Proc. Natl. Acad. Sci. USA
Vol. 90, pp. 11247–11251, December 1993
Cell Biology

A human mitochondrial ATP-dependent protease that is highly homologous to bacterial Lon protease

(protease La/mitochondria/protein degradation)

NAN WANG*, SUSAN GOTTESMAN†, MARK C. WILLINGHAM‡, MICHAEL M. GOTTESMAN*§, AND MICHAEL R. MAURIZI*

Laboratories of *Cell Biology and of †Molecular Biology, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892; and ‡Division of Anatomic Pathology, Department of Pathology and Laboratory Medicine, Medical University of South Carolina, Charleston, SC 29425

Communicated by Richard D. Klausner, September 1, 1993

ABSTRACT **We have cloned a human ATP-dependent protease that is highly homologous to members of the bacterial Lon protease family. The cloned gene encodes a protein of 963 amino acids with a calculated molecular mass of 106 kDa, slightly higher than that observed by Western blotting the protein from human tissues and cell lines (100 kDa). A single species of mRNA was found for this Lon protease in all human tissues examined. The protease is encoded in the nucleus, and the amino-terminal portion of the protein sequence contains a potential mitochondrial targeting presequence. Immunofluorescence microscopy suggested a predominantly mitochondrial localization for the Lon protease in cultured human cells. A truncated *LON* gene, in which translation was initiated at Met[118] of the coding sequence, was expressed in *Escherichia coli* and produced a protease that degraded α-casein *in vitro* in an ATP-dependent manner and had other properties similar to *E. coli* Lon protease.**

Energy-dependent proteolysis plays a key role in prokaryotic and eukaryotic cells by regulating the availability of certain short-lived regulatory proteins, ensuring the proper stoichiometry of multiprotein complexes, and ridding the cell of abnormal and damaged proteins (1, 2). Among the ATP-dependent proteases, Lon protease encoded by the *lon* gene of *Escherichia coli* was one of the first to be identified and remains the best studied (1–4). Mutants in *lon* have decreased capacity for degradation of proteins with abnormal conformations (5, 6). *In vivo, E. coli* Lon protease specifically degrades several short-lived proteins, including the cell division inhibitor SulA and a transcriptional regulator, RcsA (7). Lon homologs have been identified in both Gram-negative and Gram-positive bacteria; in *Myxococcus*, one Lon-like protein has a role in development of fruiting bodies (8–11). *E. coli* also possesses a second ATP-dependent protease, Clp, which is a multicomponent protease that degrades a variety of specific proteins as well as abnormal proteins (1, 12, 13).

Several ATP-dependent protease activities in eukaryotic cells and organelles have been described. In the eukaryotic cytosol and nucleus, ATP hydrolysis is required for both conjugation to ubiquitin and proteolysis by the 26S protease, the mostly completely characterized of the ATP-dependent proteolytic systems in eukaryotes (14). DNA sequence analysis indicates that conserved members of the Lon and Clp families may exist in eukaryotic cells (15–18); however, there appears to be little functional or evolutionary relationship between these protease families and the 26S protease (19). The autonomy of eukaryotic organelles suggests that they may have independent ATP-dependent proteolytic systems. For example, the degradation system of the endoplasmic reticulum may not involve the 26S protease (20). Energy-dependent protease activity has been detected in isolated mitochondria (21, 22) and chloroplasts (23), and enzymatic properties of these proteases suggested their similarity to the *E. coli* Lon protease.

Here, we describe the cloning and characterization of a human ATP-dependent protease¶ which is highly homologous to other bacterial *lon* gene products. We demonstrate that the human Lon protease, which is encoded in the nucleus, is expressed in all human tissues and is localized in mitochondria.

MATERIALS AND METHODS

Materials. Protein A-Sepharose and Mono Q were obtained from Pharmacia. Sequenase version 2.0 was from United States Biochemical. Polymerase chain reaction (PCR) reagents were purchased from Perkin–Elmer/Cetus.

DNA Sequencing. DNA sequencing was performed with the dideoxy chain-termination method (24). Either automatic sequencing with a Genesis 2000 system (DuPont) or manual sequencing with a Sequenase version 2.0 kit was used. Universal T7 and T3 oligonucleotide primers (Promega) as well as custom primers synthesized by the 308A DNA synthesizer (Applied Biosystems) were used to sequence both strands of *LON* cDNA.

Preparation of Antisera and Western Analysis. cDNA clone pHHCPK77 was obtained from the American Type Culture Collection. This clone, which was reported to contain sequences homologous to bacterial Lon protease (16), was mutated to insert an *Nde* I restriction site at a methionine codon near the 5′ end of the partial reading frame for the C-terminal fragment of human Lon. PCR was employed with a 5′ primer spanning the ATG and a 3′ primer positioned downstream from the *Eco*RI site in the vector. The PCR product was cut with *Nde* I and *Eco*RI and ligated to pVex11 [a derivative of the pET vectors (25), generously provided by V. Chaudhary, National Cancer Institute] cut with the same enzymes. The resulting clone, pLON/hu1, expressed a 51-kDa fragment of human Lon protein under the control of the T7 RNA polymerase in *E. coli* BL21(DE3) cells (25). The protein was induced in *E. coli* BL21(DE3) carrying pLON/hu1, and the inclusion-body fraction containing the insoluble protein was partially purified by the method of Bruggemann *et al.* (26). The washed inclusion-body protein was used to immunize rabbits as described (26). The IgG fraction of the rabbit polyclonal anti-human Lon protease was purified on a

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

§To whom reprint requests should be addressed at: Laboratory of Cell Biology, National Cancer Institute, National Institutes of Health, Building 37, Room 1B22, Bethesda, MD 20892.

¶The sequence reported in this paper has been deposited in the GenBank data base (accession no. U02389).

protein A-Sepharose column. Western blotting was performed and Amersham's ECL Western blotting reagents were used to detect the antigen.

Isolation of the cDNA Clones. A cDNA probe was made with pHHCPK77 and used to screen by filter hybridization a human hippocampal cDNA library (Stratagene, no. 936205). The cDNA inserts from the positive phage clones were rescued as phagemids by *in vivo* excision (27). The size of the cDNA inserts was determined by *Eco*RI digestion and agarose gel electrophoresis. cDNA clones containing inserts of >2 kb were further analyzed by restriction mapping and PCR. One clone, pBluescript SKII/HHL11, contained an open reading frame sufficient to encode a human Lon protease.

Subcloning and Mutagenesis of Human LON Clones in *E. coli*. The *Kpn* I–*Bam*HI fragment from pBluescript SKII/HHL11 was subcloned into the *Kpn* I–*Bam*HI site of pVEX11 to create pLON/hu2. To express the full-length human Lon protein in *E. coli*, the plasmid pLON/hu2 was cut with *Nde* I and a blunt end was made with Klenow DNA polymerase; then the linearized DNA was partially digested with *Bgl* I and a blunt end was made with T4 DNA polymerase. Ligation and transformation yielded a clone, pLON/hu3, with the initiator ATG codon positioned 12 bases from the Shine–Dalgarno sequence of the vector, as well as an unexpected mutant construct, pLON/hu4, in which the starting ATG was altered to AGG. The latter clone provided a useful control for subsequent experiments, because no Lon protein expression was obtained when the initiator codon was mutated.

Two additional mutant clones were constructed by deleting DNA coding for the N-terminal region of human Lon. Oligonucleotides homologous to the DNA sequences including either the second (Met[118]) or third (Met[200]) methionine codon altered to incorporate an *Nde* I restriction enzyme site were chemically synthesized. pLON/hu3 DNA was used as a template for the PCR with one of these primers and a primer homologous to a downstream region beyond an *Sac* II restriction site. The PCR product was cut with *Nde* I and *Sac* II and ligated to a purified fragment of pLON/hu3 cut with the same enzymes (Fig. 1). These clones contained open reading frames with 846 (pLON/hu5) or 763 (pLON/hu6) amino acids. All constructions were subsequently verified by DNA sequencing.

Immunofluorescence. For immunofluorescence localization of human Lon protease, human fetal lung WI-38, and human foreskin Detroit 532 cells grown in 35-mm plastic tissue culture dishes were fixed with 3.7% formaldehyde in phosphate-buffered saline (PBS) for 10 min at 23°C and then permeabilized with 0.1% Triton X-100 in PBS for 3 min. The cells were then incubated with affinity-purified rabbit anti-human Lon protease (2 μg/ml in normal goat globulin/0.1% saponin/PBS for 30 min at 23°C) followed by affinity-purified goat anti-rabbit IgG conjugated to rhodamine (25 μg/ml), as

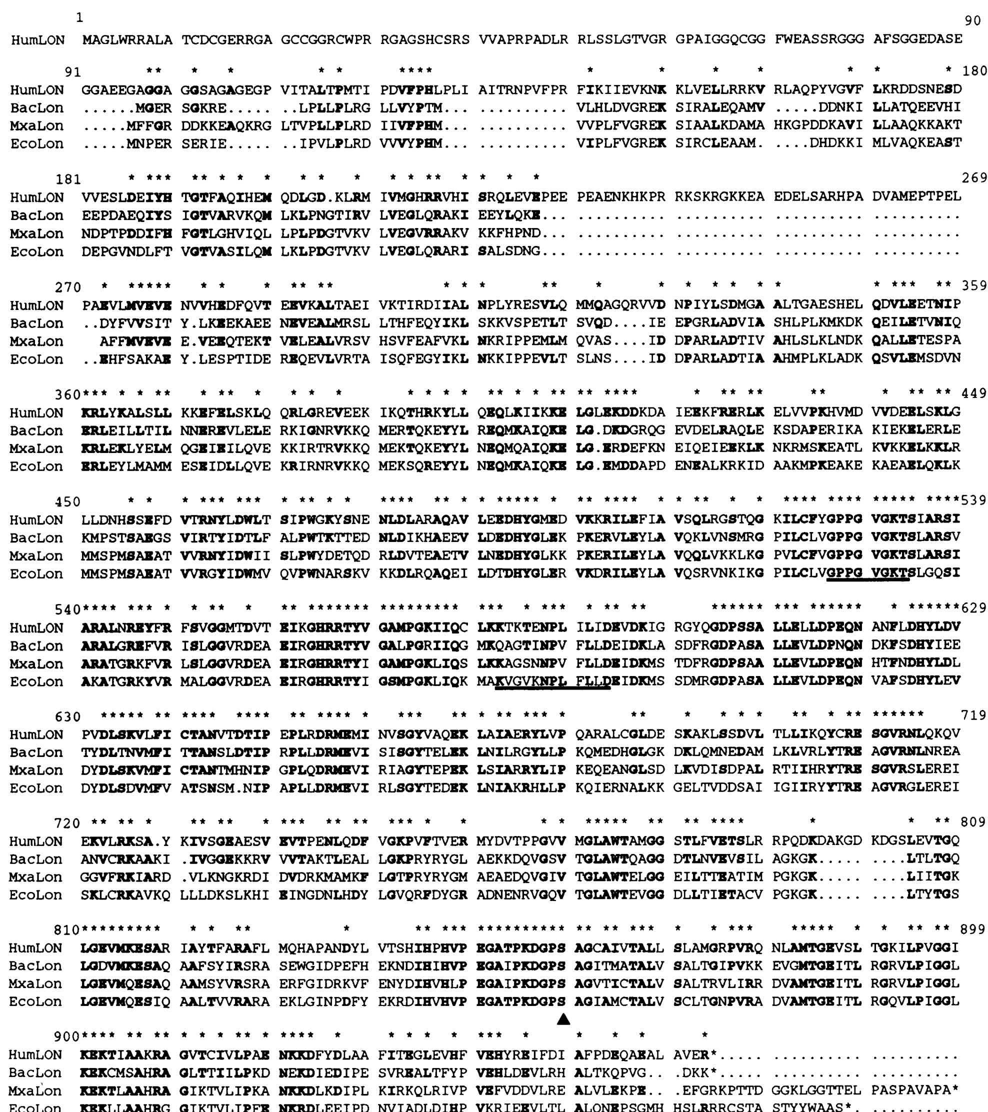


FIG. 1. Alignment of amino acid sequence of Lon proteases. The numbers enumerate the amino acid residues of Lon protease deduced from the nucleotide sequence of clone pLon/hu2. Hum, human hippocampus; Bac, *Bacillus brevis* (8); Mxa, *Myxococcus xanthus* (vegetative growth phase) (10); Eco, *E. coli* (28, 29). The stars in the number line indicate the positions in which human Lon is identical to at least one of the bacterial Lons; identical amino acids are shown in bold letters. A serine residue at the putative active site (29) is indicated by an arrowhead, and the proposed ATP-binding site, which has a Walker-type consensus motif (30), is underlined. The stars in the amino acid line indicate the stop codons.

described (31). The cells were visualized with a Zeiss Axioplan epifluorescence microscope with rhodamine filters and photographed with Kodak Tri-X film.

Protease and Peptidase Assays *in Vitro* and *in Vivo*. Lon protease was assayed at 37°C in 50 mM Tris·HCl, pH 8.0/10 mM $MgCl_2$/1 mM dithiothreitol containing [^{3}H]α-casein [7000 cpm/μg, prepared as described (32)] at 25–50 μg/ml, with or without 4 mM ATP. A unit of protease activity was defined as the degradation of 1 μg of casein per hour into trichloroacetic acid-soluble form (32). Peptidase activity using succinyl-Ala-Ala-Phe methoxynaphthylamide as a substrate was measured as described (33).

RESULTS

Sequence of the cDNA and Polypeptide Encoded by the *LON* Gene. Clone HHCPK77, a cDNA with homology to *E. coli lon*, was derived from a human brain library and was partially sequenced by Adams *et al.* (16) as part of an effort to tabulate expressed sequence tags from random human cDNAs. Our preliminary analysis of this clone confirmed its homology to bacterial Lon. pHHCPK77 contained a 1.8-kb insert and was lacking about 1.6 kb of the 5′ coding region of the *LON* open reading frame. Using a 1.8-kb *Eco*RI cDNA fragment from clone HHCPK77 as a probe, we isolated 54 cDNA clones from the cDNA library. cDNA clones containing inserts of >2 kb were further analyzed by restriction mapping and PCR. PCR was performed with an internal primer derived from pHHCPK77 cDNA with an orientation toward the 5′ end of the cDNA combined with a primer from either the T7 or the T3 promoter which flanked the cDNA insert of the phagemid. Clones confirmed by both restriction mapping and PCR were further analyzed by DNA sequencing. One clone, pBluescript SKII/HHL11, appeared to contain a complete open reading frame for a protein of the expected size of human Lon protease.

pBluescript SKII/HHL11 contained a cDNA insert of 3193 bp with an open reading frame of 2889 bases, encoding a protein of 963 amino acids with a calculated molecular weight of ≈106,000 (Fig. 1). We refer to the protein encoded by this open reading frame as human Lon protease. The context of the ATG translation start codon (CCAGTATGG) compared favorably with the consensus sequence for translation initiation in vertebrates, CC(A/G)CCATGG (34). The 3′ noncoding segment contains a variant polyadenylylation signal, ATTAAA (35), 11 bases upstream of the poly(A) tail. A computer-assisted RNA secondary structure prediction of the 450-bp 5′ segment yielded a very complex structure with 70% G+C content (data not shown), perhaps explaining the scarcity of full-length cDNAs isolated.

Homology of Human Lon Protease to Other Bacterial *lon* Gene Products. The complete predicted amino acid sequence of human Lon protease and its alignment with bacterial Lon proteases are shown in Fig. 1. This putative human Lon protease sequence showed 38%, 37%, and 36% identity with those of *B. brevis*, *M. xanthus*, and *E. coli*. Human Lon is identical to one or more of these bacterial Lon proteins at 46% of positions (excluding the N-terminal 98 amino acids of human Lon which are nonhomologous). The complete identity among the four sequences is 23%. The alignment revealed at least two highly conserved regions. The first region (amino acids 526–597 in the human Lon sequence) has ≈47% complete identity and contains the consensus sequence for ATP-binding sites (28). The second region (amino acids 844–870) shows approximately 73% complete identity and includes the serine residue at the putative proteolytic active site (29).

Lon Protease Expression in Human Tissues. Northern blot analysis showed a single band of ≈3.4 kb detectable in all the human tissues examined (Fig. 2*A*), as well as in several cell lines (data not shown). Higher levels of mRNA were detected

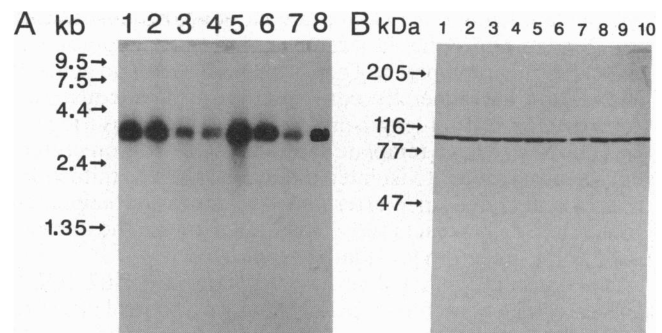


FIG. 2. Expression of the human *LON* gene. (*A*) Northern analysis of *LON* RNA in human tissues. Lanes: 1, heart; 2, brain; 3, placenta; 4, lung; 5, liver; 6, skeletal muscle; 7, kidney; 8, pancreas. (*B*) Western analysis of Lon protease in human cell lines. For each lane, 15 μg of protein of whole cell lysate was loaded and the proteins were resolved under reducing conditions in an SDS/10% polyacrylamide gel. The prestained protein molecular size standards from Bio-Rad were used to indicate the apparent mass of the antigen. Lanes: 1, monkey kidney COS-1 cells; 2, human glioma U251 cells; 3, human breast cancer MCF7 cells; 4, human cervical carcinoma HeLa cells; 5, human cervical carcinoma KB cells; 6, human kidney cancer A1212 cells; 7, human foreskin fibroblast Detroit 532 cells; 8, human embryonic lung fibroblast WI-38 cells; 9, human hepatoma BEL-7404 cells; 10, human Burkitt lymphoma CA-46 cells.

in heart, brain, liver, and skeletal muscles. Western blot analysis using an antiserum (no. 4712) prepared against a fragment of bacterially expressed human Lon (see *Materials and Methods*) revealed a major antigen band of ≈100 kDa in all the human and monkey cell lines examined (Fig. 2*B*). The level of this protein was comparable in different cell lines and paralleled the mRNA level in these cell lines (data not shown). The anti-human Lon protease antibody also recognized an antigen with similar molecular mass in rat and mouse and crossreacted with *E. coli* Lon protease (data not shown).

Immunolocalization of Lon in Mitochondria from Mammalian Cells. Antibodies against human Lon were purified on an affinity column made with the C terminal portion of human Lon and were used to localize Lon in mammalian cells by indirect immunofluorescence. Most of the fluorescence was associated with structures that appeared to be mitochondria on the basis of their cytoplasmic localization and shape (Fig. 3). Although mitochondrial targeting peptides lack a common consensus sequence, a certain bias in the composition and positional distribution of amino acids has been reported (36, 37). Targeting signals are rich in positively charged and

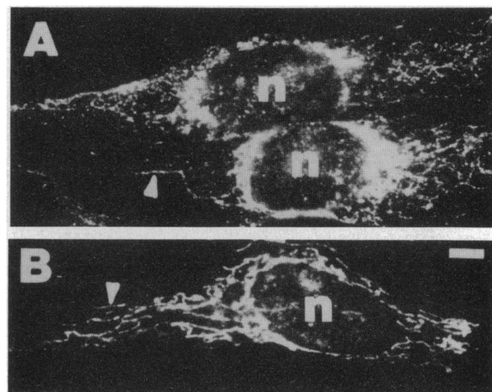
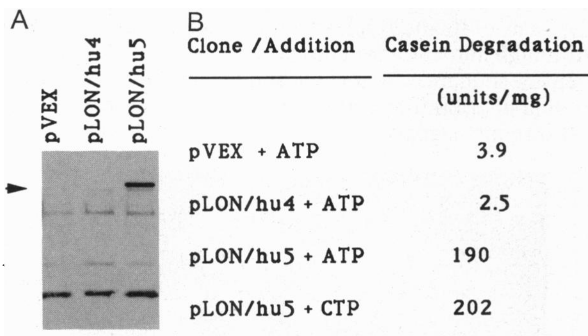


FIG. 3. Immunofluorescence localization of human Lon protease in cultured cells. Detroit 532 (*A*) and WI-38 (*B*) cells were labeled for immunofluorescence with anti-human Lon antibody. Both cell types showed a mitochondrial pattern of localization, in which individual mitochondria (arrowheads) can be seen in flattened areas of the cytoplasm. n, Nucleus. (Bar = 7.5 μm.)

hydroxylated amino acids, are devoid of acidic amino acids or extended hydrophobic stretches, and most importantly, are markedly amphiphilic. The N-terminal 60 amino acids of human Lon are generally consistent with these consensus features (Fig. 1). Full-length human *LON* RNA transcribed *in vitro* can be used as a template for translation of a Lon protein that is transported into isolated mitochondria. The mitochondrial Lon detected on Western blots or immunoprecipitated from whole cell extracts is 5–7 kDa smaller than the unprocessed form made *in vitro* (data not shown).

Expression of Human Lon in *E. coli*. *E. coli* BL21(DE3) cells carrying vector alone, pLON/hu5, or a control plasmid, pLON/hu4, were grown at 37°C and induced with 1 mM isopropyl β-D-thiogalactopyranoside for 2 hr. BL21 is naturally *lon*⁻ (38). Western blots of extracts from the cells carrying pLON/hu5 revealed a protein of about 95 kDa that crossreacted with antibody against human Lon (Fig. 4*A*). No equivalent band was observed in the extracts of cells with pLON/hu4 or the vector, pVEX (Fig. 4*A*). The amount of Lon protein obtained in induced cells was relatively low compared with other proteins expressed with the T7 system. Whether this reduced expression is related to secondary structure in the 5′ end of the mRNA is not known. Extracts of cells carrying pLON/hu5 had greatly increased ATP-dependent casein-degrading activity compared with control cells (Fig. 4*B*). Casein degradation by the cloned protease was activated by CTP as well as ATP, as has been seen with *E. coli* Lon and the rat liver ATP-dependent protease. The cloned activity was, however, completely insensitive to PinA (data not shown); PinA is a T4 phage protein that is a specific and highly potent inhibitor of the *E. coli* Lon protease (J. Hilliard, L. Simon, and M.R.M., unpublished work).

Mono Q anion-exchange chromatography of an extract of cells carrying pLON/hu5 revealed a major peak of ATP-dependent casein-degrading activity that coincided with the presence of the 95-kDa human Lon protein on Western blots (Fig. 5). Smaller amounts of ATP-dependent protease activity were detected in the flow-through and in low salt fractions from the column. These fractions also contained human Lon protease; the lower activity may be the result of partial degradation or other modification of the enzyme in cells or in extracts during preparation. The fractions with the highest ATP-dependent casein-degrading activity also showed an adenosine 5′-[β,γ-imido]triphosphate-stimulated hydrolysis of succinyl-Ala-Ala-Phe methoxynaphthylamide, a fluorogenic peptide substrate for *E. coli* Lon protease and the rat liver Lon-like ATP-dependent protease (data not shown).



Clone /Addition	Casein Degradation (units/mg)
pVEX + ATP	3.9
pLON/hu4 + ATP	2.5
pLON/hu5 + ATP	190
pLON/hu5 + CTP	202

FIG. 4. Human Lon expressed in *E. coli*. (*A*) Western analysis. *E. coli* BL21(DE3) cells carrying vector alone (pVEX), a clone with the initiator codon mutated to AGG (pLON/hu4), and the clone in which translation is initiated at Met[118] (pLON/hu5) were grown to an OD_{600} of 1.0 and induced for 2 hr with 1 mM isopropyl β-D-thiogalactopyranoside. Cell extracts were made and about 5 μg of protein from the 30,000 × *g* supernatant was loaded on an SDS/polyacrylamide gel. Human Lon (arrowhead) was detected by Western blotting using antibody raised against the C-terminal 60% of human Lon. (*B*) ATP-dependent proteolysis by *E. coli*-expressed human Lon. Extracts of clones carrying vector alone (pVEX), pLON/hu4, or pLON/hu5 were assayed for casein-degrading activity in the presence and absence of ATP or CTP. Only NTP-dependent activity is shown; all extracts expressed between 13 and 20 units of casein-degrading activity in the absence of NTP.

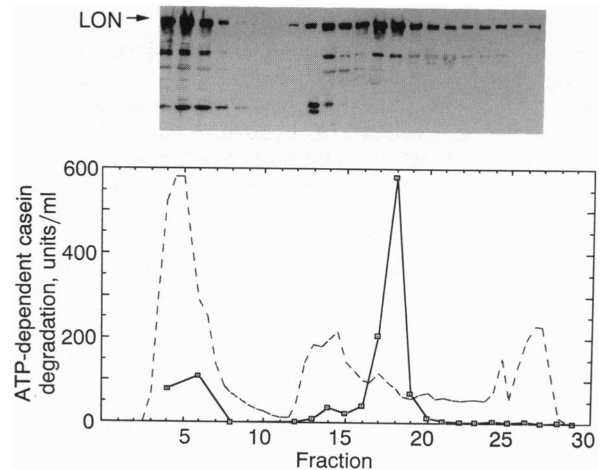


FIG. 5. Separation of Lon protease by anion-exchange chromatography on Mono Q. An extract of induced cells carrying pLON/hu5 was made, and 2 ml of soluble protein was loaded on a 5/5 Mono Q column equilibrated in 50 mM Tris·HCl, pH 7.5/1 mM EDTA/10 mM $MgCl_2$/1 mM dithiothreitol/10% glycerol. Proteins were eluted with a gradient of KCl (20 mM/ml). Fractions (0.75 ml) were assayed for casein-degrading activity with and without ATP, and the increase in activity in the presence of ATP is shown (⊡). The dashed line represents A_{280} for the eluted protein; maximum absorbance in fractions 3 and 4 was about 2.0.

DISCUSSION

Mitochondria are semi-autonomous organelles that generate ATP via oxidative phosphorylation and whose morphology and functions appear to be developmentally and physiologically regulated. Considerable interest is now directed toward understanding the possible contributions of mitochondrial abnormalities to a number of human degenerative diseases, including various myopathies, adult-onset diabetes, Parkinson disease, and Alzheimer disease (39, 40). The identification of genes for regulatory enzymes that may control developmental and physiological changes in mitochondria will greatly contribute to our understanding of mitochondrial function and failure. We report here the sequence and preliminary characterization of a human mitochondrial protein highly homologous to the bacterial Lon protease. Lon is an ATP-dependent protease that degrades a number of regulatory proteins in bacterial cells and is an important part of developmental pathways and stress responses (1).

We isolated a cDNA clone that encodes an ATP-dependent protease that is highly homologous to the *E. coli* ATP-dependent Lon protease. Northern and Western analyses suggest that adult human cells express only a single major species of Lon protease. Immunofluorescent staining of cultured cells with antibody raised against the cloned protein indicates a predominantly mitochondrial distribution for the protease. It seems likely that the Lon protease reported here is similar to the biochemically described ATP-dependent protease from rat liver (41) and bovine adrenal cortex (22), both of which were described as Lon (or protease La)-like proteases. The human Lon has a similar subunit size, and preliminary characterization of the cloned protease suggests that the enzymatic properties are similar. The rat liver protease was reported to fractionate with matrix components, and the sequence of the signal portion of the Lon

protein suggests a matrix localization. While these data argue for the presence of Lon in mitochondria, we cannot rule out the occurrence of small amounts of Lon outside of mitochondria.

Considerable progress has been made in recent years concerning the processing or maturation of proteins imported into mitochondria. Limited proteolysis of precursor proteins is not energy-dependent and is carried out by one of several well-characterized proteases (42). Turnover of mitochondrial proteins is less well understood. Mitochondrial proteins display heterogeneous rates of degradation (half-lives of 1–40 hr) which can vary according to the physiological state of the cell (43). It is likely that the alterations in structure and composition of mitochondria in response to regulatory signals or changes in nutritional conditions may depend on the differential degradation of preexisting proteins and the synthesis of new ones.

Proteins synthesized in isolated mitochondria are rapidly degraded, with half-lives of <60 min (41). There are several plausible explanations for the short half-life of mitochondrial proteins. Because many mitochondrial proteins occur in complexes with cytoplasmically synthesized proteins imported into mitochondria, newly synthesized proteins in isolated mitochondria may represent "proteins without partners," a class of proteins whose unsatisfied intersubunit bonding domains render them susceptible to rapid degradation in all cells (1, 7). Mitochondrial proteins may be more susceptible to oxidative damage owing to their proximity to the electron transport enzymes. Abnormal proteins produced in the presence of puromycin are also subject to rapid degradation in mitochondria (43). It is likely that one function of the mitochondrial Lon protease, as for the Lon protease of *E. coli*, is to degrade abnormal proteins that arise from damage to proteins, errors in synthesis, or uncoordinated synthesis of multimeric proteins. In *E. coli* and other bacteria, and for bacteriophage, Lon protease is also responsible for the degradation of specific regulatory proteins and thus plays a pivotal role in developmental pathways and stress responses (1, 9, 11). The remarkable conservation of the Lon protease in human mitochondria suggests similarly important functions for the mitochondrial enzyme, which remain to be elucidated.

Preliminary sequence information indicates that a homolog of Lon protease is also found in *Saccharomyces cerevisiae* (C. Suzuki, personal communication). Disruption of the putative Lon protease gene of yeast results in an inability to grow on nonfermentable carbon sources (C. Suzuki, personal communication; ref. 44), suggesting that mitochondrial function is impaired in the absence of Lon. Identification of the essential targets of Lon-dependent proteolysis will add to our understanding of mitochondrial function and may lead to a further understanding of mitochondrial abnormalities and their contribution to human degenerative diseases.

We thank Carolyn Suzuki for communicating her unpublished results on yeast Lon to us. We thank M. Adams for providing the sequence of the pHHCPK77 clone.

1. Gottesman, S. & Maurizi, M. R. (1992) *Microbiol. Rev.* **56**, 592–621.
2. Goldberg, A. L. (1992) *Eur. J. Biochem.* **203**, 9–23.
3. Charette, M., Henderson, G. W. & Markovitz, A. (1981) *Proc. Natl. Acad. Sci. USA* **78**, 4728–4732.
4. Chung, C. H. & Goldberg, A. L. (1981) *Proc. Natl. Acad. Sci. USA* **78**, 4931–4935.
5. Maurizi, M. R., Trisler, P. & Gottesman, S. (1985) *J. Bacteriol.* **164**, 1124–1135.
6. Goff, S. A. & Goldberg, A. L. (1987) *J. Biol. Chem.* **262**, 4508–4515.
7. Gottesman, S. (1989) *Annu. Rev. Genet.* **23**, 163–198.
8. Ito, K., Udaka, S. & Yamagata, H. (1992) *J. Bacteriol.* **174**, 2281–2287.
9. Tojo, N., Inouye, S. & Komano, T. (1993) *J. Bacteriol.* **175**, 4545–4549.
10. Tojo, N., Inouye, S. & Komano, T. (1993) *J. Bacteriol.* **175**, 2271–2277.
11. Gill, R. E., Karlok, M. & Benton, D. (1993) *J. Bacteriol.* **175**, 4538–4544.
12. Katayama-Fujimura, Y., Gottesman, S. & Maurizi, M. R. (1987) *J. Biol. Chem.* **262**, 4477–4485.
13. Hwang, B. J., Park, W. J., Chung, C. H. & Goldberg, A. L. (1987) *Proc. Natl. Acad. Sci. USA* **84**, 5550–5554.
14. Hershko, A. & Ciechanover, A. (1992) *Annu. Rev. Biochem.* **61**, 761–807.
15. Adams, M. D., Kerlavage, A. R., Fields, C. & Venter, J. C. (1993) *Nature Genet.* **4**, 256–267.
16. Adams, M. D., Dubnick, M., Kerlavage, A. R., Moreno, R., Kelley, J. M., Utterback, T. R., Nagle, J. W., Fields, C. & Venter, J. C. (1992) *Nature (London)* **355**, 632–634.
17. Gottesman, S., Squires, C., Pichersky, E., Carrington, M., Hobbs, M., Mattick, J. S., Dalrymple, B., Kuramitsu, H., Shiroza, T., Foster, T., Clark, W. P., Ross, B., Squires, C. L. & Maurizi, M. R. (1990) *Proc. Natl. Acad. Sci. USA* **87**, 3513–3517.
18. Maurizi, M. R., Clark, W. P., Kim, S.-H. & Gottesman, S. (1990) *J. Biol. Chem.* **265**, 12546–12552.
19. Dubiel, W., Ferrell, K., Pratt, G. & Rechsteiner, M. (1992) *J. Biol. Chem.* **267**, 22699–22702.
20. Klausner, R. D. & Sitia, R. (1990) *Cell* **62**, 611–614.
21. Desautels, M. & Goldberg, A. L. (1982) *J. Biol. Chem.* **257**, 11673–11679.
22. Watabe, S. & Kimura, T. (1985) *J. Biol. Chem.* **260**, 14498–14504.
23. Malek, L., Bogorad, L., Ayers, A. R. & Goldberg, A. L. (1984) *Fed. Eur. Biochem. Soc.* **166**, 253–257.
24. Sanger, F., Nicklen, S. & Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. USA* **74**, 5463–5467.
25. Studier, F. W., Rosenberg, A. H., Dunn, J. J. & Dubendorff, J. W. (1990) *Methods Enzymol.* **185**, 60–89.
26. Bruggemann, E. P., Chaudhary, V., Gottesman, M. M. & Pastan, I. (1991) *BioTechniques* **10**, 202–209.
27. Short, J. M., Fernandez, J. M., Sorge, J. A. & Huse, W. D. (1988) *Nucleic Acids Res.* **16**, 7583–7600.
28. Chin, D. T., Goff, S. A., Webster, T., Smith, T. & Goldberg, A. L. (1988) *J. Biol. Chem.* **263**, 11718–11728.
29. Amerik, A. Y., Antonov, V. K., Gorbalenya, A. E., Kotova, S. A., Rotanova, T. V. & Shimbarevich, E. V. (1991) *FEBS Lett.* **287**, 211–214.
30. Walker, J. E., Saraste, M., Runswick, M. J. & Gay, N. J. (1982) *EMBO J.* **1**, 945–951.
31. Willingham, M. C. & Pastan, I. (1985) *An Atlas of Immunofluorescence in Cultured Cells* (Academic, Orlando, FL), pp. 1–13.
32. Maurizi, M. R. (1987) *J. Biol. Chem.* **262**, 2696–2703.
33. Waxman, L. & Goldberg, A. L. (1985) *J. Biol. Chem.* **260**, 12022–12028.
34. Kozak, M. (1991) *J. Biol. Chem.* **266**, 19867–19870.
35. Gaskins, C. J., Smith, J. F., Ogilvie, M. K. & Hanas, J. S. (1992) *Gene* **120**, 197–206.
36. Hendrick, J. P., Hodges, P. E. & Rosenberg, L. E. (1989) *Proc. Natl. Acad. Sci. USA* **86**, 4056–4060.
37. von Heijne, G., Steppuhn, J. & Herrmann, R. G. (1989) *Eur. J. Biochem.* **180**, 535–545.
38. Studier, F. W. & Moffatt, B. A. (1986) *J. Mol. Biol.* **189**, 113–130.
39. Wallace, D. C. (1992) *Annu. Rev. Biochem.* **61**, 1175–1212.
40. Taylor, R. (1992) *J. Natl. Inst. Health Res.* **4**, 62–66.
41. Desautels, M. & Goldberg, A. L. (1982) *Proc. Natl. Acad. Sci. USA* **79**, 1869–1873.
42. Glover, L. A. & Lindsay, J. G. (1992) *Biochem. J.* **284**, 609–620.
43. Desautels, M. (1986) in *Mitochondrial Physiology and Pathology*, ed. Fiskum, G. (Van Nostrand Reinhold, New York), pp. 40–65.
44. Hahn, J., Pinkham, J., Wei, R., Miller, R. & Guarente, L. (1988) *Mol. Cell. Biol.* **8**, 655–663.