The Human LON Protease Binds to Mitochondrial Promoters in a Single-Stranded, Site-Specific, Strand-Specific Manner†

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ABSTRACT: LON proteases, which are ATP-dependent and exhibit ATPase activity, are found in bacteria, yeast, and humans. In *Escherichia coli*, LON is known to regulate gene expression by targeting specific regulatory proteins for degradation. The yeast and human LON proteins are encoded in the nucleus but localize to the mitochondrial matrix. In yeast, LON has been shown to be essential for the maintenance of the integrity of the mitochondrial genome. *E. coli* Lon has long been known to bind DNA, but we have only recently demonstrated that it binds preferentially to a specific TG-rich double-stranded sequence. We now show that human LON recognizes a very similar site in both the light and heavy chain promoters of the mitochondrial genome, in a region which is involved in regulating both DNA replication and transcription. Unlike *E. coli* Lon, however, human LON specifically binds to the TG-rich element only when it is presented in the context of a single DNA strand. These findings suggest that the human LON protease might regulate mitochondrial DNA replication and/or gene expression using site-specific, single-stranded DNA binding to target the degradation of regulatory proteins binding to adjacent sites in mitochondrial promoters.

The product of the *Escherichia coli lon* gene is the 87 kDa ATP-dependent Lon protease. Lon also has ATPase activity and is involved in the heat-shock response (*1*). In *E. coli*, Lon plays several important regulatory roles through the degradation of abnormal cellular proteins and targeted breakdown of several short-lived regulatory proteins (*2*−*4*). In other prokaryotes, Lon has been shown to play a role in gene regulation through the targeted degradation of transcription factors directly involved in gene expression (*5*, *6*). In eukaryotes, the yeast LON homologue has been cloned and was found to localize to the mitochondria (*7*). Yeast cells lacking LON were found to have deficient respiration and were not able to maintain a functional mitochondrial genome (*7*, *8*). Introduction of the *E. coli* Lon into these LON− cells can partially restore mitochondrial genome integrity, showing remarkable functional conservation of the LON protease between distantly related organisms (*9*). The LON protease has also been cloned in humans and found to exhibit an ATP-dependent proteolytic activity (*10*). Like yeast LON, human LON was found exclusively in the mitochondrial matrix (*11*). The function of LON in humans is not understood.

The human mitochondrial genome is a 16.6 kilobase closed circular double-stranded DNA (*12*, *13*). Replication of mitochondrial DNA (mtDNA)[1] originates bidirectionally on two separate strands: the heavy (H) strand (guanine rich) and the light (L) strand (cytosine rich). Most of the mtDNA encodes for oxidative phosphorylation genes, structural rRNAs, and tRNAs, leaving a small noncoding control region, termed the displacement (D)-loop. Within the D-loop are the two mtDNA promoters: the heavy strand promoter (HSP) and the light strand promoter (LSP) (*14*, *15*). Recognition by mitochondrial RNA polymerase of both promoters requires a transcription factor, termed mtF1 (*16*−*18*), which binds to the LSP and HSP in an orientation-independent manner to stimulate transcription (*15*). The mitochondrial RNA polymerase is unlike the nuclear RNA polymerase complex. In addition to its role in transcription, mtRNA polymerase participates in DNA replication as well as transcription (*19*). Replication of mtDNA depends largely on mtDNA polymerase (pol γ). Pol γ is in turn stimulated by a mitochondrial single-stranded binding protein (mtSSB), which binds preferentially to the single-stranded DNA of the D-loop region (*20*−*22*). Like LON, mtSSB counterparts can be found in *E. coli* and yeast. This protein is critical to mtDNA replication because its deletion in yeast leads to loss of mtDNA (*23*).

Although it has been known for some time that *E. coli* Lon binds to DNA (*24*) and that this interaction enhances

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[1] Abbreviations: mtDNA, mitochondrial DNA; HS, heavy strand; LS, light strand; D-loop, displacement loop; HSP, heavy strand promoter; LSP, light strand promoter; pol γ, mtDNA polymerase (polymerase γ); mtSSB, mitochondrial single-stranded binding (protein); a.s., antisense; s., sense; d.s., double stranded; HIV-2, human immunodeficiency virus type 2; pets, peri-ets; aP2, adipocyte P2.

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FIGURE 1: Sequence comparison of the mitochondrial heavy (HSP) and light strand promoters (LSP) with the DNA sequence of the pets element from the human immunodeficiency virus type 2 (HIV-2) enhancer. The DNA sequences of the mitochondrial promoters (*15*) and the HIV-2 enhancer (*26*, *27*) have been reported elsewhere. A vertical line between two bases denotes similar sequences between the different promoter elements. For all experiments employing single-stranded oligonucleotide probes, H(a.s.) represents the HSP coding strand and L(a.s.) represents the LSP noncoding strand. The positions of the promoter elements are as indicated.

the enzyme's proteolytic activity (*25*), a specific DNA recognition sequence has not been reported, and it had generally been believed that DNA binding by LON is a nonspecific process. We previously described (*26*) the selective affinity of *E. coli* Lon for a double-stranded DNA sequence (*26*−*29*). This element displayed remarkable similarity to the sequence of a site in the HSP and LSP of the human mtDNA (Figure 1). Due to the high degree of similarity between the *E. coli* and human LON proteases, and due to the localization of human LON to the mitochondria, we examined whether human LON might bind specifically to the mitochondrial promoter elements. Using the electrophoretic mobility shift assay (EMSA) and a newly developed S1 nuclease single-stranded footprinting assay, we demonstrate the selective affinity of human LON for a single-stranded sequence found on the mtDNA LSP and HSP. Contrary to our expectations, unlike the *E. coli* Lon which binds to the same sequence in double-stranded DNA, human LON did not bind to double-stranded DNA. These findings suggest that LON may play a role in mitochondrial gene regulation or DNA replication by binding specifically to this critical regulatory region of human mtDNA.

EXPERIMENTAL PROCEDURES

Preparation of Recombinant Human LON. The plasmid pVEX11/hLON with most of the coding region of human LON under control of a T7 promoter and anti-human LON antibodies were obtained from Dr. M. Maurizi (*10*, *11*). pVEX11/hLON was used to program rabbit reticulocyte lysate for coupled transciption/translation production of LON. For polyhistidine tagging of recombinant human LON, the *Nde*I−*Bam*HI fragment from pVEX11/hLON containing the LON cDNA was cloned into the polyhistidine-tagging proEx-1 vector (Gibco-BRL) cut with the same restriction enzymes. The resulting plasmid, proEx-1/hLON was verified by DNA sequencing. Recombinant human LON was induced to high expression levels in *E. coli* strain JM 109 using the manufacturer's suggested guidelines (Gibco-BRL). Polyhistidine-tagged recombinant human LON was purified using Ni−NTA resin (QIAGEN) following manufacturer's guidelines except with the following modifications: The wash buffer used was 50 mM Tris-HCl, pH 8.5, 1 M KCl, and 10% glycerol. The elution buffer used was 50 mM Tris-HCl, pH 8.5, 0.1 mM KCl, 10% glycerol, and 0.1 M imidazole. SDS−polyacrylamide gel electrophoresis (PAGE) and western blotting were performed using standard procedures (*30*).

Mitochondrial Extract Preparation. Mitochondrial extract was prepared from cultured human Jurkat T cells. The procedure used for isolating intact mitochondria from these cells has been described elsewhere (*31*). The mitochondria were lysed by dounce homogenization and clarified by centrifugation at 15000*g* at 4 °C. The presence of human LON was verified by Western blotting.

Electrophoretic Mobility Shift Assay (EMSA). The following oligonucleotides were used in EMSA experiments: a.s. denotes the antisense strand, s. denotes the sense strand, and d.s. represents a double-stranded oligonucleotide prepared by annealing the sense and antisense strands. The mitochondrial light strand promoter or LSP is represented by the letter L, and the mitochondrial heavy strand promoter or HSP is represented by the letter H). L(s.): 5′ACAGTCACCCCCCAACTAACACAT; L(a.s.): 5′AATAATGTGTTAGTTGGGGGGTGA; H(s.): 5′ACCCCATACCCCGAACCAACCAAA; H(a.s.): 5′GGGGTTTGGTTGGTTCGGGGTATG. The oligonucleotides were end-labeled using T4 polynucleotide kinase (New England BioLabs) in the presence of [γ-^{32}P]ATP and purified over a Sephadex G-50 column (Pharmacia) prior to use. The binding buffer used contained 20 mM Tris-HCl, pH 7.5, 50 mM KCl, 10 mM $MgCl_2$, 1 mM $CaCl_2$, 10% glycerol, 0.1 mg/mL bovine serum albumin, 10 000 cpm of labeled probe per reaction, and bromophenol blue. For experiments employing purified recombinant human LON, 50 ng was used per individual reaction. The binding reaction was performed at room temperature for 10 min prior to electrophoresis using a Tris−glycine−EDTA (TGE) gel.

S1 Nuclease Footprinting Assay. A 54 base pair oligonucleotide containing the mitochondrial light strand promoter (LSP) was synthesized using the following sequence: 5′AGTGGGAGGGGAAAATAATGTGTTAGTTGGGGGGTGACTGTTAAAAGTGCATAC. This oligonucleotide was end-labeled using T4 polynucleotide kinase (New England BioLabs) in the presence of [γ-^{32}P]ATP and purified over a Sephadex G-50 column (Pharmacia) prior to use. A G+A ladder was generated using piperidine following modification of A and G residues by methylation. The binding reactions were performed either with or without 100 ng of purified recombinant human LON in a 50 μL volume containing 20 mM Tris-HCl, pH 7.5, 50 mM KCl, 1 mM $ZnSO_4$, 10 mM $MgCl_2$, 5% glycerol, 2 mg/mL bovine serum albumin, and 30 000 cpm of end-labeled probe. Following incubation at room temperature for 10 min, 1 μL of a 1:100 dilution of S1 nuclease (Gibco-BRL) in the supplied enzyme dilution buffer was added to the reaction and allowed to incubate at room temperature for an additional 5 min. (For reactions containing LON, the S1 nuclease was not diluted prior to use so as to compensate for the proteolytic breakdown of S1 nuclease by the active human LON protease.) The nuclease activity was terminated by the addition of 50 μL of stop buffer (20 mM EDTA, 1% SDS, 200 mM NaCl, 250 μg/mL tRNA). Digested DNA fragments were extracted with phenol−chloroform, ethanol-precipitated, and analyzed using a 12% sequencing gel.

RESULTS

Sequence Comparison between the Human Immunodeficiency Virus Type 2 (HIV-2) Enhancer Peri-ets (pets)

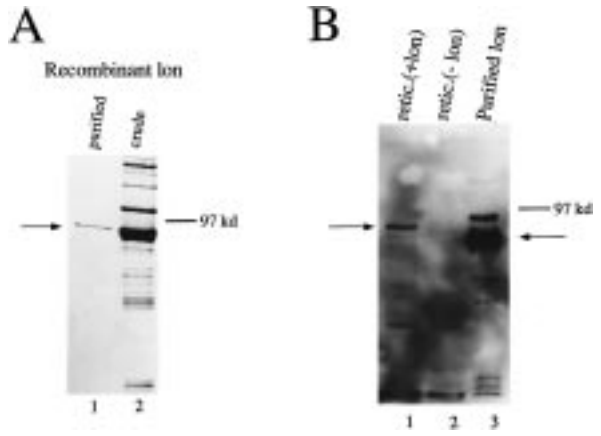

FIGURE 2: Recombinant LON was prepared in *E. coli* as described under Experimental Procedures. In (A), Coomassie staining following SDS−PAGE indicated the purity of the recombinant polyhistidine-tagged LON preparation. In lane 2, several bands were seen in crude *E. coli* extracts after induction to express human LON to high levels, but, following purification using Ni−NTA resin, only an 88 kDa band remained (lane 1). In (B), the identity of this purified recombinant human LON was confirmed using Western blotting (lane 3). A positive control for the Western blotting was made by coupled in vitro transcription and translation of rabbit reticulocyte lysates (Promega) programmed with a LON cDNA (lane 1) or unprogrammed (lane 2).

Element and the Mitochondrial Heavy (HSP) and Light (LSP) Chain Promoters. During our experiments to clone a mammalian transcription factor binding to the peri-ets, or pets, element of the HIV-2 enhancer (*32*), we noticed highly specific binding to the same site from an *E. coli* protein. Following purification and biochemical characterization, we identified this *E. coli* factor as the bacterial Lon protease (*26*). An ATP-dependent protease very similar to bacterial Lon had been cloned in humans and was found to localize to the mitochondria (*10*, *11*). Interestingly, the DNA sequence of the two major promoter elements of the human mitochondrial genome, the HSP and LSP, showed remarkable resemblance to the HIV-2 pets element, especially within the bases that are conserved between the LSP and HSP (Figure 1).

Production and Isolation of Human LON. The *E. coli* Lon protease has an affinity for both single-stranded and double-stranded DNA. As the human LON protease is 36% identical with its *E. coli* counterpart (*4*), we wished to determine whether human LON was also capable of sequence-specific binding to the mitochondrial promoter elements. To do this, we produced a recombinant version of the human LON protease in *E. coli*. To separate recombinant human LON from inherent *E. coli* LON and other contaminating proteins, we tagged our recombinant human LON with polyhistidine and purified it to homogeneity. Coomassie staining (Figure 2A, lane 1) and Western blotting (Figure 2B, lane 3) confirmed the purity and identity of our preparation.

Selective Binding of Human LON to Specific Single-Stranded DNA Sequences Contained within the LSP and HSP. Using the recombinant human LON we had prepared, we demonstrated selective affinity of human LON for single-stranded DNA depending on its DNA sequence. In EMSA experiments, while human LON was able to bind to the LSP noncoding (a.s.) and HSP coding (a.s.) single-stranded DNA elements (Figure 3, lanes 2 and 4), it had no affinity for the complementary strand DNA (lanes 1 and 3), or for double-stranded DNA containing the same sequences (lanes 5 and 6). The HSP coding (a.s.) and the LSP noncoding (a.s.) DNA sequences are conserved at several bases (see Figure 1). Having shown that LON has a selective single-stranded affinity for the sequences contained within the LSP noncoding element, we sought to demonstrate that such binding activity is seen in human mitochondria and to further test whether LON is capable of site-specific recognition of the single-stranded LSP element. First, we examined whether a protein is present in human mitochondria which recognizes the LSP antisense sequences containing the TG-rich element. As can be seen in Figure 4A, while there is some nonspecific binding, site-specific binding activity for this single-stranded element is seen in whole mitochondrial extracts, albeit at a lower affinity than is seen with recombinant LON (Figure 4B). Using recombinant LON, we found that single-stranded binding to the LSP noncoding DNA probe can be competed by the addition of unlabeled LSP noncoding oligonucleotide (Figure 4B, lanes 2 and 3), and, to a lesser extent, with the HSP coding oligonucleotide (lanes 8 and 9). However, the inclusion of the HSP noncoding, LSP coding oligonucleotide (lanes 4−7), or doubled-stranded DNA (lanes 10 and 11), does not compete away the binding of human LON for the LSP noncoding oligonucleotide. These studies demonstrate that human LON binds selectively to the single-stranded LSP, which contains a TG-rich element which is very similar to the HIV-2 pets site.

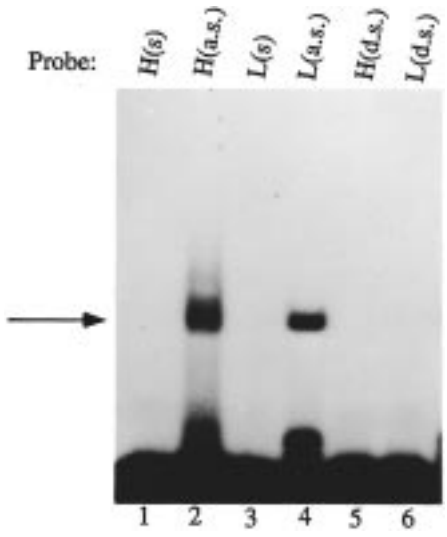

FIGURE 3: Electrophoretic mobility shift assay (EMSA) was used to determine the sequence-specific affinity of LON for single-stranded DNA. Purified recombinant human LON was incubated with various single-stranded and double-stranded ^{32}P- labeled oligonucleotides as indicated. H represents the mitochondrial heavy strand promoter (HSP) DNA sequence, and L represents the mitochondrial light strand promoter (LSP) DNA sequence. Antisense DNA sequence is abbreviated as a.s., and sense as s. (See Experimental Procedures for DNA sequences used.) A band-shift complex was observed when using the antisense strand probes (lanes 2 and 4) as indicated by the arrow in the figure. This retardation was not seen when the sense strand probes (lanes 1 and 3) were used or when double-stranded DNA probes were used (lanes 5 and 6).

S1 Nuclease Single-Stranded DNA Footprinting Assay. Having demonstrated that human LON could bind to short pieces of single-stranded oligonucleotide containing specific DNA sequences, we then developed a footprinting assay, much like the commonly used DNase I footprinting assay,

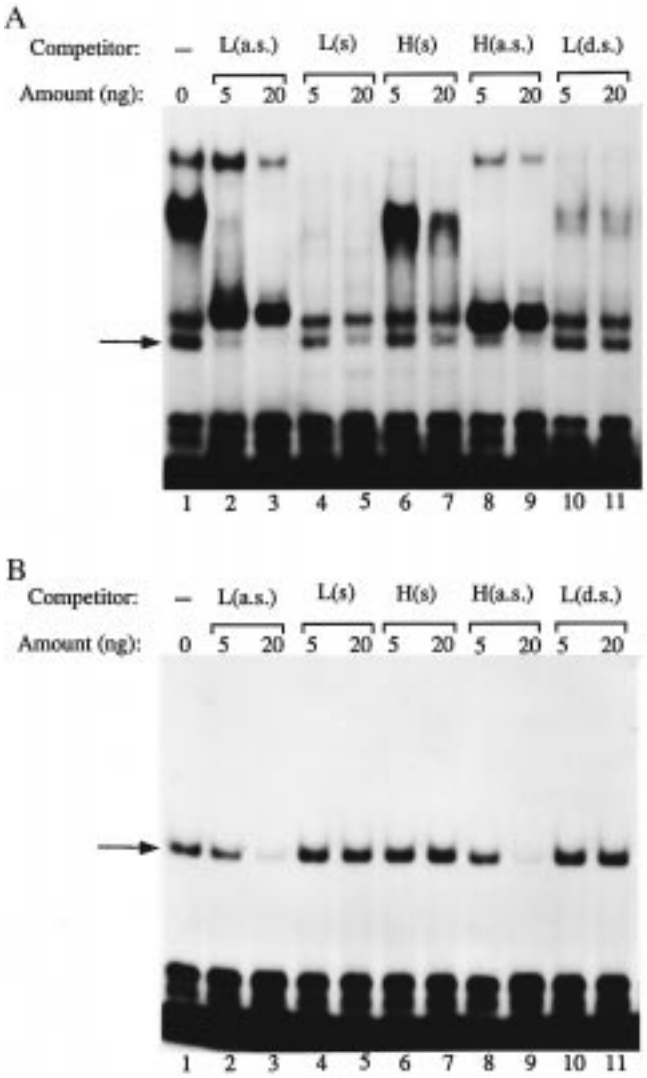

FIGURE 4: In (A), mitochondrial extract was used in EMSA to determine if specific binding is seen to the mitochondrial light strand promoter element, or L(a.s.), containing the TG-rich sequence. Several band-shift complexes were observed with this crude extract preparation containing many different proteins, but a single band shift (indicated by arrow) demonstrated sequence-specific binding. This band was efficiently competed away with the L(a.s.) (lanes 2 and 3) and the H(a.s.) (lanes 8 and 9) oligonucleotides, but much less so with the L(s.) (lanes 4 and 5) and H(s.) (lanes 6 and 7), and little if at all by the double-stranded oligonucleotide (lanes 10 and 11). In (B), the single-stranded, sequence-specific affinity of LON for the antisense strand DNA of the mitochondrial light strand promoter, or L(a.s.), was demonstrated using EMSA. Purified recombinant human LON, when incubated with the L(a.s.) probe, produced a band shift (lane 1) as indicated by the arrow in the figure. Addition of unlabeled L(a.s.) oligonucleotides competed with the probe for binding (lanes 2 and 3), but addition of unlabeled sense strand DNA (lanes 4−7) or double-stranded DNA (lanes 10 and 11) did not compete away binding to the probe. Some competition was also seen when the H(a.s.) oligonucleotide, which is very similar in sequence to the L(a.s.) probe, was used (lanes 8 and 9).

except that as binding to single-stranded DNA was to be assessed, S1 nuclease under the appropriate reaction conditions was used in place of DNase I. This experiment confirmed that recombinant human LON was able to bind to the sequence in the LSP noncoding strand that is conserved between the HIV-2 pets, LSP noncoding, and HSP coding

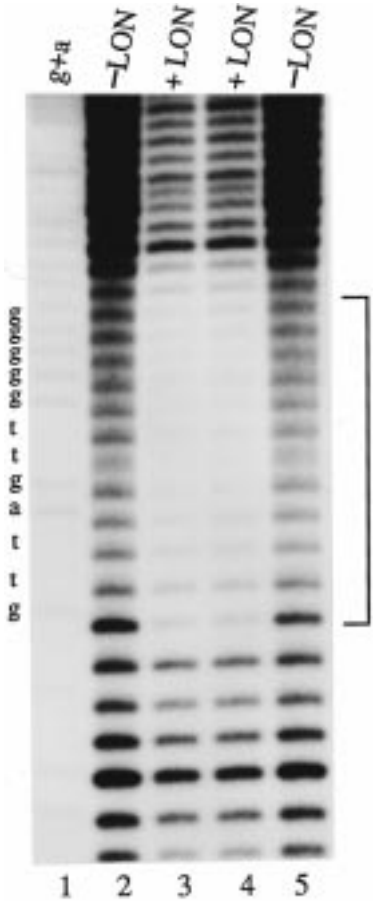

FIGURE 5: Single-stranded, sequence-specific binding of human LON to the mitochondrial light strand promoter (LSP) was demonstrated using the S1 nuclease footprinting assay. In the absence of LON, S1 nuclease cleaved 5′ end-labeled single-stranded DNA to generate fragments of DNA with varying sizes (lanes 2 and 5). Inclusion of recombinant LON (lanes 3 and 4) with the single-stranded DNA containing the LSP binding site prior to nuclease treatment protects a region as indicated by the bracket in the figure. A G+A ladder generated using the single-stranded probe is shown in lane 1. Bases protected from nuclease digestion are printed to the left.

elements (Figure 5, lanes 2 and 5 vs lanes 3 and 4) and protect this region from nuclease digestion. To our knowledge, this is the first report of a successful single-stranded DNA footprinting assay. This novel experiment further confirms our observation of single-stranded, site-specific DNA binding by human LON.

DISCUSSION

The finding that human LON can bind to an important regulatory region of mtDNA suggests that this multifaceted protease plays a role in mitochondrial gene regulation and/or DNA replication. It is most tempting to speculate that LON functions similarly to AEBP1, a site-specific DNA binding protease responsible for transcriptional repression (*33*). This carboxypeptidase binds to the mouse adipocyte P2 (aP2) promoter to repress aP2 gene expression. It was demonstrated that the proteolytic activity of AEBP1 is directly responsible for the repression function. It is possible that human and *E. coli* LON function in an analogous manner. While prokaryotic Lon is known to specifically target transcription factors for degradation, the mechanism by which it targets these proteins is unknown (*5*, *6*). Whether

or not eukaryotic LON also specifically degrades certain transcription factors remains to be shown.

Human LON could mediate gene expression and/or DNA replication by targeting the breakdown of other single-stranded binding proteins binding adjacent to the LON binding site on the D-loop. One potential target for LON proteolysis is the mtSSB protein, or a protein which interacts with it. mtSSB has been demonstrated to induce conformational changes to the mtDNA, thereby causing a change in the rate of replication/transcription (*34*−*36*). Interestingly, studies performed with yeast demonstrate that both LON and mtSSB are required for proper mtDNA maintenance, as deletion of either one will lead to loss of mtDNA integrity. Further, both LON and mtSSB bind to the same regulatory region, where RNA and DNA polymerase activities, and hence transcription and DNA replication, are tightly linked (*19*). Therefore, human LON might be involved in maintaining the proper functioning of mtSSB, either by direct interaction or through degradation of another factor which affects mtSSB.

Our previous studies demonstrated that *E. coli* Lon, previously thought to bind DNA without regard to sequence, is a site-specific DNA binding protein (*26*). While *E. coli* Lon binds to double-stranded DNA, data presented in the current study show that human LON, while recognizing a very similar sequence, will not bind to double-stranded DNA, but rather to a single-stranded DNA element in a site-specific, strand-specific manner. Thus, while evolution has preserved the DNA binding function of Lon proteins, the human mitochondrial LON has evolved a different mechanism for interacting with potential regulatory sites in promoters. Further studies to characterize the functional significance of site-specific binding by human LON to single-stranded mtDNA will provide insights into the function of this intriguing protease and its role in maintaining the mitochondrial genome.

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