

CLK-1 protein has DNA binding activity specific to O_L region of mitochondrial DNA

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Received 10 January 2002; revised 20 February 2002; accepted 3 March 2002

First published online 14 March 2002

Edited by Horst Feldmann

Abstract Mutations in the *clk-1* gene of *Caenorhabditis elegans* extend worm life span and slow down a variety of physiological processes. Here we report that *C. elegans* CLK-1 as well as its mouse homologue have DNA binding activity that is specific to the O_L region of mitochondrial DNA. DNA binding activity of CLK-1 is inhibited by ADP, and is altered by mutations that extend nematode life span. Our results suggest that, in addition to its enzymatic function in ubiquinone biosynthesis, CLK-1 is involved in the regulation of mtDNA replication or transcription. © 2002 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.

Key words: Aging; Mitochondrion; O_L-hairpin; *Caenorhabditis elegans*; *clk-1*

1. Introduction

clk-1 mutants of *Caenorhabditis elegans* are characterized by extended life span and slowdown of developmental processes and rhythmic behaviors [1]. CLK-1 is a mitochondrial protein that is required for ubiquinone (UQ) biosynthesis in yeast [2,3] and worms [4]. *clk-1* mutants are unable to synthesize UQ and accumulate a biosynthetic precursor demethoxyubiquinone (DMQ). Complementation experiments in *Escherichia coli* [5], and homology modeling [6] suggested that CLK-1 is a hydroxylase that converts DMQ into 5-hydroxy-UQ. This enzymatic function does not explain, however, many aspects of *clk-1* phenotype. Such as, average slowdown of physiological processes is often accompanied by an increase in variability, i.e. some *clk-1* worms can develop even faster than the wild type (reviewed in [7]). In addition, *clk-1* mutants cannot properly adjust to changes in temperature, and when embryos are shifted to higher or lower temperature they continue to develop at a rate corresponding to the temperature at which their mothers were grown. All phenotypes of *clk-1* mutants are fully rescued when homozygous mutants originate from a mother, carrying a wild copy gene, a phenomenon called maternal rescue, which implies that a very low amount of protein stored in the oocyte is sufficient for function [1]. Knock out studies in mice suggested that CLK-1 is necessary for embryonic development, rather than for mitochondrial

respiration [8]. All these phenotypes suggest that in addition to its enzymatic function in UQ biosynthesis CLK-1 has a regulatory function. It has been proposed that CLK-1 functions as a metabolic regulator that coordinates mitochondrial and nuclear function [7].

To test whether CLK-1 is involved in the regulation of mtDNA transcription or replication we examined whether CLK-1 can bind mtDNA in vitro. We have found that *E. coli* expressed CLK-1 has DNA binding activity which is specific to O_L region of mtDNA. The binding activity is down regulated in the presence of ADP, but not ATP. CLK-1 proteins carrying e2519 (missense mutation) and qm30 (C-terminal truncation) mutations, that are known to extend life span in *C. elegans*, have altered DNA binding efficiency and respond differently to ATP and FAD.

2. Materials and methods

2.1. Plasmids

Full length *C. elegans* and mouse *clk-1* cDNAs were amplified with the following pairs of primers: GGcatatgTTCGGTGT-AATAACCCGTGGAGC (*clk1*-sens) and GGggatccTCAAATTTT-CTCAGCAATCGCAATAGC (*clk1*-anti); GCacatATGAGCGCC-GCCGGAGCC-ATAGC (mouse-sens) and AGAggatccTAAACCTTTCTGATAAATATATGG (mouse-anti). PCR products were digested with *NdeI*–*Bam*HI and cloned into the same sites of the expression vector pTXB1 (New England Biolabs), the resulting plasmids were named pTXB-CLK1 and pTXB-mCLK1, respectively. e2519 mutations (E148 to K substitution) were introduced into pTXB-CLK1 using site-directed mutagenesis kit (Stratagene) with the following oligonucleotides: CAAGATTACGTGATGAGAGCTTCATCATATGATATGG (e2519-sens) and CCAGTATCATGATGATG-AAGCTtCTCATCACGTAATCTTG (e2519-anti). qm30 mutation (deletion of 35 amino acids at the C-terminus) was prepared by PCR with the following primers: *clk1*-sens and cgtgGGATCctcatcaagtGATGATGAAGCTCCTCATCACG (qm30tail-sens). PCR product was digested with *NdeI*–*Bam*HI and cloned into the same sites of pTXB1 vector. All the CLK-1 derivatives for deletion analysis of CLK-1 protein were prepared by PCR, digested with *NdeI*–*Bam*HI and cloned into the same sites of pTXB1. The following pairs of primers were used: *clk1*-sens and GGggatccTTATAGAAGTGCT-GAACCGACACCG (TRC1-anti) for TRC1; GGcatatgGGAAAA-GAAGTGCAATGGC (TRC2-sens) and *clk1*-anti for TRC2; CATcatatgGATACTGGAGTAGAACACGATGG (qm30tail-sens) and *clk1*-anti for qm30tail; GAacatatgATTGGACAACATTATAAT-GATCAATTGAA (mini2-sens) and qm30-anti for M30; TRC2-sens and ATGggatcctcaCTCCTCATCACGTAATCTTGAG (mini2-anti) for TM; mini2-sens and mini2-anti for MM; *clk1*-sens and CATggatcctcaATGTTCTTTCTCCTCATCCCAC (mini1-anti) for TRC1mini; GCCcatatgGGGAAGGAAGGAGCAATGGCCT-GC (MT-sens) and CAGggatccttaGTGGTGTTCAGCTCCTCAT-CGC (M30-anti) for mouse MT; GAacatatgATCGTAATCACTA-CAACAACCAG (Mmini-sens) and M30-anti for mouse MM. Internal deletion of one DNA binding site (delta) was constructed using site-directed mutagenesis kit (Stratagene) with the primers GT-

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CCctcGAGTTCTTCAACTGCAATTGTACAAGC (dmini-anti) and TGATctcgagCTTCATCATCATGATACTGGAGTAG (dmini-sens), and pTXB-CLK1 template. The resulting PCR product was digested with *Xho*I, self-ligated and transformed into *E. coli*. The resulting plasmid named pTXB-delta was used as a template for *clk-1* construct lacking both DNA binding sites (delta2). pTXB-delta2 was constructed using the same strategy as pTXB-delta: pTXB-delta was amplified with the following primers: GATggtaccAAAGAACATT-TAGATACAATGGAAAG (dmtrel-sens) and ATCggtaccAAGCT-CTCCAGCATGATCAAC (dmtrel-anti), the PCR product was digested with *Kpn*I and circularized. All the constructs were verified by sequencing of the *clk-1* region in both directions.

2.2. Protein extracts

Recombinant CLK-1 proteins were expressed in *E. coli* strain BL21. Cells were grown at 37°C to OD₅₉₅ 0.5, IPTG was added to a concentration of 0.5 mM and growth was continued for 7 h at 25°C. Cells were cooled down and collected by centrifugation. Pellets were sonicated in the buffer containing 150 mM NaCl, 50 mM KCl, 5 mM MgCl₂, 25 mM HEPES pH 7.6 and the complete protease inhibitor set (Roche). Following sonication, extracts were centrifuged at 12 000 × *g* for 15 min. Glycerol was added to the supernatants to a concentration of 10% and extracts were frozen in small aliquots in liquid nitrogen. Protein amounts were quantified using Bio-Rad protein assay.

2.3. Electrophoretic mobility shift assay (EMSA)

High performance liquid chromatography-purified oligonucleotides were purchased from Gibco BRL. Probes were end-labeled using T4 polynucleotide kinase and purified on G25 MicroSpin columns from Pharmacia. Ten µg of protein extract (unless otherwise indicated), 0.5 µg poly(dI-dC) and 0.2 µg bovine tRNA were incubated for 10 min at 25°C in a buffer consisting of 25 mM HEPES pH 7.6, 100 mM NaCl, 50 mM KCl, 10% glycerol, 1 mM DTT, 0.6 mM MgCl₂ in 20 ml volume. Then 100 000 cpm of 32P-end-labeled probe was added and incubation was continued for 20 min at 25°C. The following oligonucleotides were used as probes: TTTGAGCACGTTAGTACATTATTTTAATAATGTGCTAATA (O_L-hairpin); aaactatgatctcgACATTATTTTcgagggtcatattat (O_L-mut.stem); TTTGAGCACGTTAGTAacggcgggTAATAATGTGCTAATA (O_L-mut.ring); aaactatgatctcgAacggcgggTcgagggtcatattat (O_L-mut.all); TTTGAGCACGTTAGTACATTATTTTcgagggtcataAATA (O_L-no.hairpin); TAAGACTTCTACCGCCATTTTTCGCGGCGGTAGAAAGTAGAT (O_L-mouse); ATATTATATTTAT-ATTGATAAATAATATTTTATAAATTAT (OH CR repeat); CGGGAATTCATATGCCGTTCTCTATCACCACACC (K); double-stranded oligo was prepared by annealing of K and complementary oligo anti-K GGTGTGGTGATAGAGAACGGCATA-TGGAATTCCTCG; AGATAATAATTCTAGATAGTTTACACG (O_H-sens). Fragments of *C. elegans* mtDNA containing O_L and O_H regions were amplified by PCR from the total genomic DNA with following primers: TAAGTGTGTTTACTAATAATCTAGAG (O_L-sens) and CTCTAATCAAACGCAATCCTCTCTG (O_L-anti) for O_L region; AGATAATAATTCTAGATAGTTTACACG (O_H-sens) and TAATAATATCTTAGCAACCCAAATGC (O_H-anti) for O_H region. TCGCTTTTATTACTCTATATGAGCGTC (O_L cont-sens) and GGTACAAGTAAAAATGATACTTCAGAGG (O_L cont-anti) for ND1 region, ATCTCAGACTGATACTGTGACCAAATAC (O_H cont-anti) and AATGGTATTTAATCCACTTT-TATTGTGAG (O_H cont-sens) for COX1 region, negative control for O_H.

For supershift reactions anti-CLK-1 antiserum was added, and the reaction was incubated on ice for 30 min. The reaction was run on a 4.5% native acrylamide gel in 1 × TBE buffer at 4°C. In competition reactions, cold competitor was added prior to the addition of protein. Following electrophoresis, gels were dried under vacuum at 80°C and subjected to autoradiography. Quantification of the amount of shifted probe was performed using Bio-Rad densitometer. Goat anti-*C. elegans* CLK-1 polyclonal antibodies were purchased from Santa Cruz Biotechnology Inc. The mixture of CLK-1(cN-20) and CLK-1(cC-14) antibodies was concentrated to 1 µg/µl prior to use. Rabbit anti-mouse CLK-1 antiserum was obtained from the laboratory of S. Hekimi [9].

We were unable to test the DNA binding activity of purified CLK-1 because in GST or polyhistidine CLK-1 fusions the DNA binding activity was altered. CLK-1 protein fused to GST lost its DNA binding activity, while addition of His tag changed the specificity of DNA

binding, and the His-tagged CLK-1 acquired strong affinity to any single-stranded DNA.

3. Results and discussion

3.1. *C. elegans* CLK-1 and its mouse homologue bind O_L region of mtDNA

To study the involvement of CLK-1 in the regulation of mtDNA transcription or replication we tested whether CLK-1 can bind mitochondrial DNA. Nematode mtDNA has two non-coding regions [10] that contain putative O_H and O_L origins of mtDNA replication (Fig. 1A). Therefore, we hypothesized that these two regions are the likely candidates for binding of regulatory proteins. The cDNA for *C. elegans* CLK-1 was expressed in *E. coli* and total cell extract was used in EMSA with four different probes comprised of PCR fragments containing the whole O_L and O_H (D-loop) regions with some adjacent DNA, and two control fragments amplified from the coding region of mtDNA. The retarded band was observed only with O_L containing probe (data not shown). To define more precisely the minimal sequence bound by CLK-1 we performed EMSA with oligonucleotide probe containing only the proposed hairpin structure from the O_L region (Fig. 1A). Efficient binding was observed with the extract containing CLK-1 (Fig. 1B), while no retarded band was present with the extract from cells containing empty vector or vector with unrelated protein. To test whether mouse homologue of CLK-1 (mCLK-1) has the same activity cDNA for mCLK-1 was expressed in *E. coli* and tested in EMSA with the oligonucleotide probe to mouse O_L-hairpin (Fig. 1A). The binding activity similar to that of worm CLK-1 was observed (Fig. 1B) which is consistent with the previous reports of functional conservation among CLK-1 homologues [11]. Antibodies to *C. elegans* and mouse CLK-1 supershifted the retarded complexes (Fig. 1C), which further confirms that the formation of shifted bands is specifically due to the CLK-1.

CLK-1 did not have affinity to hairpin structures within tRNA and bovine tRNA has been routinely used as unspecific competitor in EMSA assays. In order to study whether CLK-1 has specificity to DNA or RNA, RNA oligo corresponding to O_L-hairpin was tested. RNA O_L was recognized by CLK-1, although with much lower efficiency than DNA O_L oligo (data not shown).

In order to examine in more detail the sequence specificity of CLK-1 DNA binding, the following oligonucleotides were used as probes. To study whether CLK-1 recognizes hairpin structure or a specific sequence within the O_L-hairpin we mutagenized the stem region, loop region, both regions but maintained the hairpin conformation, or changed one half of the stem sequence to complementary, thereby destroying the ability to form hairpin. We also used several oligos as additional negative controls such as: an oligo corresponding to inverted repeats (CR) from O_H region, an oligo to protein coding region of mitochondrial DNA (O_H-sens), an unspecific oligo (K), and oligo K annealed to the complementary oligo anti-K to form a double-stranded fragment. Gel retardation assays were performed in two ways for each probe: as direct assays where various probes were labeled and mixed with protein extracts (Fig. 2) and as competition assays where various probes were used as competitors in a binding reaction of CLK-1 and labeled O_L-hairpin probe (data not shown). Both types of assay gave the same results; CLK-1 did not

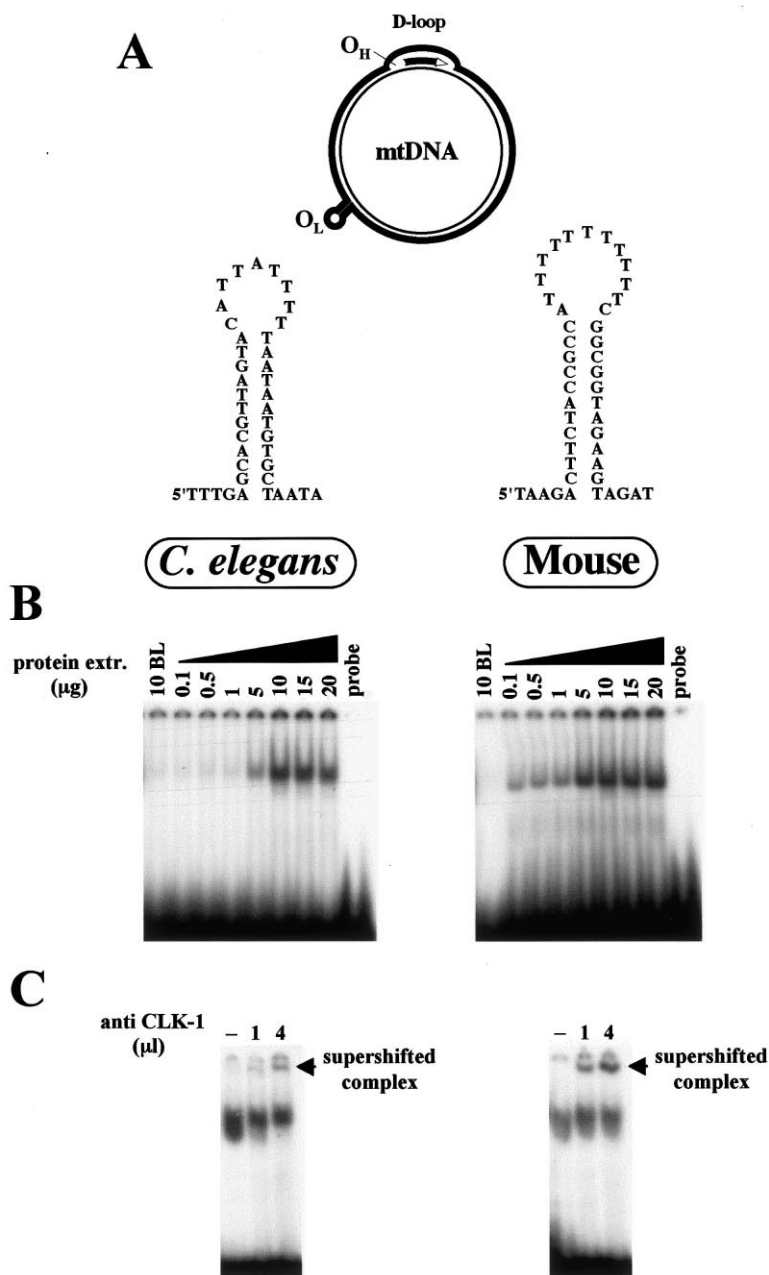


Fig. 1. DNA binding activity of CLK-1 protein. A: Schematic representation of the mitochondrial genome and O_L-hairpin structure in *C. elegans* and mouse mtDNA. The mtDNA light and heavy strands are depicted as heavy and thin lines, respectively. D-loop region contains transcription start site and the origin of replication of heavy strand (O_H). The sequence of the D-loop region is AT-rich and contains several repeats called CR. The light strand origin of replication (O_L) is located two thirds of genomic distance away from O_H on the mtDNA molecule. The DNA sequence in this region has the potential to form hairpin structure. B: EMSA assays with increasing amounts of *C. elegans* CLK-1 (left panel) and mouse CLK-1 (right panel), and an oligonucleotide probe corresponding to *C. elegans* or mouse O_L-hairpin, respectively. The amount of protein extract from bacteria expressing CLK-1 is indicated. This extract was supplemented with protein extract from bacteria containing empty vector to the final amount of 10 μg. The first lane (10 BL) represents EMSA with protein extract lacking CLK-1 and the last lane (probe) shows EMSA without protein extract. C: Supershift of *C. elegans* (left panel) and mouse (right panel) CLK-1/O_L complexes with anti-CLK-1 antibodies. Supershifted complexes are indicated by arrow. The indicated amounts of antiserum were added to EMSA reactions and incubated for additional 30 min on ice.

bind O_L with mutated stem, hairpin of a different sequence or any other oligonucleotide unrelated to O_L. The binding was retained when the loop region or hairpin conformation were modified. These data indicate that CLK-1 recognizes the sequence at the stem of the hairpin, and that hairpin conformation is not required for binding. Furthermore, since CLK-1

recognized both O_L and 'no hairpin'-O_L probe (Fig. 2) as well as a longer PCR product containing O_L region, used in initial experiments, it can be concluded that CLK-1 binds O_L sequence in both single- and double-stranded forms. O_L is thought to acquire hairpin conformation only during replication when replication fork passes this region and exposes the

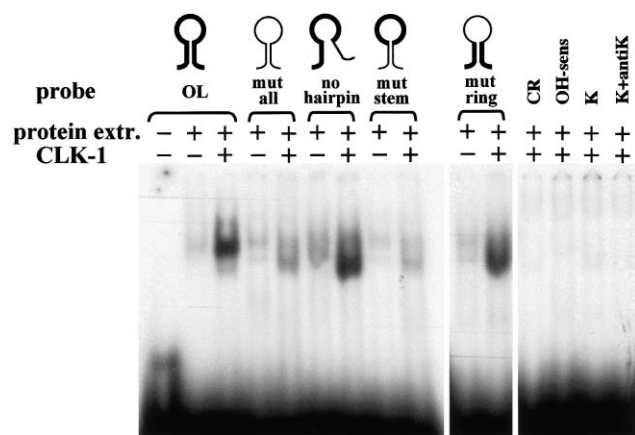


Fig. 2. EMSA assays with mutated O_L -hairpin probes and other non- O_L probes. For each probe (except CR, OH-sense, K and K+anti-K) two binding reactions were performed, one with protein extract from bacteria containing empty vector and another with protein extract containing *C. elegans* CLK-1. The structure of the mutated O_L -hairpin probes is shown above the corresponding lanes. Wild type and mutated sequences are depicted as thick and thin lines, respectively. For sequences of the probes see Section 2.

parental H-strand as a single strand. Therefore, this result indicates that *in vivo* CLK-1 can be bound to O_L region in the absence of replication.

3.2. Localization of the DNA binding sites within the CLK-1

In order to identify DNA binding domain(s) within CLK-1 protein we introduced several deletions into CLK-1 cDNA, expressed deleted proteins in *E. coli* and tested in gel retardation assays. The results of these experiments are summarized in Fig. 3. All the CLK-1 constructs were expressed at approximately similar levels, except for the two constructs containing only N-terminal half of the protein (TRC1 and TRC1mini) which were expressed at the lower level.

Previous sequence analysis revealed the presence of two tandemly repeated domains named TRC1 and TRC2 within CLK-1 protein [11]. When expressed separately both TRC1 and TRC2 domains were able to bind O_L probe. This observation provides the first experimental evidence that TRC1 and TRC2 are two functional domains within CLK-1 protein. Using deletion analysis we localized DNA binding activity between amino acids 114 and 148 in TRC2, and between amino acids 1 and 63 in TRC1. The fragment of mouse CLK-1 from amino acid 144 to 182 which corresponds to amino acids 114–148 fragment in worm CLK-1 was also sufficient for binding of mouse O_L . O_L binding regions are predicted to be composed of two α helices connected by a loop [11], which is reminiscent of a DNA binding motif. According to the structural model of CLK-1 proposed by Rea [6] and Stenmark et al. [5] these regions are located on the surface of the protein, which is consistent with the DNA binding function. Loop regions between α helices often exhibit high variability; despite the latter, both loops within the O_L binding regions are highly conserved in animalia (from *C. elegans* to mammals), while yeast and bacterial CLK-1 homologues contain various insertions and deletions within the loops [6]. Therefore we hypothesize that in lower organisms CLK-1 has only single func-

tion in UQ biosynthesis, while in higher eukaryotes it evolved an additional regulatory DNA binding function.

3.3. DNA binding activity of CLK-1 is sensitive to ADP

We examined the effect of various energy cofactors which

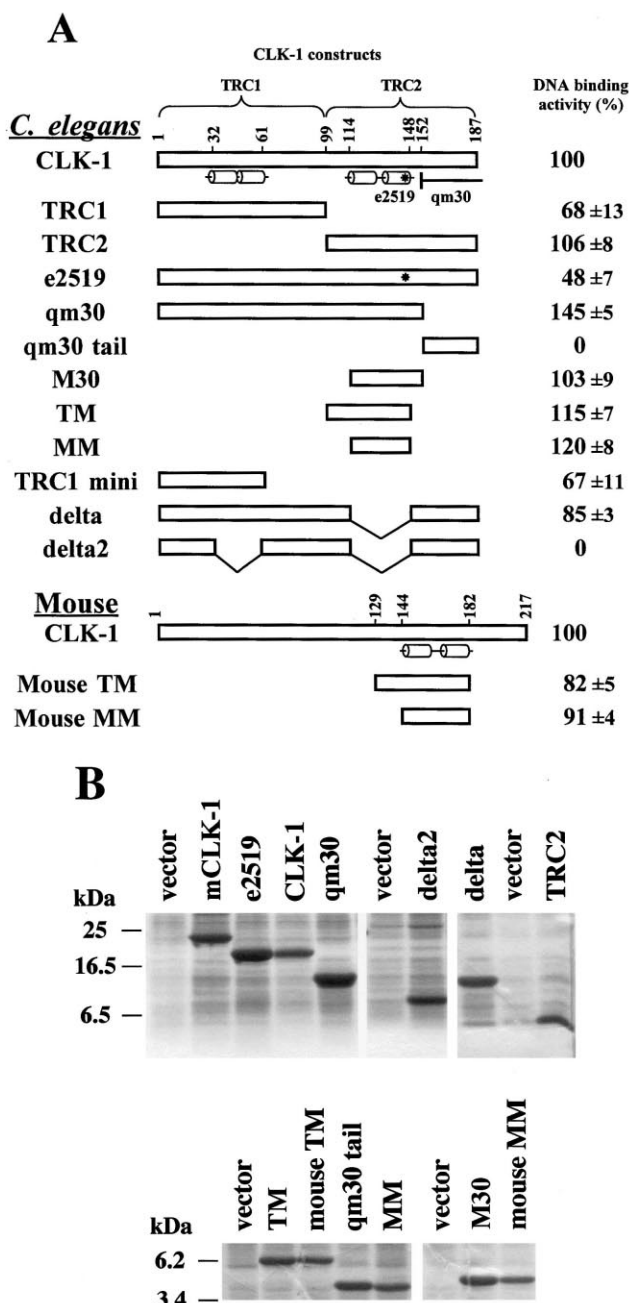


Fig. 3. Localization of DNA binding activity within CLK-1. A: At the top, a CLK-1 protein is shown (aa 1–187) with the coordinates of deletion break points. Potential α -helices responsible for DNA binding are depicted as rods. Position of e2519 point mutation is indicated by a star. qm30 C-terminal deletion is also indicated. Various deletion constructs are shown below. Deletion constructs were expressed in *E. coli* and their DNA binding activity was tested in EMSA with O_L -hairpin probe. Similar analysis was performed for mouse CLK-1, as shown at the bottom of the panel. B: Expression of the recombinant CLK-1 proteins described in (A). 15 μ g of *E. coli* protein extract was loaded in each lane, separated by Tris-glycine SDS-PAGE (upper panel) or Tris-tricine SDS-PAGE for very small proteins (lower panel) and stained with coomassie blue.

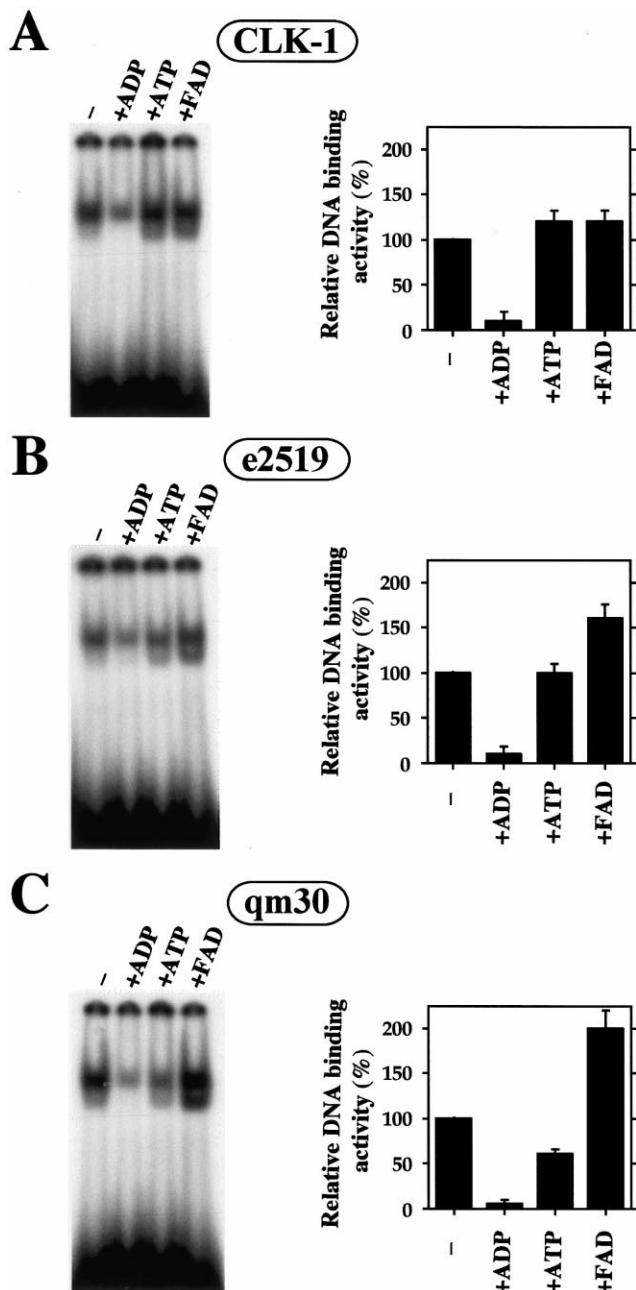


Fig. 4. CLK-1 mutants, e2519 and qm30, that extend worm life span respond differently to the addition of ADP, ATP and FAD. EMSA reaction mixtures containing the wild type CLK-1 (A), e2519 (B) and qm30 (C) were supplemented with 2 mM ADP, ATP or FAD prior to the addition of O_L -hairpin probe. The supershifted bands were quantified by densitometry. DNA binding activity in the presence of ADP, ATP and FAD is expressed relative to the control reactions (-) without additional energy cofactors, in which the level of DNA binding was taken as 100%. The average of at least three independent experiments is shown. Error bars represent standard deviations.

are present in the mitochondria on the DNA binding activity of CLK-1. Two mM concentration of ATP, ADP, NAD^+ , NADH or FAD was added to the binding reactions containing CLK-1 protein extract and O_L -hairpin probe, the same chemicals were also added to the control reactions with protein extracts lacking CLK-1 to rule out stimulatory effect on

any other protein(s) from the extract. In the control set of experiments no retarded band was observed after addition of any of the tested energy cofactors. In the binding reactions containing CLK-1 (Fig. 4A) the most striking effect was observed in the presence of ADP. Addition of ADP strongly inhibited (~ 10 times) DNA binding activity of CLK-1. ATP and FAD caused minor ($\sim 20\%$) stimulation of DNA binding activity of CLK-1 (Fig. 4A). Addition of NAD^+ or NADH did not affect the binding activity of CLK-1 (data not shown). This result suggests that the discovered DNA binding activity of CLK-1 is tightly regulated by ADP. Moreover, this regulation is specific to ADP but not other ADP-based molecules, such as ATP, NAD^+ , NADH or FAD. The sensitivity of CLK-1 to ADP is consistent with the proposed role of CLK-1 [7,12] as the regulator of mitochondrial activity. We hypothesize that CLK-1 may regulate mitochondrial activity according to the amount of ADP in the cell.

3.4. e2519 and qm30 mutations alter the DNA binding activity

e2519 and qm30 mutations in CLK-1 extend the worm life span. e2519 is a single amino acid substitution of glutamic acid to lysine at position 148 and has weaker phenotype than qm30 which is the deletion of 35 amino acids at the C-terminus [11]. To test the effect of these mutations on DNA binding we introduced the corresponding mutations into CLK-1 cDNA, expressed them in *E. coli* and tested in gel retardation assays with O_L -hairpin probe. Both e2519 and qm30 mutations altered the DNA binding activity of CLK-1. e2519 mutation reduced the efficiency of binding to approximately 48% of the wild type protein, while qm30 mutation increased the binding to approximately 145% of the wild type protein (Fig. 3). DNA binding activity of the mutant proteins was repressed by ADP, similarly to the wild type, however, their response to ATP and FAD was altered (Fig. 4B,C). ATP had inhibitory effect ($\sim 40\%$) on qm30, and no effect on e2519, while it had minor stimulatory effect ($\sim 20\%$) on wild type protein. FAD had strong stimulatory effect on both qm30 and e2519 ($\sim 100\%$ and $\sim 60\%$, respectively), while it had only minor ($\sim 20\%$) stimulatory effect on wild type CLK-1. The differences from the wild type in response to ATP and FAD were stronger with qm30. Importantly, e2519 is known to have weaker phenotype in worms than qm30, which correlates with the degree in which their DNA binding activity differs from the wild type. This provides further indication that newly described DNA binding activity of CLK-1 is involved in the life span determination.

3.5. Possible role of CLK-1 at O_L

It has been well demonstrated in mammalian mitochondria that O_L region serves as an origin of replication; however, it is not known what regulatory elements it contains. It has been shown recently that in aging human tissues O_L sequence is frequently deleted in the mtDNA that underwent clonal expansion [13]. Thus O_L might contain elements that regulate normal partitioning and replication of mtDNA, and deletion of these sequences causes clonal expansion of mitochondria with deleted mtDNAs. It is possible that CLK-1 has such a regulatory function. We speculate that CLK-1 is involved in the regulation of mtDNA replication and adjusts it according to ADP concentration in the mitochondria. Another possibility is that CLK-1 can affect mitochondrial transcription either via its effect on replication, as transcription and replication of

mtDNA are two dependent processes, or by stabilizing or destabilizing the formation of O_L-hairpin.

The connection between DNA binding activity of CLK-1 and its role in UQ biosynthesis is unclear. It has been proposed, however, that bifunctionality is a common feature of mtDNA-associated proteins [14]. For example, RPO41, encoding the mtRNA polymerase, functions in mtDNA stability through its N-terminal domain, which is independent of the transcription domain [15]. Furthermore, it has been demonstrated that heat shock protein hsp60 and Krebs cycle protein Kg2p bind mtDNA and play a role in DNA maintenance [14]. CLK-1 is likely to be another example of a bifunctional protein that has enzymatic function in UQ biosynthesis and also regulates mtDNA metabolism.

Acknowledgements: We thank Dr. Olivia Pereira-Smith for critically reading the manuscript, Dr. Nicolai Timchenko for support and encouragement, Dr. Chang-Su Lim for helpful suggestions and Dr. Shane Rea for advice on CLK-1 molecular model.

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