 105,865. A putative polyadenylation signal (AATAAA), which is common to eukaryotic mRNAs, was located 29 nucleotides upstream from the poly(A) addition site. The amino acid sequences determined by automated Edman degradation of the intact p112 protein and of peptides from the bovine and rat proteins are present in the open reading frame deduced from the nucleotide sequence of the human cDNA (Figure 1B, continuous lines), indicating that the sequences are highly conserved between human, bovine, and rat, although there are several substitutions of amino acid residues (dotted lines). The isoelectric point (pI) of p112-L was calculated to be 5.12 by the method of Skoog and Wichman (1986).

The nucleotide sequence of the p112-S cDNA clone is identical to that of p112-L shown in Figure 1B, with the exception of a 170-bp deletion in the coding region between nucleotides 135 and 304 (shown as a box in Figure 1B). The p112-S clone contains an open reading frame of 2451 bp that encodes 817 amino acids with a calculated molecular weight of 90,512 and a calculated pI of 5.47. We concluded that the ATG located at nucleotides 409 to 411 of the p112-L cDNA is the initiation codon of p112-S, because a stop codon, TGA, was found approximately 200 bp downstream in frame with the ^{1}ATG^{3} of p112-L. Therefore the deduced amino acid sequence is identical to that of the p112-L clone except it lacks 136 amino acids in the N-terminal region because of the 170-bp deletion.

The C-terminal region extending from lysine 838 to glutamic acid 870 of p112-L contains a "KEKE" motif, a hydrophilic region rich in "alternating" positively (lysine) and negatively (glutamate) charged amino acids (Realini *et al.*, 1994b). This motif has been identified in a variety of proteins, including some 20S proteasome subunits, the α subunit proteasome activator, PA28 (Realini *et al.*, 1994a; Ahn *et al.*, 1995), and some other PA700 subunits including p40 (Tsurumi *et al.*, 1995). Although the significance of KEKE motifs is unknown, they have been proposed to promote the association between proteins in which they are found (Realini *et al.*, 1994b). A short KEKE-rich region also exists from lysine 821 to lysine 827. These two KEKE-sequence regions are doubly underlined in Figure 1B.

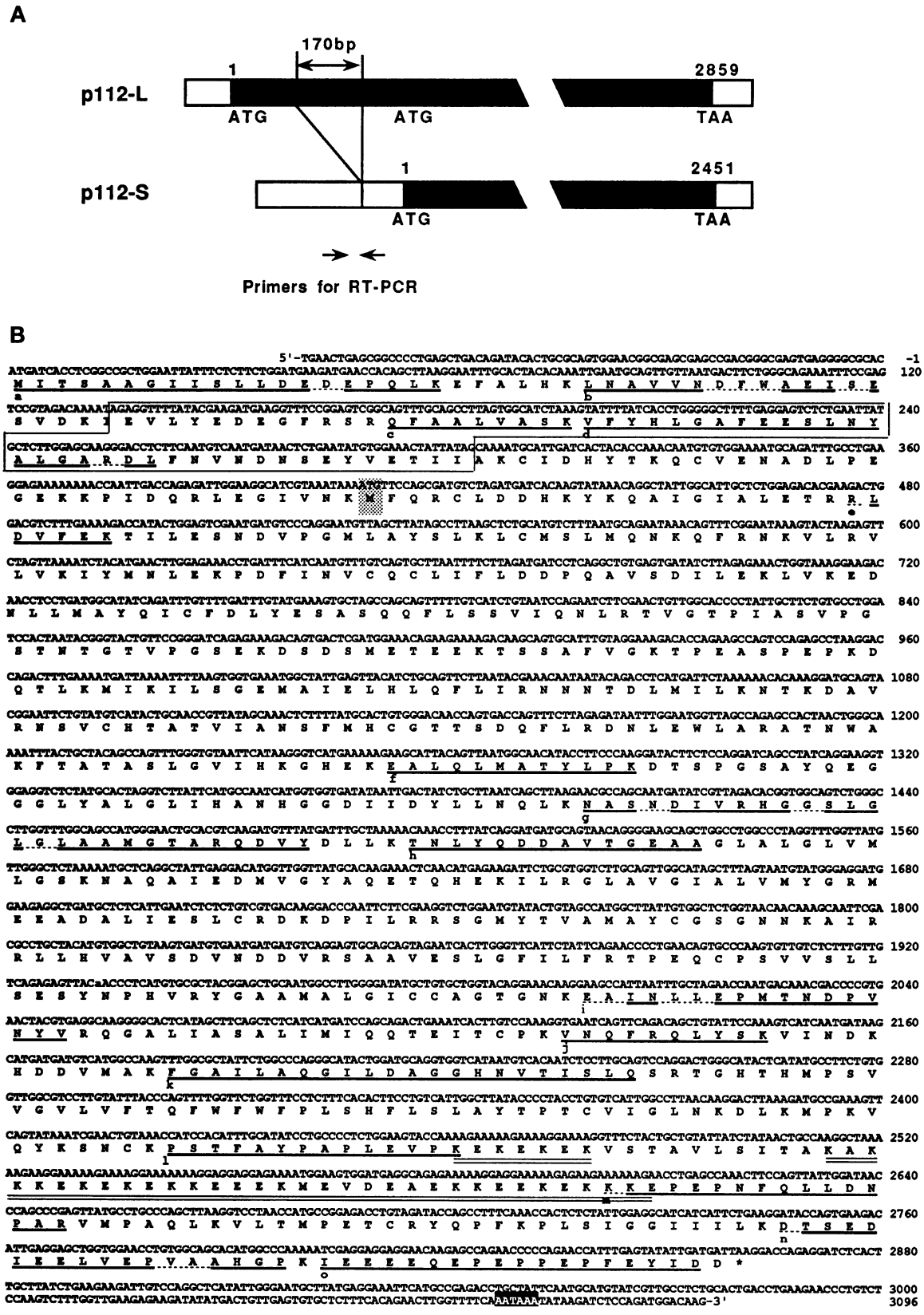
To determine the tissue specificity of p112 gene expression we examined the levels of p112 mRNA in various human tissues. On Northern blot analysis, one hybridization band of about 3.5 kb was observed in RNAs from various human tissues (Figure 2A). Although p112 mRNA was expressed in all tissues examined, it was particularly high in heart and skeletal muscle. The high level of mRNAs in these muscle tissues also was observed for the 20S proteasome subunits, X and Y (Tsurumi *et al.*, 1995). We also examined the mRNA levels of three other proteins: EF1α, G3PDH, and β-actin. The mRNA amounts of EF1α are apparently comparable in various human tissues. However, the mRNA levels of G3PDH and β-actin, like that of p112, were significantly higher in heart and skeletal muscle than in other examined tissues. Because Northern blot analysis cannot discriminate which type of p112 mRNA, namely p112-L and p112-S, is expressed, we performed RT-PCR analysis with primers corresponding to sequences upstream and downstream of the 170 bp-deletion (see Figure 1A). Figure 2B shows that two PCR products with expected sizes were detected with poly(A)$^{+}$ RNA from human renal carcinoma KPK13 cells and normal adult kidney. Expression of the shorter mRNA encoding p112-S was much higher than that of the longer mRNA for p112-L. However, the physiological significance of the existence of two mRNAs for p112 remains unclear at present.

Chromosomal Mapping of p112 by In Situ Hybridization

To investigate localization of the *p112* gene on the human chromosome, the full length p112 cDNA clone in a pBluescript SK^{-} was used as a probe for in situ hybridization. In the 100 typical R-banded (pro)metaphase plates examined, 7% exhibited complete twin spots on both homologues, 18% were incomplete single and/or twin spots on either or both homologues, and no spots were detectable in the others (75%). The signals were located on the q37.1–q37.2 band of chromosome 2. No twin spots were observed on other chromosomes. The *p112* gene could therefore be assigned to the q37.1–q37.2 region of human chromosome 2 (Figure 3, a–d).

Similarity to Yeast SEN3 Gene Product

Computer analysis showed that human p112 is highly homologous to a previously reported protein, the product of the *S. cerevisiae SEN3* gene (42% identity and 60% similarity). *SEN3* encodes a factor affecting tRNA splicing endonuclease, whose sequence was submitted to the GenBank by DeMarini *et al.* (accession number L06321) (Figure 4) and recently published (DeMarini *et al.*, 1995). Similarities are observed throughout almost their entire sequences, suggesting that the two proteins have similar functions (see below). The molecular size of the p112 resembles that of


A
170bp
1
2859
p112-L
ATG
ATG
TAA
1
2451
p112-S
ATG
TAA
Primers for RT-PCR
B
5'-TGAACTGAGCGGCCCCTGAGCTGACAGATACACTGCGCAGTGGAACGGCGAGCGAGCCGACGGGCGAGTGAGGGGCGCAC -1
ATGATCACCTCGGCCGCTGGAATTATTTCTCTTCTGGATGAAGATGAACCACAGCTTAAGGAATTTGCACTACACAAATTGAATGCAGTTGTTAATGACTTCTGGGCAGAAATTTCCGAG 120
M I T S A A G I I S L L D E D E P Q L K E F A L H K L N A V V N D F W A E I S E
a
b
TCCGTAGACAAAATAGAGGTTTTATACGAAGATGAAGGTTTCCGGAGTCGGCAGTTTGCAGCCTTAGTGGCATCTAAAGTATTTTATCACCTGGGGGCTTTTGAGGAGTCTCTGAATTAT 240
S V D K I E V L Y E D E G F R S R Q F A A L V A S K V F Y H L G A F E E S L N Y
c
d
GCTCTTGGAGCAAGGGACCTCTTCAATGTCAATGATAACTCTGAATATGTGGAAACTATTATAGCAAAATGCATTGATCACTACACCAAACAATGTGTGGAAAATGCAGATTTGCCTGAA 360
A L G A R D L F N V N D N S E Y V E T I I A K C I D H Y T K Q C V E N A D L P E
GGAGAAAAAAAACCAATTGACCAGAGATTGGAAGGCATCGTAAATAAATGTTCCAGCGATGTCTAGATGATCACAAGTATAAACAGGCTATTGGCATTGCTCTGGAGACACGAAGACTG 480
G E K K P I D Q R L E G I V N K M F Q R C L D D H K Y K Q A I G I A L E T R R L
e
GACGTCTTTGAAAAGACCATACTGGAGTCGAATGATGTCCCAGGAATGTTAGCTTATAGCCTTAAGCTCTGCATGTCTTTAATGCAGAATAAACAGTTTCGGAATAAAGTACTAAGAGTT 600
D V F E K T I L E S N D V P G M L A Y S L K L C M S L M Q N K Q F R N K V L R V
CTAGTTAAAATCTACATGAACTTGGAGAAACCTGATTTCATCAATGTTTGTCAGTGCTTAATTTTCTTAGATGATCCTCAGGCTGTGAGTGATATCTTAGAGAAACTGGTAAAGGAAGAC 720
L V K I Y M N L E K P D F I N V C Q C L I F L D D P Q A V S D I L E K L V K E D
AACCTCCTGATGGCATATCAGATTTGTTTTGATTTGTATGAAAGTGCTAGCCAGCAGTTTTTGTCATCTGTAATCCAGAATCTTCGAACTGTTGGCACCCCTATTGCTTCTGTGCCTGGA 840
N L L M A Y Q I C F D L Y E S A S Q Q F L S S V I Q N L R T V G T P I A S V P G
TCCACTAATACGGGTACTGTTCCGGGATCAGAGAAAGACAGTGACTCGATGGAAACAGAAGAAAAGACAAGCAGTGCATTTGTAGGAAAGACACCAGAAGCCAGTCCAGAGCCTAAGGAC 960
S T N T G T V P G S E K D S D S M E T E E K T S S A F V G K T P E A S P E P K D
CAGACTTTGAAAATGATTAAAATTTTAAGTGGTGAAATGGCTATTGAGTTACATCTGCAGTTCTTAATACGAAACAATAATACAGACCTCATGATTCTAAAAAACACAAAGGATGCAGTA 1080
Q T L K M I K I L S G E M A I E L H L Q F L I R N N N T D L M I L K N T K D A V
CGGAATTCTGTATGTCATACTGCAACCGTTATAGCAAACTCTTTTATGCACTGTGGGACAACCAGTGACCAGTTTCTTAGAGATAATTTGGAATGGTTAGCCAGAGCCACTAACTGGGCA 1200
R N S V C H T A T V I A N S F M H C G T T S D Q F L R D N L E W L A R A T N W A
AAATTTACTGCTACAGCCAGTTTGGGTGTAATTCATAAGGGTCATGAAAAAGAAGCATTACAGTTAATGGCAACATACCTTCCCAAGGATACTTCTCCAGGATCAGCCTATCAGGAAGGT 1320
K F T A T A S L G V I H K G H E K E A L Q L M A T Y L P K D T S P G S A Y Q E G
f
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G G L Y A L G L I H A N H G G D I I D Y L L N Q L K N A S N D I V R H G G S L G
g
CTTGGTTTGGCAGCCATGGGAACTGCACGTCAAGATGTTTATGATTTGCTAAAAACAAACCTTTATCAGGATGATGCAGTAACAGGGAAGCAGCTGGCCTGGCCCTAGGTTTGGTTATG 1560
L G L A A M G T A R Q D V Y D L L K T N L Y Q D D A V T G E A A G L A L G L V M
h
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L G S K N A Q A I E D M V G Y A Q E T Q H E K I L R G L A V G I A L V M Y G R M
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E E A D A L I E S L C R D K D P I L R R S G M Y T V A M A Y C G S G N N K A I R
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S E S Y N P H V R Y G A A M A L G I C C A G T G N K E A I N L I E P M T N D P V
i
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N Y V R Q G A L I A S A L I M I Q Q T E I T C P K V N Q F R Q L Y S K V I N D K
j
CATGATGATGTCATGGCCAAGTTTGGCGCTATTCTGGCCCAGGGCATACTGGATGCAGGTGGTCATAATGTCACAATCTCCTTGCAGTCCAGGACTGGGCATACTCATATGCCTTCTGTG 2280
H D D V M A K F G A I L A Q G I L D A G G H N V T I S L Q S R T G H T H M P S V
k
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V G V L V F T Q F W F W F P L S H F L S L A Y T P T C V I G L N K D L K M P K V
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Q Y K S N C K P S T F A Y P A P L E V P K E K E K E K E K V S T A V L S I T A K A K
l
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K K E K E K E K E K K E E E K M E V D E A E K K E E K E K K K E P E P N F Q L L D N
m
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P A R V M P A Q L K V L T M P E T C R Y Q P F K P L S I G G I I I L K D T S E D
n
ATTGAGGAGCTGGTGGAACCTGTGGCAGCACATGGCCCAAAAATCGAGGAGGAGGAACAAGAGCCAGAACCCCCAGAACCATTTGAGTATATTGATGATTAAGGACCAGAGGATCTCACT 2880
I E E L V E P V A A H G P K I E E E E Q E P E P P E P F E Y I D D *
o
TGCTTATCTGAAGAAGATTGTCCAGGCTCATATTGGGAATGCTTATGAGGAAATTCATGCCGAGACCTGCTATTCAATGCATGTATCGTTGCCTCTGCACTGACCTGAAGAACCCTGTCT 3000
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Figure 1.

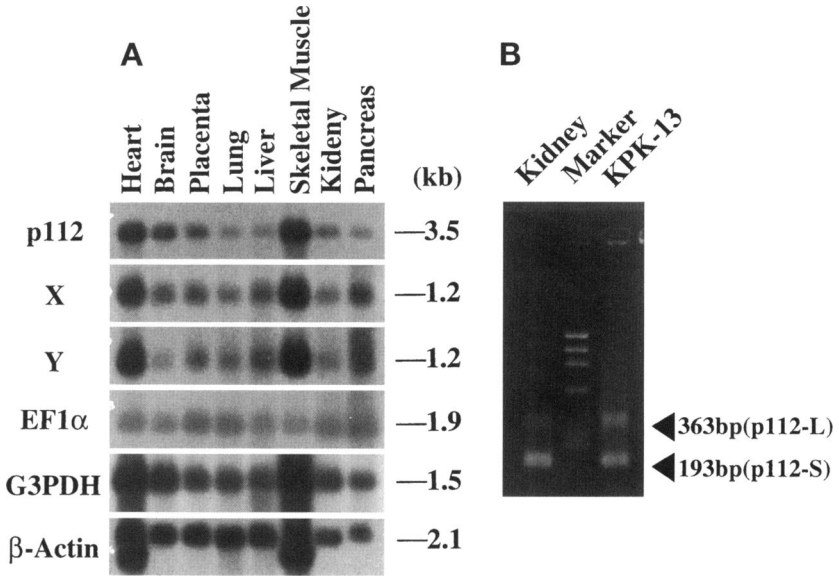

Figure 2. Expression of p112 mRNA in various human tissues. (A) RNA blot hybridization with poly(A)$^+$RNAs from various human tissues. Amount of mRNAs encoding p112, X and Y of the human 20S proteasome, EF1α, G3PDH, and β-actin were analyzed by RNA blot hybridization. The length of each mRNA is shown (in kb) on the right. (B) Detection of two types of mRNAs coding p112-L and p112-S by RT-PCR using poly(A)$^+$RNAs from human renal carcinoma KPK-13 cells and normal kidney. For details of primers used, see MATERIALS AND METHODS.

Sen3p (104 kDa), and its charge (pI = 5.12) differs only slightly from that of Sen3p (pI = 5.87). Two KEKE motifs appearing in human p112 have also been conserved in Sen3p. DeMarini *et al.*, (1995) showed that Sen3p is a regulatory subunit of the yeast 26S proteasome complex. This finding is in accord with the identification of p112 as a subunit of PA700, the complex that binds to the 20S proteasome to form the 26S proteasome.

Isolation of Mutants whose Growth Is Dependent on the Presence of NIN1, One of which Carries the SEN3 Mutation

The *S. cerevisiae NIN1* gene encodes a protein that is a counterpart of human p31, another regulatory subunit of the 26S proteasome, and which is required for normal cell cycle progression. The C-terminal truncated mutant *nin1–1* shows temperature-sensitive growth (Kominami and Toh-e, 1994; Kominami *et al.*, 1995). To screen mutations that, when combined with the *nin1–1* mutation, result in lethality, we exploited the color assay method developed by Koshland *et al.* (1985). Thus, TM1–4B[pDL120] growing at 25°C produces white sectors in the red colonies because pDL120 is dispensable at this temperature, whereas the same strain growing at 37°C develops homogeneous red colonies because pDL120 is essential under this condition. TM1–4B[pDL120] cells were treated with ethylmethanesulfonate as described in MATERIALS AND METHODS and then were spread on YPD plates. After incubation at 25°C for several days, colonies that did not produce white sectors were isolated and saved. Five independent mutants were isolated. One of them, TM1–4B-14[pDL120] was further analyzed in this study. To facilitate cloning experiments, pDL120 in the TM1–4B-14[pDL120] was replaced with pHN19 (P_{GAL1}-*NIN1 LEU2 TRP1 ARS1 CEN4*), resulting in TM1–4B-14[pHN19] whose growth is dependent on galactose even at 25°C.

To clone the gene responsible for the synthetic lethality with the *nin1–1* mutation in TM1–4B-14 [pHN19], the yeast genomic library based on YCUp4

Figure 1 (cont). Nucleotide sequence and predicted amino acid sequence of 26S proteasomal p112. (A) Organization of two isoforms of cDNA coding for the human 26S proteasomal subunit p112, which are tentatively termed p112-L and p112-S, in order of size. The nucleotide sequences of clone p112-L and clone p112-S were identical except for a 170-bp insertion/deletion at the position indicated. The solid boxes show the coding regions. The open boxes show 5′ and 3′ noncoding regions. To expand the N terminal regions the solid boxes are represented with interruptions. The putative translational initiation codon ATG and the termination codon TAA are indicated. The arrows show positions of primers used for RT-PCR analysis. (B) Nucleotide sequence and predicted amino acid sequence of the human 26S proteasomal p112-L. Nucleotides are numbered starting from A of the translational initiation codon ATG. The asterisk shows the termination codon. The possible polyadenylation signal AATAAA is boxed in black. Continuous underlines show the amino acid sequences corresponding to those obtained by Edman degradation of the purified p112 and its peptide fragments from bovine erythrocytes (a, b, c, d, e, f, h, i, k, m, and n) and rat liver (g, j, l, and o). The amino acids shown by dotted lines were not identical with those found by chemical analyses of either rat or bovine p112, or were amino acids not identified by the chemical analysis. The 170-bp insertion distinguishing p112-L from p112-S clones is boxed and the putative translational initiation codon ATG of p112-S clone is shadowed. The lysine-glutamate rich regions (KEKE motifs) are indicated with double underlines.

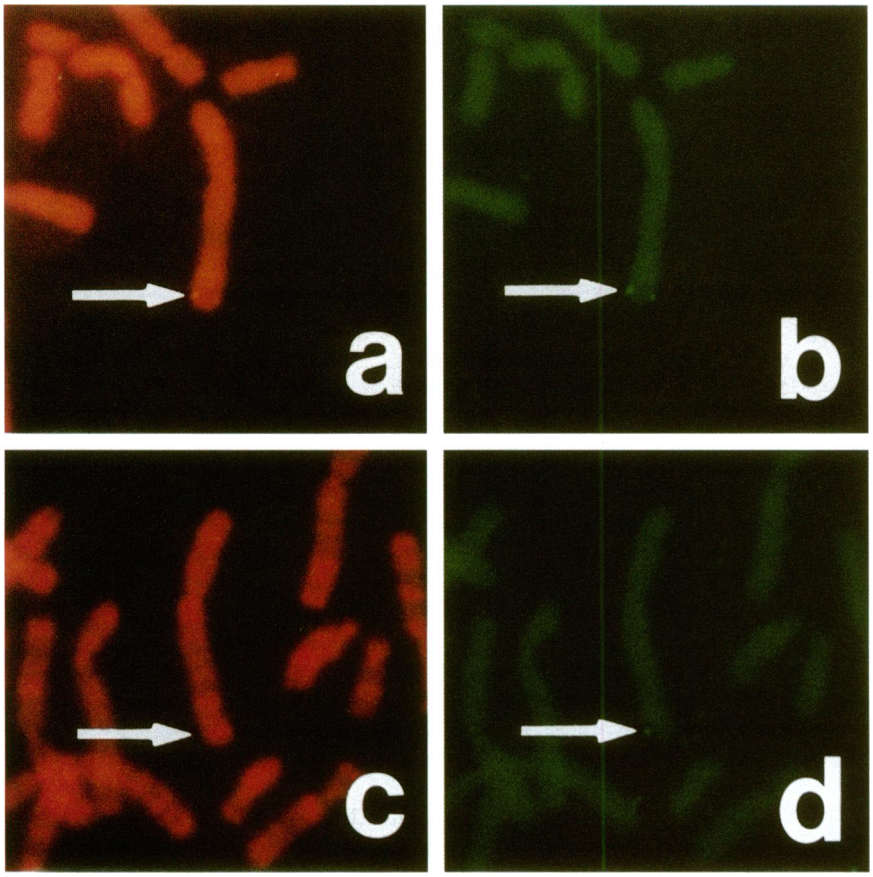

Figure 3. Chromosomal localization of the human 26S proteasomal p112 gene. Partial R-banded metaphase plates after FISH with the p112 cDNA. The panels of a/b and c/d show different plates. Filter combinations are as follows: a and c, Nikon B-2A; b and d, Nikon B-2E. Arrows indicate the signals on 2q37.1–q37.2.

(Fujita, unpublished data) was introduced into TM1–4B-14[pHN19] cells and screened for Ura^+ transformants on SC-URA plates. After confirming that the transformants were unable to lose the YCUp-plasmid by testing fluoro orotic acid sensitivity in glucose medium, plasmid DNAs other than pHN19 were recovered from them. Two kinds of plasmid were isolated. One of them contained *NIN1*. The other plasmid, when introduced into TM1–4B-14 [pDL120-*ura3*], caused white sectors at 25°C and was designated YCUp-SEN3 for reasons described below. A 3-kb *Sal*I–*Hin*dIII fragment was cloned between the *Sal*I and *Hin*dIII sites of pBluescript KS^- and a part of the nucleotide sequence of the cloned segment was determined by the dideoxy chain termination method. The sequence thus determined was found to be a part of that of the *SEN3* gene (DeMarini *et al.*, 1995; accession number L06321). Because the *SEN3* ORF occupies a major part of the cloned DNA and because the insertion of the *URA3* gene into the *Hin*dIII site localized within the *SEN3* ORF disrupted the complementation activity of the plasmid (Toe-h, unpublished data), we concluded that the activity complementing the synthetic lethal defect of TM1–4B-14[pHN19] resides in the *SEN3* gene. YCUp-SEN3 and YCUp4 were separately introduced into TM1–4B-14[pHN19], whose growth is dependent on galactose. Four independent transformants from each transformation experiment were streaked on YPD or YPGal plates, followed by incubation at 25°C for 3 days. All the transformants tested grew on YPGal plates, whereas strains possessing YCUp-SEN3 grew on YPD but those with YCUp4 did not (Figure 5).

High Copy of SEN3 Suppresses nin1–1

The 4.5-kb *Bam*HI fragment containing the *SEN3* gene was excised from YCUp-SEN3 and inserted at the *Bam*HI site of YEp24 (2 μm ori, *URA3*) to obtain YEp-SEN3. YEp-SEN3, pHN4 (*NIN1*), and YEp24 were separately introduced into the *nin1–1* strain YK109. A representative from each transformation experiment was streaked across a YPD plate followed by incubation at 25, 30, 35, and 37°C for 3 days. As shown in Figure 6, the *nin1–1* strain YK109 cells carrying YEp24 grew normally at 30°C, but were unable to grow at 35 and 37°C. However, YK109 cells containing the YEp-SEN3 plasmid grew at 35°C but not at 37°C, indicating that a high copy number of *SEN3* partially suppresses *nin1–1*. In contrast, a high copy of *NIN1* did not sup-

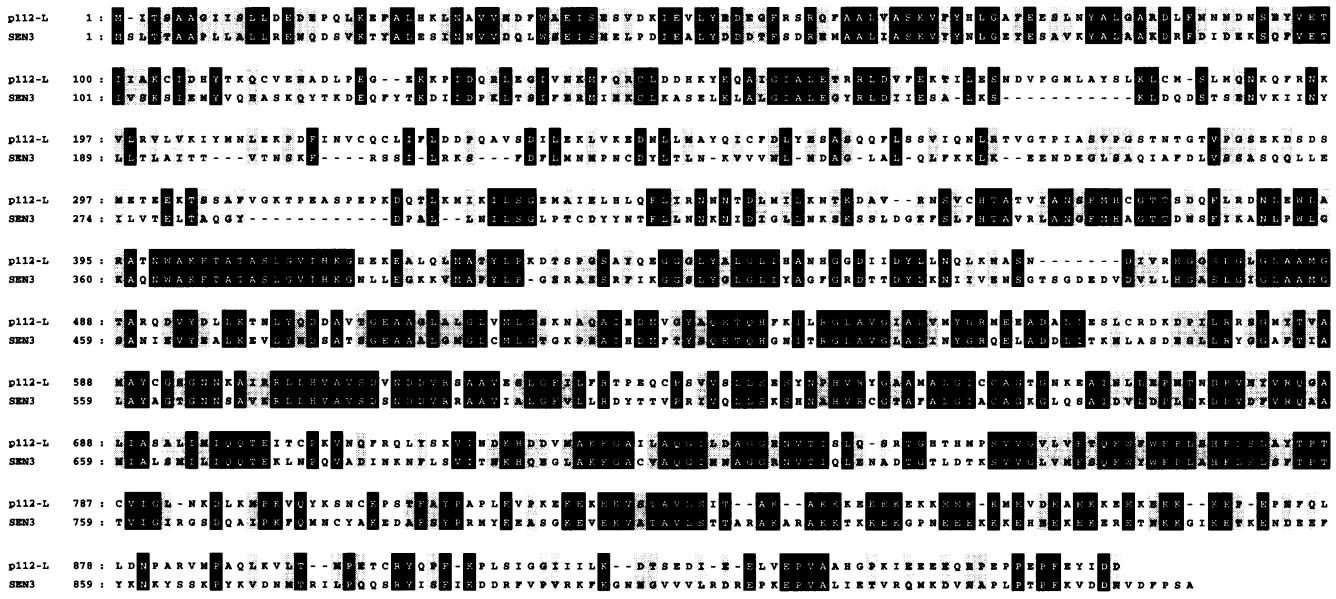

Figure 4. Alignment of amino acid sequences of human 26S proteasomal subunit p112 and *S. cerevisiae* Sen3p. The one-letter amino acid notation is used. Gaps (-) have been inserted to achieve the maximum homology. Identical and conserved amino acid residues are shown by black and shaded boxes, respectively. Conserved amino acids are defined as reported by Needleman and Wusch (1970): A, S, T, P, and G; N, D, E, and Q; R, K, and H; I, V, L, and M; and F, Y, and W.

press the temperature-sensitive phenotype of the *sen3* disruptant YAT1886, which will be described in the following section (Toe-h, unpublished data).

Disruption of SEN3 Gene

To examine whether the *SEN3* gene is an essential gene or not, it was disrupted as follows. First, the 3-kb *Bam*HI–*Sal*I fragment possessing most of the *SEN3* gene was inserted between the *Bam*HI and *Sal*I sites of pBluescript KS^+. The resultant plasmid contains a unique *Hin*dIII site within the *SEN3* ORF. The *SEN3* ORF was disrupted by inserting the 1.1-kb *Hin*dIII fragment containing *URA3* into the *Hin*dIII site (Figure 7A). Plasmid DNA thus constructed was digested with *Bam*HI and *Sal*I and then used as donor DNA for transformation of W303D from Ura^- to Ura^+. Correct disruption was confirmed by Southern hybridization (Toe-h, unpublished data). The heterozygous diploid cells were sporulated and dissected. Four viable spore clones were recovered from most of the asci dissected; the segregation ratio of Ura^+:Ura^- was 2:2, and Ura^+ clones always showed temperature-sensitive growth (Toe-h, unpublished data). These results suggest that *SEN3* is not essential for growth (however, see DISCUSSION), and the Ura^+ segregants, W1646–1C and W1646–1D, were saved for further use.

When W1646–1D was crossed to YK109 and tetrads were dissected, *sen3*::*URA3 nin1–1* recombinants were

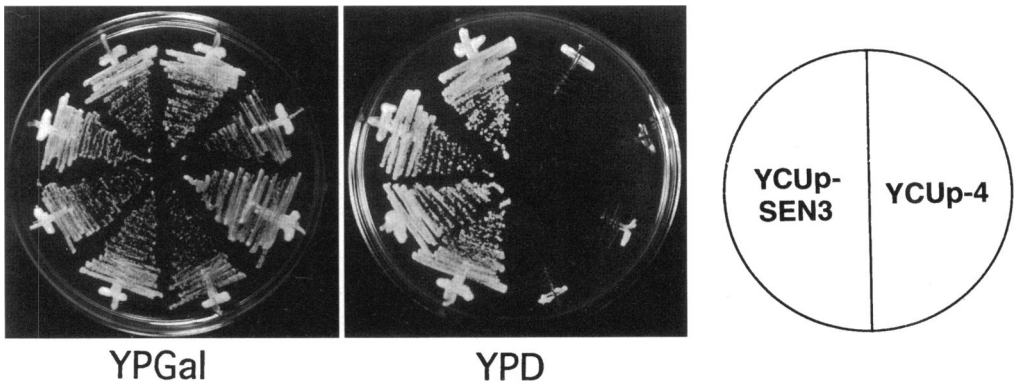

Figure 5. The *SEN3* gene suppresses lethality of TM1–4B-14[pHN19]. YCUp-SEN3 or YCUp4 were introduced into TM1–4B-14[pHN19] by selecting Ura^+ transformants. Four transformants from each transformation experiment were streaked across YP-Gal and YPD plates and incubated at 30°C for 3 days.

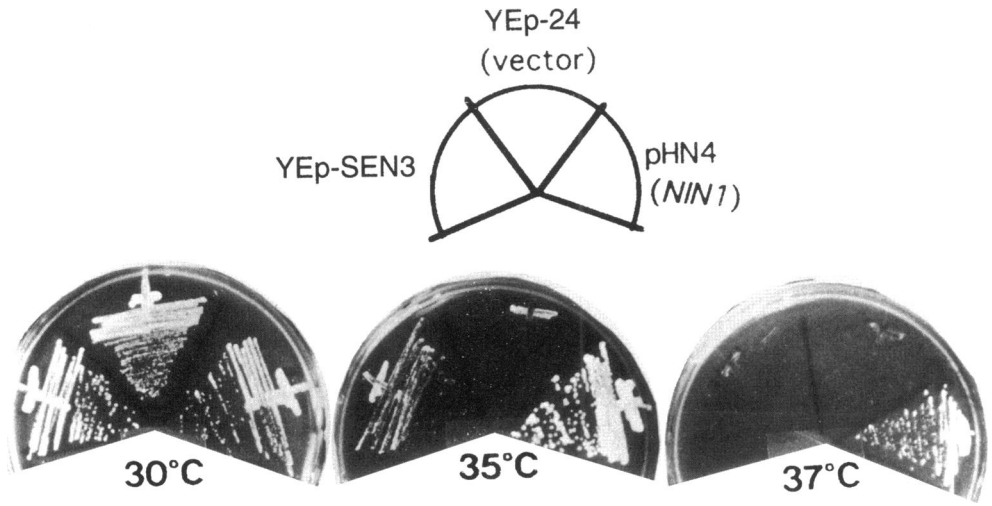

Figure 6. High copy of *SEN3* suppresses *nin1–1*. Each of YEp-SEN3, pHN4, and YEp24 was introduced into the YK109 (*nin1–1*) strain by selecting Ura$^+$ transformants. One representative from each transformation experiment was streaked across YPD and incubated at 30°C, 35°C, and 37°C for 3 days.

frequently found among segregants. To our surprise, a *sen3::URA3 nin1–1* double mutant strain was found not to be lethal. A cross between TM1–4B-14[pHN19] and W1710–1B gave rise to asci having two or more spores growing on YPD, indicating that synthetic lethality of TM1–4B-14 is due to more than two mutation in addition to the *nin1–1* mutation. On the other hand, when TM-4B-14[pHN19] was crossed with YAT2011, the ability to grow on YPD segregated in a 2:2 ratio. This result can be most simply explained by assuming that one of the mutations in TM1–4B-14 is an allele of *SEN3*. Furthermore, the *SEN3* gene on a

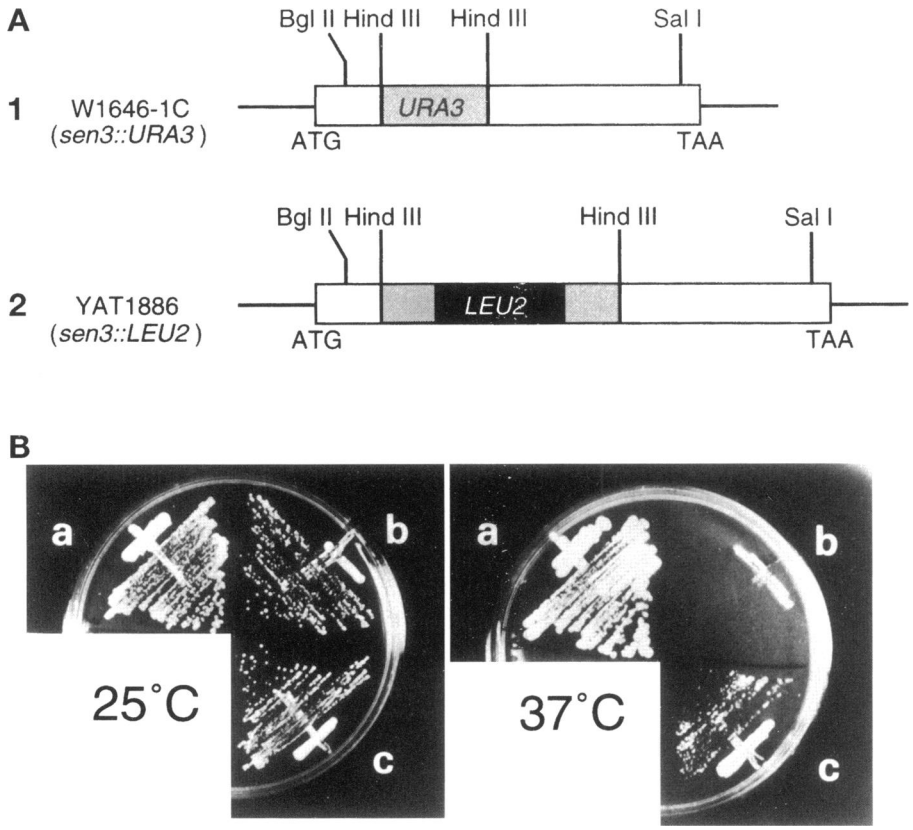

Figure 7. The p112-L cDNA complements temperature-sensitive growth of a strain that inherited the *sen3::LEU2* allele. (A) Predicted chromosomal structure of the *SEN3* gene containing the inserted *URA3* (top) or *ura3::LEU2* marker gene (bottom). Strains bearing these genes were named W1646–1C and YAT1886, respectively. For details, see text. (B) Each of YEp-SEN3 (a), TOp59 (b), and pAT540 (c) was introduced into the YAT1886 by selecting Ura$^+$ transformants. A representative from each transformation experiment was streaked across the SC-Ura plate and incubated for 3 days at 25°C (left plate) and 37°C (right plate).

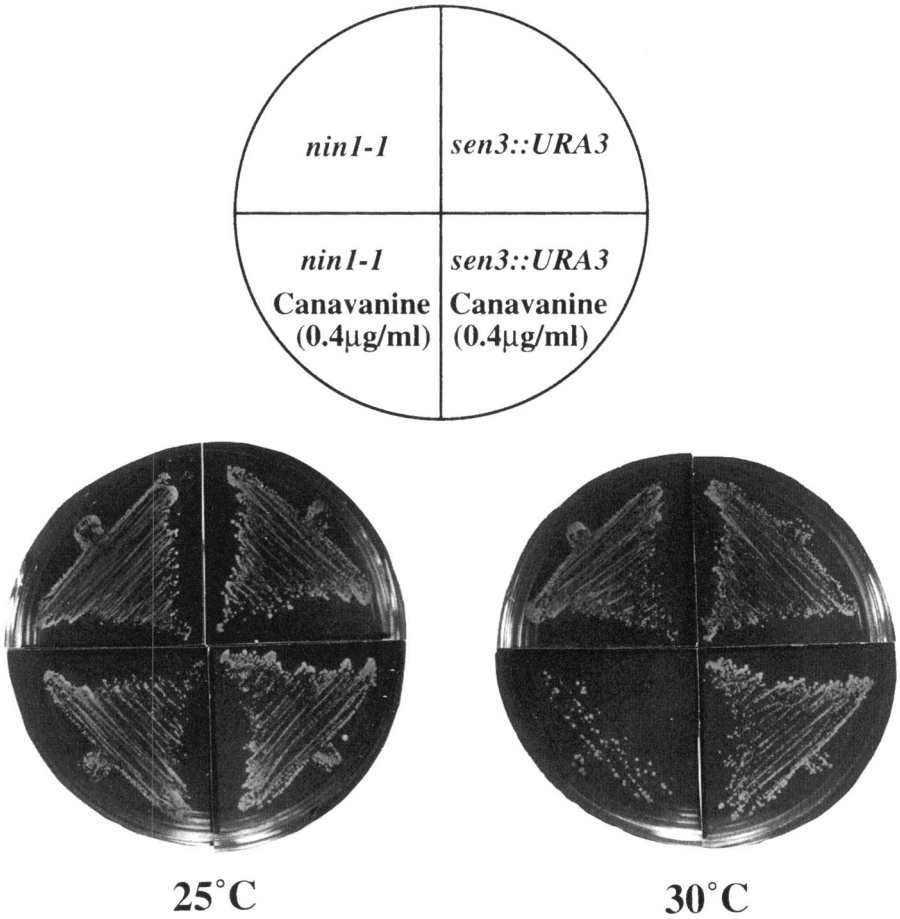

Figure 8. Effects of elevated temperature and canavanine application on growth of *nin1–1* and *sen3* disruptant cells. YK109 (*nin1–1*) and W1646–1C(*sen3*::*URA3*) strains were streaked onto SC-agar plates (0.68% yeast nitrogen base without amino acids, 2% glucose, and 0.04% adenine, and supplements as required) with or without canavanine at the final concentration of 0.4 μg/ml. Plates were incubated at 25°C (left panel) or 30°C (right panel) for up to 3 days.

low copy vector complemented the growth defect shown by TM1–4B-14 as shown in Figure 5, it is clear that *SEN3* is involved in the synthetic lethal phenomenon. These results suggest the existence of an unidentified gene whose coexistence with *sen3*::*URA3* and *nin1–1* is necessary for synthetic lethality.

High Expression of p112 Suppresses the Temperature Sensitivity of a sen3 Disruptant

The *URA3* gene of W1646–1C was disrupted by replacement transformation using the DNA fragment containing the *ura3*::*LEU2* gene from our stock plasmid. The resulting strain was designated YAT1886 (Figure 7A). For complementation, pAT540 was introduced into YAT1886 by selecting Ura^+ transformants, which in turn were streaked across YPD plates followed by incubation at 25°C or 37°C. The transformants grew at 37°C (Figure 7B). Thus, the p112-L cDNA complements temperature-sensitive growth of a strain that inherited the *sen3*::*LUE2* allele. However, transfection of p112-S cDNA by a similar method did not complement temperature-sensitive growth of *sen3*-defective cells (Toe-h, unpublished data). These results indicate that the p112-L, but not p112-S, gene functions as the *SEN3* gene in yeast.

Effects of Canavanine and Cadmium Stresses on Growth of a sen3 Disruptant and the nin1–1 Strain

As in the *nin1–1* strain, elevated temperature stress resulted in defective growth of *SEN3* disrupted cells. We also examined the effect of other stresses. Application of the amino acid analogue canavanine was first examined. As shown in Figure 8, 0.4 μg/ml of canavanine had no effect on cell viability either in cells bearing a *sen3*::*URA3* allele (W1646–1C) or in *nin1–1* cells (YK109) at 25°C, at the permissive temperature. However, canavanine caused a growth defect of YK109 at 30°C without affecting proliferation of wild-type cells (Simizu and Tanaka, unpublished data) or of W1646–1C.

Recently, resistance to cadmium, a potent poison for living cells, was found to be mediated by the ubiquitin pathway, because this proteolysis is activated in response to cadmium exposure (Jungmann *et al.*, 1993).

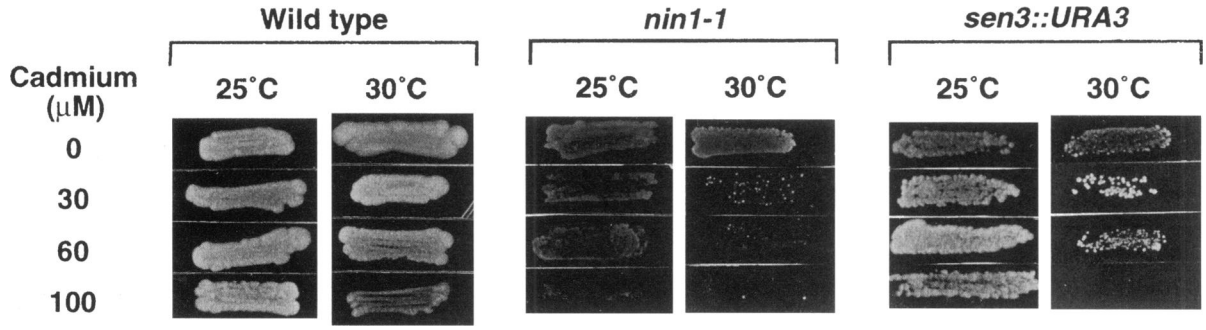

Figure 9. Cadmium hypersensitivities of mutants of the 26S proteasome genes. Experimental conditions were the same as those in Figure 8, except that various concentrations of cadmium as indicated were added instead of canavanine, and incubated at 25°C or 30°C.

Moreover, mutants in the 20S proteasome were shown to be hypersensitive to cadmium (Jungmann *et al.*, 1993). Therefore, we examined the effect of cadmium on mutants in the 26S proteasome genes. As shown in Figure 9, 100 μM of cadmium caused defective growth in YK109, but not in W1646–1C cells at 25°C. However at 30°C, cadmium suppressed the proliferation of YK109 and *sen3* disruptant in a dose-dependent manner, being very sensitive in YK109 cells (Figure 9). Exposure to cadmium had no effect on the proliferation in the wild-type cells (Simizu and Tanaka, unpublished data). These effects suggest that dysfunction of the 26S proteasome is responsible for stress-dependent growth arrest.

Proteolysis in a sen3 Disruptant and the nin1–1 Strain

Previously we reported that the YK109 cells abnormally accumulate polyubiquitin-conjugated proteins at the restrictive temperature (Kominami *et al.*, 1995). Therefore, we examined whether disruption of the *SEN3* gene also results in accumulation of ubiquitinated proteins. For this analysis, we employed a monoclonal antibody, FK1, which reacts specifically with polyubiquitin chains ligated to proteins, but does not react with monoubiquitinated proteins or with free ubiquitin (Fujimuro *et al.*, 1994). As shown in Figure 10, when *SEN3*-deficient cells (YAT1886) were cultured at 37°C, multiple high molecular weight, immunoreactive bands appeared in a time-dependent fashion in the *sen3* disruptant cells, but not in the wild-type cells. These results clearly indicate that the disruption in the *SEN3* gene promotes the accumulation of considerable amounts of polyubiquitinated cellular proteins, and that a functional Sen3p subunit within the 26S proteasome is required for the normal degradation of ubiquitin-protein conjugates in vivo.

One proteolytic pathway mediated by ubiquitination is the N-end rule pathway, in which destabilization of proteins is governed by an amino-terminal amino acid (Varshavsky, 1992). Mutants defective in the chymotryptic activity of the yeast proteasome fail to degrade substrates of the N-end rule pathway (Richter-Ruoff *et al.*, 1992; Seufert and Jentsch, 1992). To test the involvement of Nin1p and Sen3p in the ubiquitin-dependent N-end rule pathway, we analyzed the stability of short-lived β-galactosidase (βgal) fusion proteins in the *nin1–1* strain (YK109) and the *sen3* disruptant (YAT1886). Cells were transformed with the plasmids expressing the differently engineered Ub-X-βgal proteins, where "X" indicates any amino acid residue. To obtain the proteolytic stabilization of the metabolically unstable X-βgal proteins, we determined their degradation rates by employing pulse-chase experiments. Wild-type and mutant cells

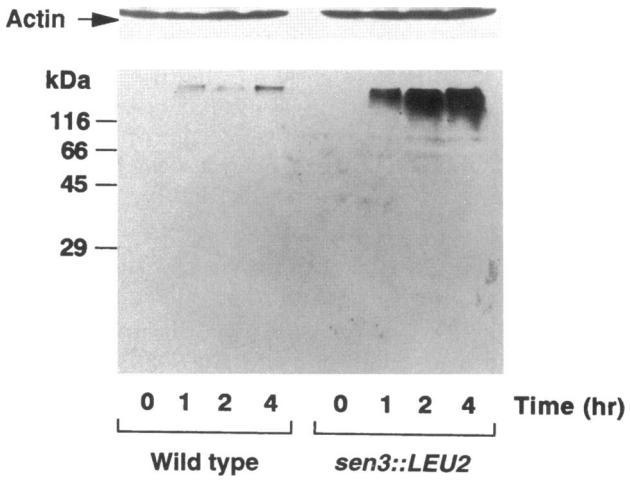

Figure 10. Immunoblot analyses of polyubiquitinated proteins in the *sen3* disruptant at a restrictive temperature. Early exponential cells of *sen3* disruptant cells, YAT1886 (*sen3::LEU2*), and the wild-type strain grown in SC medium at 25°C were transferred to 37°C. Cells were taken out at indicated times after the shift and processed for immunoblot analysis with monoclonal antibody FK1 specific for multi-ubiquitin chains (lower panel). Immunoblot analysis of actin was measured with the same membrane (upper panel).

were briefly labeled with [^{35}S]methionine, and cell lysates were subjected to immunoprecipitation using antibodies specific to βgal. Labeled and precipitated proteins were separated by SDS-PAGE, followed by fluorography. As shown previously (Bachmair *et al.*, 1986), the metabolically stable test protein Ala-βgal remained stable in wild-type cells during the test period (30 min) whereas proteins known to be metabolically unstable in wild-type cells, Arg-βgal, Leu-βgal, and Tyr-βgal, disappeared rapidly with time (Figure 11A, upper panel); the expression of Arg-βgal was quite low in this strain for unknown reasons. In contrast, the test proteins were almost completely stabilized in the *nin1–1* mutant cells at the restrictive temperature (Figure 11A, lower panel). Similar marked stabilization of Arg-βgal and Leu-βgal were observed in *sen3*-disrupted cells unlike wild-type cells at the restrictive temperature (Figure 11B). These results suggest that the 26S proteasome is a principal protease responsible for in vivo proteolysis mediated by the ubiquitin and N-end rule pathway, and that a functional Sen3p subunit is required for this process.

Disruption of SEN3 Leads to Defective Nuclear Protein Translocation

In addition to the *SEN3* gene, we have isolated the *SON1* gene by the synthetic lethal screen with the *nin1–1* mutant strain (our unpublished observations). *SON1* was previously identified as an extragenic suppressor of *sec63* alleles that impair the transport of proteins into the nucleus (Nelson *et al.*, 1993). The genetic interaction shown by the synthetic lethality between the *son1* disruptant and the *nin1–1* mutation suggests that Son1p may be another subunit of the 26S proteasome. These findings led us to consider the possibility that other regulatory genes of the 26S proteasome may be also responsible for nuclear protein transportation. Therefore, we examined whether mutations of *p112/SEN3* and *p31/NIN1* genes affect nuclear translocation of proteins. The transfection of plasmid pTG102 had no effect at the restrictive temperature in the nuclear translocation in YK109 (*nin1–1*) cells or wild-type cells (Figure 12, upper panel). In contrast, as shown in Figure 12, at elevated temperature (30°C, upper panel), but not at 25°C (lower panel), a major defect of the nuclear transport of a reporter gene product was observed in the *sen3* disruptant cells (YAT1886), suggesting that the 26S proteasome containing Sen3p may be involved in the nuclear transport system. Cell proliferation had no significant effect on the *nin1–1* cells or *sen3* disruptant cells at 30°C, as shown in Figure 8, although the mutant cells had a flattened shape. It is unknown whether the change of cell shape indirectly affects nuclear transport of proteins (see DISCUSSION).

DISCUSSION

In the present study, we have isolated and sequenced a human cDNA encoding p112, the largest regulatory subunit of the 26S proteasome (Figure 1). We have identified two cDNAs for the p112, termed p112-L and p112-S, which were expressed differentially in normal kidney and in renal carcinoma cells, suggesting distinct roles for these two types of p112 species. The nucleotide sequences of two cDNAs are identical, except for a 170-bp deletion in p112-S; these results suggest that the two mRNAs might be generated by alternative splicing from a single gene. Consistent with this suggestion, a single chromosomal locus of the human p112 gene was mapped to 2q37.1–q37.2 by

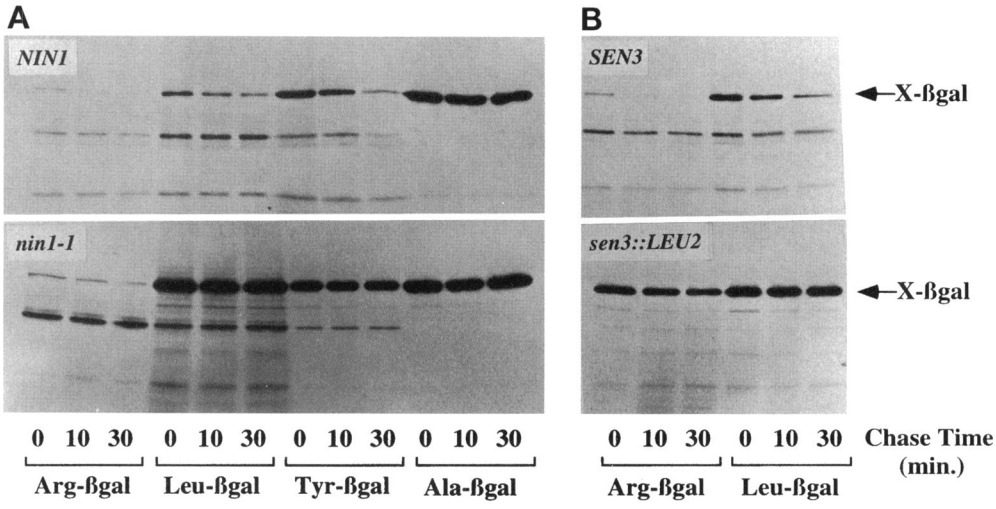

Figure 11. Metabolic stability of X-β-galactosidases in wild-type and *nin1–1* and *sen3* mutant cells. (A) Wild-type and YK109 (*nin1–1*) strain, upper and lower panels, respectively. (B) Wild-type and YAT1886 (*sen3::LEU2*) strain, upper and lower panels, respectively. Wild-type and mutant cells were grown, labeled with [^{35}S]methionine at 25°C. Labeling was done for 10 min and thereafter cells were chased with nonradioactive methionine (10 mM) for the indicated times at the restrictive temperature (37°C). Immunoprecipitation, SDS-PAGE, and fluorography were carried out as described in MATERIALS AND METHODS.

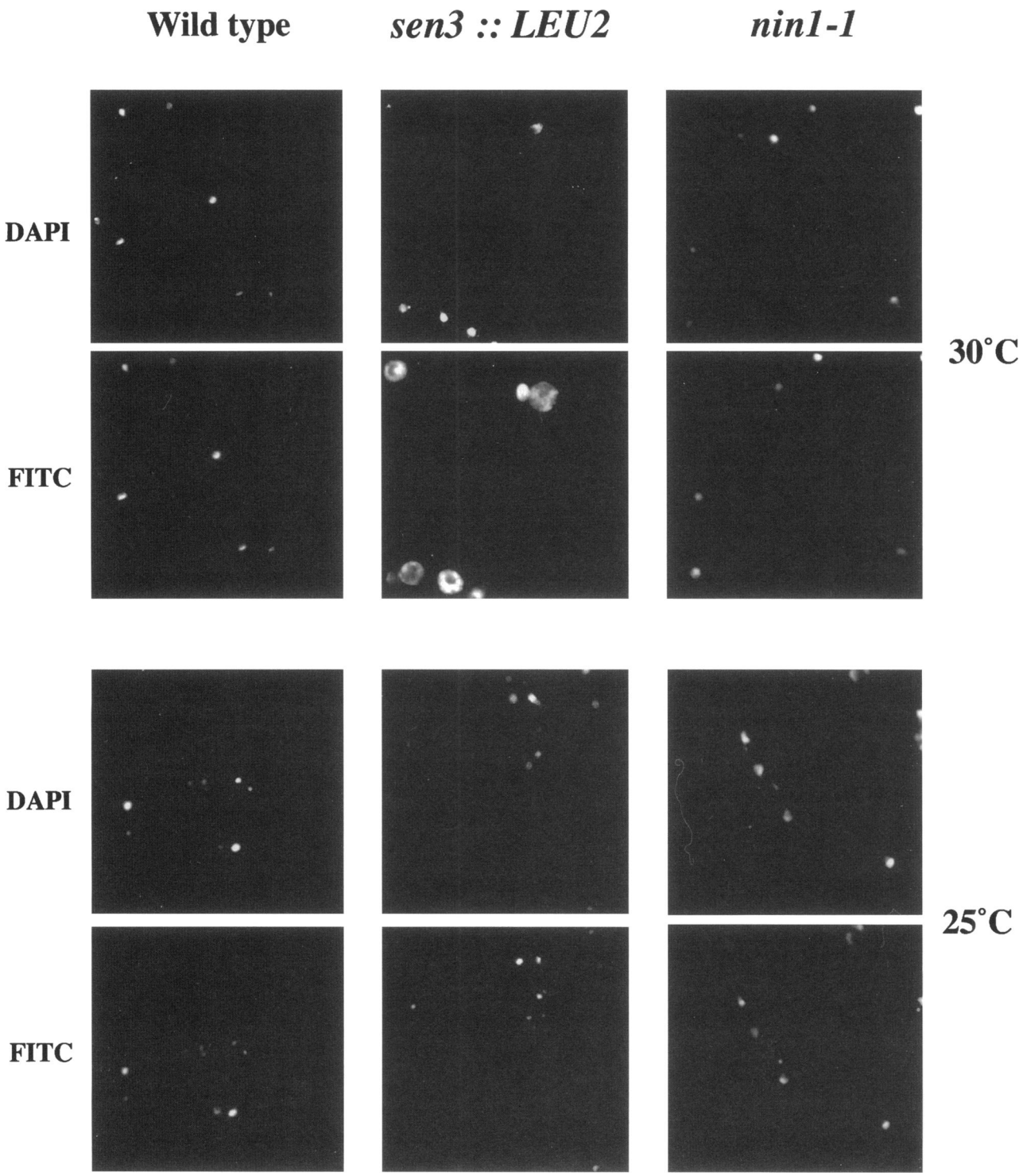

Figure 12. Nuclear protein translocation in wild-type and *nin1–1* mutant and *sen3*-disrupted cells. W303D (wild type), YAT1886 (*sen3*::*LEU2*), and YK109 (*nin1–1*) cells were cultured at 25°C and 30°C. For details of nuclear transportation assay of model protein, see MATERIALS AND METHODS. The transported protein (lower panel) and nucleus (upper panel) were stained by fluorescein isothiocyanate and 4,6-diamino-2-phenylindole, respectively.

in situ hybridization analysis (Figure 3). This chromosomal location differs from that of the regulatory subunit p40 (Tsurumi *et al.*, 1995) as well as loci of various genes encoding subunits of the 20S proteasome (Akioka *et al.*, 1995; Hisamatsu *et al.*, 1996). Previously, using two-dimensional electrophoretic peptide mapping analysis with lysylendopeptidase, we reported that two similar proteins with apparent molecular masses of 110 kDa and 90 kDa were present in the purified regulatory complex of the rat liver 26S proteasome, although most other components showed different peptide maps (Ugai *et al.*, 1993). We had thought that the smaller 90-kDa protein might be generated from the larger 110-kDa subunit by limited proteolysis. The present findings, however, suggest that these large and small subunits may correspond to the p112-L and p112-S proteins, respectively, and that each p112 subunit translated from these two mRNAs is probably assembled into a 26S proteasome complex. It is likely that two types of 26S proteasomes, one containing p112-L and the other p112-S, are present in cells, but the functional difference and physiological role of proteasomes with these two types of p112 remain to be resolved. The presence of two iso-subunits from the human 20S proteasome, PROS-30 (C2) and C6-I subunits, has also been reported (Pereira *et al.*, 1992; Ni *et al.*, 1995).

The primary structure of p112 strongly resembles that of Sen3p, a yeast protein, suggesting that Sen3p may be a homologue of p112 (Figure 4; DeMarini *et al.*, 1995). Surprisingly, disruption of the chromosomal gene encoding Sen3p had no effect on growth at the permissive temperature, indicating that Sen3p does not play a vital role in cell proliferation of yeast (Figure 7). The nonessential character of the *SEN3* gene in cell viability differs markedly from the genes for various yeast 20S proteasomal subunits, which are essential (Hilt and Wolf, 1995; Tanaka, 1995). Nevertheless, the *sen3* disruptant cells revealed a temperature-sensitive growth phenotype. Transfection of human p112 cDNA ligated with a high copy expression vector suppressed the growth defect of the *sen3* disruptant at the restrictive temperature, implying that the human p112 is a functional homologue of yeast Sen3p (Figure 7). In contrast, DeMarini *et al.* (1995) have reported that disruption of *sen3* gene causes lethality. Although the reason for these discrepant findings is unknown, it may be due to a difference of genetic background of the yeast. Analogously, we recently found that disruption of *Nas1* gene encoding a yeast homologue of human p97, the second largest subunit of the 26S proteasome, resulted in both lethal and nonlethal effects, depending on genetic background of the cell types used (Tsurumi, Kato, DeMartino, Toh-e, and Tanaka, unpublished data).

The *SEN3* gene was isolated as a factor exercising post-transcriptional control of *SEN1*, which is required for the activity of yeast tRNA-splicing endonuclease, which is encoded by the *SEN2* gene. In fact, abnormal accumulation of tRNA intermediates was detected in cells carrying the defective *sen3* gene (DeMarini *et al.*, 1995). Sen3p protein may contribute to regulation of the level of Sen1p, and Sen3p may exercise this function as an integral component of the 26S multisubunit proteasome complex, in a fashion analogous to the proteasome's regulation of the stability of various protein factors affecting the transcriptional control (Ciechanover, 1994). It is of particular interest that the *SON1* gene, like *SEN3*, was also identified in a synthetic lethal screen with the *nin1–1* mutant gene (Toe-h, unpublished data). Moreover, disruption of *son1* and *sen3* is also synthetic lethal (Toe-h, unpublished data), suggesting that Son1p is physically associated with Nin1p and Sen3p, perhaps as a subunit of the PA700 regulator complex. *SON1*, like *SEN3*, must be a nonessential gene, as reported previously (Nelson *et al.*, 1993). It is interesting that the *SON1* gene was isolated by extragenic suppressors of mutations of *npl1–1* (equivalent to *sec63–101*) (Nelson *et al.*, 1993). *NPL* (nuclear protein translocation) genes identified in mislocalization of a nuclear-targeted fusion protein have been cloned. Thus it is not surprising that a *sen3*::*URA3* allele negatively regulates the nuclear translocation of a model protein (Figure 12). In addition, very recently the *SON1* gene was identified as a gene named *UFD5*, one of five distinct genes whose mutations perturb the proteolytic pathway that recognizes ubiquitin as a degradation signal, termed the UFD pathway (ubiquitin-fusion degradation pathway) (Johnson *et al.*, 1995). *UFD5* was found to be essential for activity of both the UFD and N-end rule pathways. If the Son1p is a component of the 26S proteasome, the involvement of Son1p in the UFD pathway might be elucidated.

The *SEN3* gene was identified in a synthetic lethal screen using the *nin1–1* mutant gene (Figure 5), which encodes a truncated form of Nin1p lacking approximately 100 residues from the C-terminus of the wild-type protein. Nin1p is a yeast homologue of human p31, a subunit of PA700 (Nisogi *et al.*, 1992; Kominami *et al.*, 1995). Despite the fact that *SEN3* was found in the process of screening mutations showing synthetic lethality with *nin1–1*, a *sen3*::*URA3 nin1–1* strain did not show a clearly synthetic lethal phenotype, suggesting that unknown gene(s) may be involved in the appearance of the synthetic lethal phenotype between these two genes. A genetic interaction between *SEN3* and *NIN1* is also supported by the finding that a high dosage of the *SEN3* gene substantially suppressed the temperature-sensitive growth defect seen in the *nin1–1* mutant cells (Figure 6). These findings also suggest that Sen3p and Nin1p subunits are physically associated with each other. These genetic analyses might serve as the basis for obtaining information about the

molecular assembly of the proteasomal 26S protease complex.

The present study showed that Sen3p is involved in proteolysis mediated by the ubiquitin pathway. Indeed *sen3*-disrupted cells showed abnormal accumulation of polyubiquitinated cellular proteins at the restrictive temperature (Figure 10), a feature also observed in *nin1–1* temperature-sensitive mutant cells (Kominami *et al.*, 1995). A *sen3* disruptant, as well as a *nin1–1* mutant, was also defective in the N-end rule pathway. However, the *nin1–1* strain was much more sensitive to various stresses than the *sen3* disruptant. For example, exposure to canavanine had no significant effect on growth of *sen3* disrupted cells, but *nin1–1* cells were hypersensitive to this treatment (Figure 8). Jungmann *et al.* (1993) reported that resistance to cadmium is mediated by ubiquitin-dependent proteolysis, and exposure to this poison induces expression of genes encoding ubiquitin and ubiquitin-conjugating enzymes. This observation is consistent with the present findings that *sen3* disruptant and *nin1–1* mutant cells, unlike their wild-type counterparts, are unable to recover for proliferation upon exposure of the cadmium stress. Interestingly, the dysfunction of Nin1p appears to be more severe in response to various stress responses than that of Sen3p. However, we observed that nuclear protein transport was impaired specifically in *sen3*-disrupted cells (Figure 12). It is unclear why mutations of two different subunits of the 26S proteasome, Sen3p and Nin1p, induce different phenotypes, but perhaps these subunits serve for distinct recognition of the target proteins whose roles in cellular function may differ. It is possible that cellular dysfunctions induced by mutations of these two genes might be unrelated to a general impairment of proteolysis involved in the N-end rule pathway. This possibility is consistent with the finding that no significant phenotypic abnormality was observed after deletion of the *UBR1* gene, whose product is a key enzyme in the N-end rule pathway (Bartel *et al.*, 1990). Therefore, the 26S proteasome, with Sen3p and Nin1p as subunits, plays a critical role in the destruction of specific proteins that are closely involved in the cell growth.

Recently, DeMarini *et al.* (1995) reported the primary structure of Sen3p and a functional analysis of the *SEN3* gene in yeast. They showed that Sen3p played an important role in the ubiquitin-dependent proteolytic pathway in yeast and demonstrated that Sen3p copurified with the yeast 26S proteasome. These results are in excellent agreement with those described in the current report. Here, we extend the analysis of the *SEN3* gene in several important ways. First we have cloned the gene for a human protein, the p112 subunit of the 26S proteasome, and determined its chromosomal location. The human p112 has a high degree of sequence similarity to the yeast Sen3p and is therefore its presumptive structural homologue. Second, we have shown that human p112 can suppress defects in *SEN3*-deficient cells, indicating that the human protein is a functional homologue of Sen3p. Finally, we have demonstrated that Sen3p has distinct functional roles from other subunits of the regulatory complex of the 26S proteasome (e.g. Nin1p). This finding will provide the basis for future detailed analysis of the relative functions of the multiple subunits of this regulatory complex in the process of intracellular proteolysis.

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