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ISOLATION AND CHARACTERIZATION OF THE HC8 SUBUNIT GENE OF THE HUMAN PROTEASOME+

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SUMMARY: For study of the molecular basis of regulation of proteasome gene expression, we isolated the gene encoding the α-type HC8 subunit of the human proteasome. About 2.3 kb of the 5' flanking region of this gene was tested for promoter function by chloramphenicol acetyltransferase assay. This analysis revealed that CAAT and TATA boxes, but not a GC box, are essential for its promoter activity. These results differed from previous findings that the genes for the α-type HC3 and β-type HC5 subunits of the human proteasome have a TATA-less promoter and that two or three GC boxes function as the promoter sequences (Tamura, T. *et al.* (1994) J. Mol. Biol. 244, 1117-1124) . We mapped the HC8 gene at q23 on human chromosome 14, which differs from the chromosomal locations of nine other proteasomal subunit genes mapped so far.

The proteasome is a multicatalytic protease complex which has a sedimentation coefficient of 26S and catalyzes an energy-dependent, extra-lysosomal proteolytic pathway responsible for selective elimination of proteins with aberrant structures and naturally occurring short - lived proteins related to metabolic regulation and cell-cycle progression (1-3). The 26S proteasome is composed of a 20S catalytic core and a 22S regulatory complex (3). Eukaryotic 20S proteasomes have a molecular mass of 700 to 800 kDa and consist of a set of over 15 kinds of polypeptides of 21 to 32 kDa (4, 5). Recent findings indicate that all eukaryotic 20S proteasome subunits can be classified grossly into two subfamilies, α and β, by their highly similarities with either the α or β -subunits of the archaebacterium, *Thermoplasma acidophilum* (3). It is interesting to know how these gene families are expressed coordinately and whether these genes are a conserved or diverse family. Previously, we isolated two human proteasome genes encoding the α -type HC3 and β -type HC5 subunit (6). Analyses of their promoter sequences revealed the absence of TATA and

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CAAT elements and the existence of two or three GC boxes that function coordinately as promoters of the two genes. Differences in the exon-intron organizations of these genes, however, suggest that they diverged at an early stage of evolution.

Here, we report the cloning of another gene encoding the α-type human 20S proteasome subunit HC8, and show that its CAAT and TATA boxes function as promoters, in contrast to the promoters of the HC3 and HC5 genes. We also describe the chromosome mapping of this gene by fluorescence *in situ* hybridization.

MATERIALS AND METHODS

Isolation of the HC8 gene: For isolation of genomic DNA clones of the HC8 subunit of the human proteasome, 10^6 plaques of a human genomic DNA library constructed in an EMBL-3 bacteriophage vector were screened with either the full length cDNA of the HC8 subunit (7), or its 5' or 3' region, as a probe. One of the positive clones included an insert of approximate 14.5 kb covering the entire sequence of HC8 cDNA. This DNA insert was subcloned into pBluescript II and approximate 2.5 kb of its 5' flanking region including part of the first exon was sequenced.

Construction of plasmids for CAT assay: To construct a fusion gene of the 5' flanking region of HC8 gene and the chloramphenicol acetyltransferase (CAT) gene, we used the promoterless CAT plasmid pSV001CAT constructed from pSV00CAT, which was kindly provided by Dr. E. Araki (Kumamoto University), as a reporter gene. We first synthesized a series of deletion mutants of the 5' flanking region of the HC8 gene by the PCR technique. The primers used for amplification were as follows: upstream primers, nucleotide from -2325 to -2306, -2061 to -2042, -698 to -679, -387 to -368, -298 to -279, -149 to -130, and -125 to -106; downstream primer, from nucleotide -44 to -25 (nucleotides are numbered from the 5' end of the previously cloned HC8 cDNA (7)). These DNA fragments synthesized by PCR were fused with the pSV001CAT. For construction of deletion plasmids of CAAT and/or TATA sequences, pHC8-149CAT plasmid DNA was double-digested with suitable restriction enzymes (*Sac*I, *Spe*I and/or *Eco*47III) and ligated.

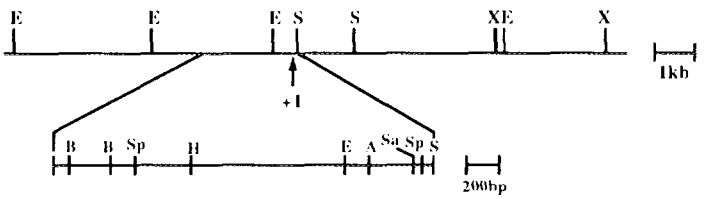
Transfection and CAT assay: For testing promoter activities, human HeLa cells (Japanese Cancer Research Resources Bank-Cell) were cultured in Dulbecco's modified Eagle's minimum essential medium supplemented with 10 % fetal bovine serum at 37 ° C. The cells were grown to a subconfluent state and then transfected by the calcium phosphate precipitation method according to the method described by Gorman (8), using a total of 13 μg of DNA mixture containing 10 μg of the HC8 and CAT fusion gene plasmid and 3 μg of an internal standard, pCH110 (Pharmacia LKB Biotechnology) bearing the galactosidase gene under the control of the simian virus 40 early promoter. Other procedures were as described previously (6).

Mapping of the HC8 gene by fluorescence *in situ* hybridization: Chromosome spreads were obtained from phytohemagglutinin-stimulated blood lymphocytes of a healthy donor after thymidine synchronization and bromodeoxyuridine incorporation by the method of Takahashi *et al.* (9, 10). A genomic DNA fragment of HC8 (6.0 kb *Eco* RI fragment including the first exon, see Figure 1) was labeled with biotin-16-dUTP (Boehringer Mannheim) by nick translation, and *in situ* hybridization was performed according to Lichter *et al.* (11) in the presence of COT-1 DNA (GIBCO BRL, Gaithersburg, MD) as a competitor. The hybridized probe was detected with FITC-conjugated avidin (Boehringer Mannheim) without any amplification step. Chromosomes were counterstained with 0.2 μg/ml propidium iodide for R-banding. Fluorescence signals were imaged using a Zeiss Axioskop epifluorescence microscope equipped with a cooled Charge Coupled Device (CCD) camera (Photometrics, PXL 1400). Image acquisition was performed on a Macintosh Quadra 840 AV computer with the software program IPLab (Signal Analytics Co.). The images were merged and processed using Adobe Photoshop 2.5J (Adobe Systems Inc.) to obtain clear signals in a glossy print. FITC and propidium iodide images were shown in black and white, respectively. The merged images were directly printed with Fuji Pictrography 3000 from a Macintosh computer.

RESULTS AND DISCUSSION

For isolation of the gene encoding subunit HC8 of the human proteasome, we screened 10^6 plaques of a human genomic DNA library constructed in an EMBL-3 bacteriophage vector using HC8 cDNA (7) as a probe. Of several positive clones, one clone which hybridized with the 5' and 3' probes of the HC8 cDNA was obtained. The restriction map and partial sequence indicated that the entire HC8 gene including approximately 7 kb of the 5' flanking region was covered by the genomic segment of this clone. Figure 1 shows the restriction map of the HC8 gene and the partial sequence of the 5' flanking region. Numbers of exons and their organization have not been determined. There was a TATA box and CAAT box sequence, and a GC box sequence at -51, -90 and -225, respectively.

To determine the promoter sequence of the HC8 gene, we examined the expression of HC8-CAT fusion genes in HeLa cells. pSV2CAT and pSV001CAT were used as positive and negative controls, respectively. The CAT activity of HeLa cells transfected with pHC8 -2325CAT was 15.6 % of that of cells transfected with pSV2CAT (Figure 2). The promoter activity was much higher than that of the HC3 gene, but lower than that of the HC5 gene (6). Deletion of the region from -2325 to -2061 resulted in slightly higher expression, indicating the presence of a weak negative element in this region. Further deletion up to -125 did not cause marked change in the promoter activity. This finding showed that the GC box present at -225 to -221 is not likely to function as a promoter of the HC8 gene. Striking decrease of the promoter activity was found on deletion of the CAAT box and/or TATA box. These results indicate that the promoter element of the HC8 gene is different from those of the HC3 and HC5 genes, which have no CAAT or TATA box in their 5' flanking regions. Previously we also found that there is a negative regulatory element between -439 and -256 in the 5' flanking region of the HC3 gene which is also effective against other promoters (6). The HC8 gene and the HC3 gene are both members of the α -type subfamily, but there is no similar negative element between -2325 and -125 in the 5'-flanking region of the HC8 gene.



ATTACATTAGAAATGGGGGTATGCTCTGTTCACGGCTTTTTCTTGAACACGTGGAATATACTAGCGCTCAAAAAATATCTTTAATCAGACTTATCTTTAACTCTTACCAAAAAGCGTTAT -589
ACTCATAGAAAGAGCACTAATACTTCCGTAAAATTCTAAGCTGAATTCAAACACGATACCCTTTAAGTGAAACTTCACGAGAGCCTGAGCACAAGGAGGTGAAGTGGATGCCATCAAGAT -469
TCTGGGTCAGGGTCGGGACGCGGAGGAAGACAGGTTACTTCGCGCCTACTGCTTTTTCTACTTTTTGTGAGAGTATACCAGCATCTCTCCCTTTGGCTTAAAGAAAGAATAAAGTGAGGA -349
GAGAATAATAATATAAAAGATAAATAATAATTCCAAGGAAAGGAATAGAGCAGCTTCTCAGATACCACGACGCCAAGCGGCTCAAAGTACGCGAACCGAAGAGGAGCCTTTGCTTTCCCC -229
AACGCCCCCGTCCATTGCTTCTGCACATGCGCGCCGCCAGCCTCTCGCGGCCCGCTTTTTTCCCACAATACTCCTCGTCCTGGGGGCCGGAGCCGCGGAACGAGCCACAGAGAAAAAGGTT -109
ATTGGTTGGCAGTTCAGCGAATGAGCGGGCCTGTTACTAGTTTGCGGCATCCTGTGGGATAGGGGAAGCGCTCCGGGCCTGGAATCCCTACGCGTCCCTTTGGGTTTAGCACGATGAGCT 12
+1 M S

Figure 1. Restriction map and nucleotide sequence of the promoter region of the subunit HC8 gene. The arrow indicates the 5' end of HC8 subunit cDNA, which was numbered as +1. Sites of the restriction endonucleases *Eco* RI (E), *Sac* I(S), *Xho*I (X), *Bst*XI (B), *Spe*I (Sp), *Hin* cII (H), *Acc*I (A), and *Sac* II (Sa) are shown. The sequenced region corresponding to the 5'-noncoding region including the translational initiation codon ATG is expanded. The transcribed region is boxed and the predicted amino acid sequence of exon 1 is shown below the nucleotide sequence. The GC, CAAT and TATA boxes are boxed in black.

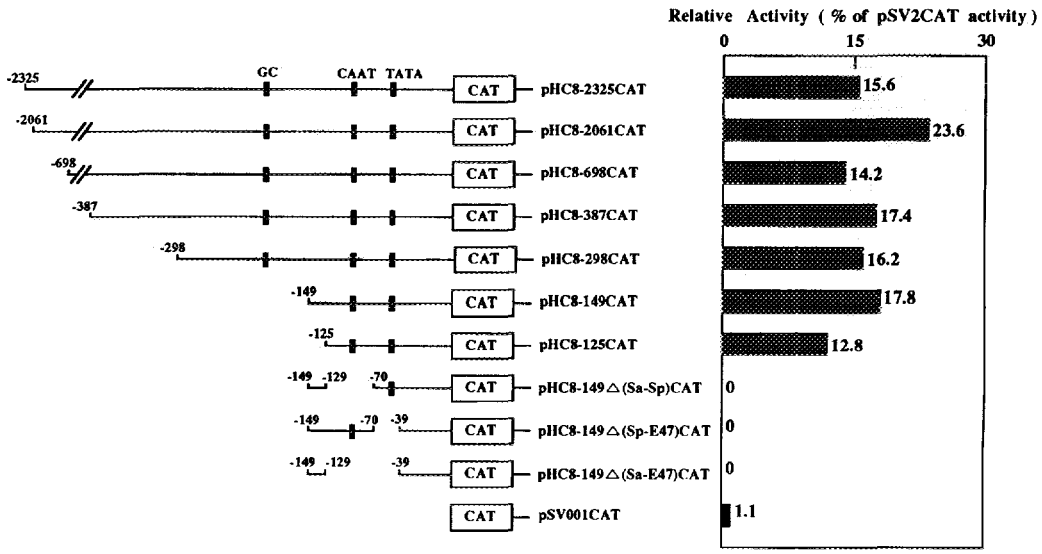


Figure 2. Promoter activity of the 5' flanking region of the HC8 gene. Relative CAT activities obtained with HC8 promoter-containing constructs are expressed as percentages of CAT activity obtained with pSV2CAT. pSV001CAT was used as a control. Experiments were repeated at least 3 times with essentially the same results and representative results are shown.

We have not yet identified any specific consensus region common to the sequences of the upstream regions of the genes for the HC8, HC3 and HC5 proteasome subunits.

For determination of whether a set of the proteasome genes is clustered, the localization of each subunit gene must be determined. The chromosomal localizations of several genes encoding human proteasome subunits have already been determined, as listed in Table 1. We determined the localization of the HC8 subunit gene of the human proteasome by fluorescence *in situ* hybridization (FISH) of prometaphase cells using a 6.0 kb human HC8 genomic fragment as a probe. Comparison of the fluorescence signals and the banding

Table 1. Chromosomal localizations of genes encoding human proteasome subunits

Name	Chromosomal localization	Reference
α-Type subunit		
PROS30(HC2)	11q15.1	Bey (12)
HC3	6q27	Okumura *et al.***
HC8	14q23	this paper
PROS27	14q13	Bey (12)
β-Type subunit		
HC5	7p12-p13	Okumura *et al.***
MB1(X)	14q11.2	Belich *et al.*(13)
Delta(Y)	17p13	Belich *et al.*(13)
LMP2	6p21	Monaco (14)
LMP7	6p21	Monaco (14)

**Okumura, K., Nogami, M., Taguchi H., Hisamatsu, H. and Tanaka K. (submitted).

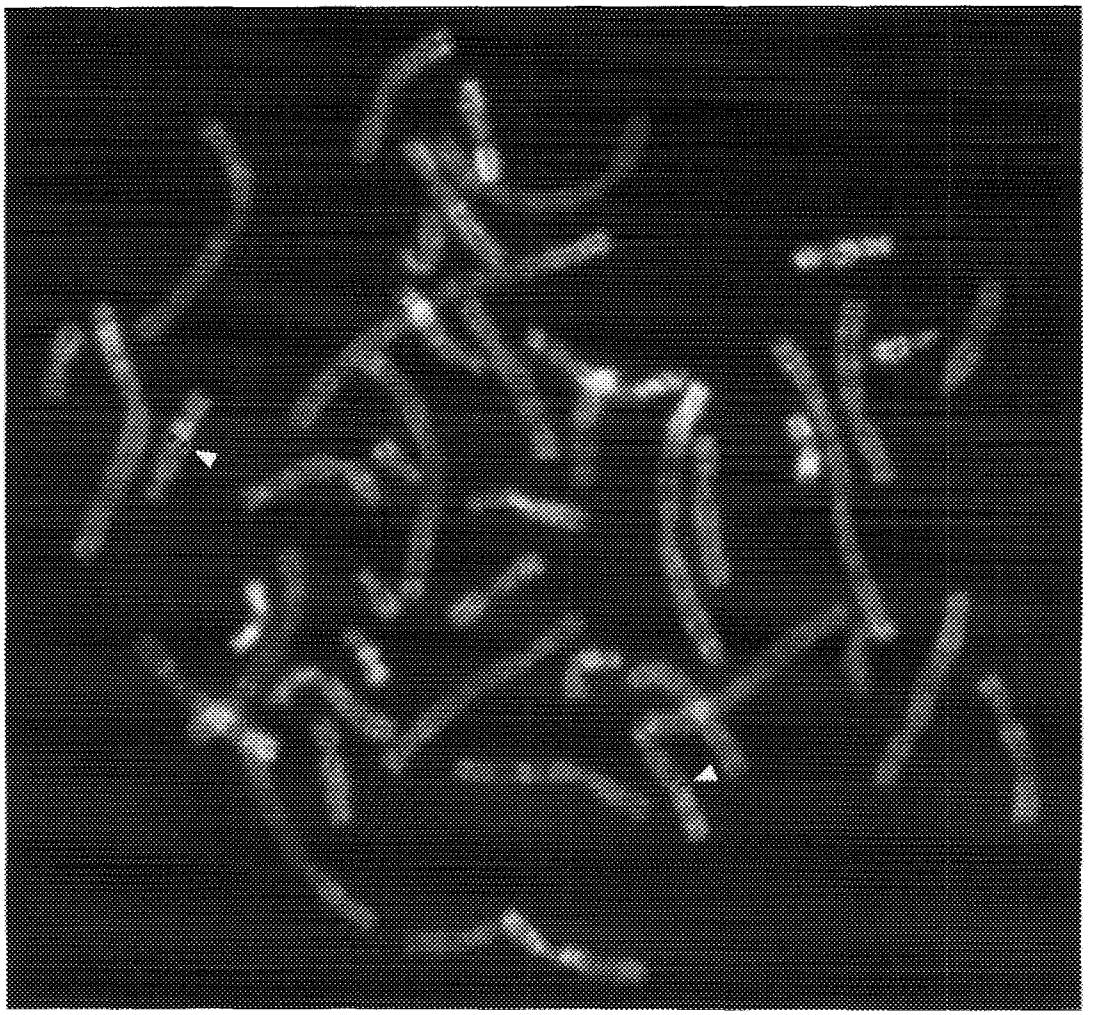


Figure 3. Chromosomal location of the HC8 subunit gene of the human proteasome by FISH. Fluorescence *in situ* hybridization was carried out using a 6.0-kb genomic DNA fragment of HC8 as a probe. Fluorescence signals on the R-banded chromosomes are indicated by arrowheads. The images were processed with Adobe Photoshop 2.5J (Adobe Systems Inc.), to obtain clear signals in a glossy print. FITC and propidium iodide images were shown in black and white, respectively. Typical fluorescence dots for the HC8 gene were observed on chromosome band 14q23. The images were directly printed with a Fuji Pictography 3000 from Macintosh computer.

patterns of the chromosomes indicated that the HC8 gene is located on chromosome band 14q23 (Figure 3). This result was based on observations on more than twenty prometaphase chromosomes. No consistent fluorescence signals were observed on other chromosomes.

Unlike eukaryotic proteasomes, the archaebacterial proteasome consists of only two kinds of polypeptides with significant homology named the α and β-subunits (16). Eukaryotic proteasomes have been classified into two subfamilies by their higher similarities to either the α and β-subunit of the archaebacterial proteasome (3). Two β-type subunit genes, LMP2 and LMP7, whose expressions are induced by interferon-γ, are located in the class II region

of chromosome 6p21. These findings are related to previous results suggesting possible involvement of the proteasome in the antigen-processing pathway (3, 14). But except for the LMP2 and LMP7 genes, the genes encoding human proteasome subunits have all been mapped to different chromosomes or different regions of the same chromosome. It is unknown whether the same chromosome assignment of the genes for HC8, Pros27 and MB1(=X) is meaningful. Determination of the exon-intron organizations of these genes may provide a clue to this problem. Further studies are necessary to clarify the evolutions of eukaryotic proteasome subunit genes and the mechanisms regulating the coordinated expression of the genes for the multiple subunits of the proteasome.

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