J. Biochem. 2011;149(5):581–589 doi:10.1093/jb/mvr008

JB THE JOURNAL OF BIOCHEMISTRY

Biochemical properties of *Caenorhabditis elegans* HMG-5, a regulator of mitochondrial DNA

Received November 29, 2010; accepted January 14, 2011; published online January 21, 2011

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***Caenorhabditis elegans* HMG-5, which is encoded by F45E4.9, contains two high mobility group (HMG) box domains and shows sequence similarity with mammalian mitochondrial transcription factor A (TFAM). In this study, using soaking RNA interference, we found that knockdown of HMG-5 reduced the amount of mtDNA in P0 hermaphrodites, suggesting it as functional orthologue of mammalian TFAM. We also examined the biochemical property of HMG-5 in mammalian cells and *in vitro*. We found that HMG-5 localized to the mitochondria in human cultured cells and was included in the NP-40-insoluble fraction in which mtDNA and TFAM were enriched. By immunoprecipitation analysis, HMG-5 was found to associate with human mitochondrial DNA (mtDNA) in the cells. *In vitro* binding experiment also showed that HMG-5 binds to *C. elegans* mtDNA and plasmid DNA, indicating its feature as a non-specific DNA-binding protein. Furthermore, it was found that HMG-5 can interact with itself. These results demonstrate that HMG5 shares similar biochemical properties with mammalian TFAM as a nucleoid factor. HMG-5 could be a good candidate for investigating mtDNA metabolism in multicellular organisms.**

Keywords: *Caenorhabditis elegans*/HMG-5/mitochondrial DNA/nucleoid/TFAM.

Abbreviations: *C. elegans*, *Caenorhabditis elegans*; DAPI, 4,6-diamidino-2-phenylindole; D-loop, displacement loop; FISH, fluorescent *in situ* hybridization; GST, glutathione S-transferase; HMG, high mobility group; mtDNA, mitochondrial DNA; mtSSB, mitochondrial single-stranded DNA-binding protein; POLG, mtDNA polymerase gamma; RNAi, RNA interference; rRNA, ribosomal RNA; TFAM, mitochondrial transcription factor A.

Mitochondria are intracellular organelles that are found in nearly all eukaryotes and provide the majority of cellular ATP through oxidative phosphorylation. Mitochondria also play an important role in apoptosis and are involved in signal transduction during cell proliferation (*1*) and many other metabolic tasks, such as steroid synthesis (*2*). These important functions demonstrate that mitochondria are essential for maintaining the health of an organism over its lifetime. Mitochondria contain their own independent genome, which is composed of mitochondrial DNA (mtDNA). The human mitochondrial genome is a 16.6 kb circular structure of double-stranded DNA, which encodes 37 genes: two ribosomal RNA (12S rRNA and 16S rRNA), 22 transfer RNA and 13 proteins (components of respiratory chain subunits).

mtDNA is packaged into protein–DNA complexes called mitochondrial nucleoids. These nucleoid components are considered to regulate the metabolism of mtDNA (*3*), for example, the mtDNA-binding proteins are thought to play roles in mtDNA maintenance and segregation (*4*). The core nucleoid protein, mitochondrial transcription factor A (TFAM), is a non-histone type protein that shows homology to the DNA-binding high mobility group (HMG) proteins found in nuclear chromatin. TFAM contains two HMG box domains, which are evolutionally conserved from yeast to humans. Mammalian TFAM is necessary for the transcription, replication and maintenance of mtDNA copy number (*5*), and the latter function is conserved in yeast (*6*). In mammals, the amount of mtDNA is directly proportional to the total TFAM protein level (*7*, *8*). Recently, we identified a novel function of human TFAM; it is required for symmetric segregation of mtDNA in cultured cells (*9*). However, it is unclear whether this regulation occurs in normal cells or organisms.

Caenorhabditis elegans is widely used for researching development, ageing and longevity. The *C. elegans* genome contains a large number of human orthologues, enabling it to be used as a model of human diseases. The *C. elegans* mtDNA genome is 13.8 kb nucleotides in length, which is smaller than its counterparts in humans and other typical eukaryotes (*10*). The *C. elegans* mtDNA genome encodes 36 genes but lacks the ATP8 gene found in human mtDNA, which encodes a subunit of ATP synthase (complex V). The mtDNA copy number of *C. elegans* is regulated during development, and mtDNA amplification is necessary for reproduction (*11*). Recently, putative mitochondrial nucleoid proteins have been shown to regulate mtDNA copy number in *C. elegans* (*12*, *13*), however, the metabolism of mitochondrial nucleoids in *C. elegans* is largely unknown.

In this study, we focused on *C. elegans* HMG-5 (F45E4.9), which contains two HMG-box domains

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and shows sequence similarity with human TFAM. RNA interference (RNAi) induced knockdown of HMG-5 resulted in a reduction of the mtDNA copy number in *C. elegans* P0 hermaphrodites. Here, from our biochemical and immunohistological analysis, HMG-5 was found to be localized to the mitochondria in mammalian cultured cells. HMG-5 was contained in the nucleoid fraction in these cells and was demonstrated to interact with mtDNA non-specifically. Furthermore, HMG-5 was shown to interact with itself as well as human TFAM. These results demonstrate that HMG5 shares similar biochemical properties with mammalian TFAM as a nucleoid factor. We propose that HMG-5 is a good candidate for investigating mtDNA metabolism in multicellular organisms and related human diseases.

Materials and Methods

Caenorhabditis elegans strains and conditions

The N2 (Bristol, UK) strain was used as the wild-type. The worms were cultured at 20°C as described (*14*).

Sequence comparisons and alignments

Amino acids sequences were obtained from WormBase and NCBI. The WormBase accession numbers of the sequences were as follows: *C. elegans hmg-5*: WBGene00001975, *C. briggsae hmg-5*: WBGene00028287, *C. remanei hmg-5*: WBGene00062466 and the NCBI accession numbers of the sequences were as follows: *Homo sapiens TFAM*: NP_003192, *Mus musculus TFAM*: NP_033386, *Drosophila melanogaster TFAM*: NP_524415, *Anopheles gambiae TFAM*: XP_316944 and *Saccharomyces cerevisiae Abf2*: NP_013788. Identities and similarities were calculated using Align, and multiple sequence alignments were performed using ClustalW2 and the open software suite of the European Bioinformatics Institute, UK (http://www.ebi.ac.uk/Tools/sequence.html).

Plasmid construction

The coding region of HMG-5 was amplified by PCR from the cDNA of the wild-type *C. elegans* N2 strain. The PCR products were introduced into the mammalian expression vector pEF4/Myc-His B (Invitrogen) or the bacterial expression vector pGEX-4T-3 (Amersham Biosciences).

Cell culture and transfection

HeLa cells were cultured as previously described (*15*). Transfection was performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions.

Immunocytochemistry

HeLa cells were plated on 35-mm poly-L-lysine-coated glass-bottomed dishes (Matsunami Glass Ind.). At 24 h after transfection, mitochondria were stained with MitoTracker Red CM-H_2XRos (2.5 μM, Molecular Probes) for 30 min. The cells were then fixed for 20 min at room temperature with 4% paraformaldehyde and 0.4% Triton X-100 in PBS. The cells were probed with anti-Myc antibody (BD Biosciences, mouse monoclonal antibody) as previously described (*15*). Fluorescent images were captured and analysed with a μRadiance^{TM} Laser Scanning Confocal Microscope System (Bio-Rad).

Western blotting

Samples were separated by electrophoresis on SDS-polyacrylamide gels (12% acrylamide) and electrophoretically transferred to a nitrocellulose membrane (Hybond ECL, GE Healthcare). The membrane was then probed with antibodies and detected with an enhanced chemiluminescence system (GE Healthcare), as previously described (*15*). The following primary antibodies were used: anti-Myc (1 : 1000; BD Biosciences), anti-TFAM (1 : 100; Santa Cruz Biotechnology) and anti-P32 (1 : 200; BD Biosciences).

Preparation of mitochondria and immunoprecipitation

Mitochondria were prepared from HeLa cells as previously described (*16*). To assess NP-40-solubility, mitochondria, which contain 200–300 μg of protein, were suspended in TES buffer (10 mM Tris–HCl, pH 7.4, 1 mM EDTA, 0.25 M sucrose and 0.5% NP-40) on ice for 30 min. After centrifugation at 20,000 *g* and 4°C for 30 min, the NP-40-insoluble pellets and -soluble supernatant were separated and subjected to the immunoblot or DNA-extraction analysis. The mitochondrial pellet from cells expressing HMG-5-Myc or not was extracted with Nucleoid IP buffer (10 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.5% NP-40 and 0.5% BSA). Immunoprecipitation was carried out by incubation of the extract with 1 μg of Myc monoclonal antibody and protein G-Sepharose (GE Healthcare) at 4°C for overnight. After washing the beads three times with Nucleoid IP buffer minus the BSA, they were extracted with 2× sample buffer for western blotting or digestion buffer (10 mM Tris–HCl, pH 8, 100 mM NaCl, 25 mM EDTA and 0.5% SDS) for DNA preparation. DNA was extracted by the standard Proteinase K digestion method.

Preparation of mitochondrial membrane fractions

The preparation of membrane fractions containing *C. elegans* mitochondria was performed as described previously (*17*) with minor modifications. The worms were washed three times with PBS and once with TNE (20 mM Tris–HCl, pH 7.4, 150 mM NaCl and 2 mM EDTA) buffer. After being washed, the worms were then suspended in two volumes of TNE buffer and disrupted using a Teflon homogenizer. The crude lysate was then centrifuged at 1,500*g* and 4°C for 10 min, and the supernatant was further centrifuged at 100,000*g* for 30 min. The pellets containing mitochondrial membrane fractions were suspended and extracted with the Nucleoid IP buffer.

Glutathione S-transferase pull-down assay

pGEX-4T-3-HMG-5 was transformed into the *Escherichia coli* BL21 strain. The expression of glutathione S-transferase (GST)-HMG-5 was induced with 0.1 mM isopropyl 1-thio-β-D-galactopyranoside (IPTG) at 22°C for 5 h, and the fusion proteins were affinity purified with glutathione-Sepharose 4B (GE Healthcare). Myc-tagged HMG-5 was synthesized *in vitro* using the TNT T7 Quick Coupled Transcription/Translation System (Promega). One microgram of GST or GST-HMG-5 was mixed with *C. elegans* membrane fractions, *in vitro*-translated HMG-5-Myc or pUC19 plasmid DNA, in Nucleoid IP buffer, and then affinity purified with glutathione-Sepharose 4B.

PCR and quantitative PCR

For semi-quantitative PCR of the human mtDNA, the displacement loop (D-loop) region (nucleotides 16,029–599, 1142 bp) was amplified. LightCycler-FastStart DNA Master SYBR Green I (Roche) was used for the quantitative PCR together with LightCycler (Roche). To produce a standard curve, 0.5, 1, 2, 4 and 8 ng of the HeLa cell genomic DNA were used. For the amplification of human mtDNA (132 bp), the following primer sets were used: forward, 5′-GCCTGCCTGATCCTCCAAAT-3′ (nucleotides 14,862–14,881), reverse, 5′-AAGGTAGCGGATGATTCAGCC-3′ (nucleotides 14,973–14,993). For the amplification of *C. elegans* mtDNA (whole genome size: 13,794 bp, amplified size: 188 bp) and 18S rRNA fragments (198 bp), the following primer sets were used: mtDNA: forward, 5′-CTTTTATTACTCTATATGAGCGTC-3′ (nucleotides 1,821–1,844), reverse, 5′-AACAAAAGAAATTCCTGGTACAAG-3′ (nucleotides 1,985–2,008), 18S rRNA: forward, 5′-CAGACCAAACGTTTTCGGACGTTG-3′, reverse, 5′-TTGGACGTGGTAGCCGTTTCTAAG-3′. The *C. elegans* DNA template used for the quantitative PCR was prepared as follows. The worms were incubated with lysis buffer (10 mM Tris–HCl, pH 8, 50 mM KCl, 2.5 mM $MgCl_2$, 0.45% NP-40, 0.45% Tween-20, 0.01% gelatin and 100 μg/ml Proteinase K) for 2.5 h at 50°C, and then the proteinase reaction was inactivated by incubation for 10 min at 95°C. Next, the lysate was appropriately diluted and used as the template. The genomic DNA prepared from *C. elegans* by the standard Proteinase K digestion method was used to construct standard curves for mtDNA amplification (0.5, 1, 2, 4 and 8 ng of the DNA) and 18S rRNA gene amplification (1, 2, 4, 8 and 16 ng of the DNA). Quantitative PCR was performed three times using the templates.

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RNA interference

Soaking RNAi was carried out as described previously (*18*). cDNA for HMG-5 or GFP containing T7 promoter sequences at its 5′- and 3′-termini was amplified by PCR with following primers: HMG-5: forward, 5′-CTAATACGACTCACTATAGGGATGTTGGGAA CAATTTCA-3′, reverse, 5′-CTAATACGACTCACTATAGGGTT GATCTGCATTTTCTTTC-3′, GFP: forward, 5′-CTAATACG ACTCACTATAGGGTAAAGGAGAAGGACTTTTC-3′, reverse, 5′-CTAATACGACTCACTATAGGGCAAGAATGTTTCCAT CTTC-3′. Using these cDNA as templates, dsRNA were prepared by *in vitro* transcription with T7 RNA polymerase. L4 hermaphrodites were soaked in the dsRNA solution (2.5 μg/μl) for 24 h and then transferred onto freshly seeded plates every 12 h. Offspring were counted after removing the P0 hermaphrodites. After 48 h incubation, the P0 hermaphrodites were sacrificed and subjected to the quantitative PCR.

DAPI staining

Whole mount staining with 4,6-diamidino-2-phenylindole (DAPI) was performed as described previously (*14*), and fluorescent images were captured with an Axio Observer D1 system (Carl Zeiss).

Combined immunofluorescence and fluorescent in situ *hybridization*

The detailed method was described in (*9*, *15*). Briefly, after fixation of the cells, the cells were stained with anti-Myc antibody and probed with mtDNA probe, sequentially.

Results

HMG-5 maintains the copy number of mtDNA in C. elegans

The *C. elegans* HMG-5 contains two HMG-box motifs and similar domain structure with human TFAM (Fig. 1). The amino acid sequence of HMG-5 shows 23.3% identity and 42.3% similarity with human TFAM by pair wise alignment. Human TFAM contains two conserved tryptophan residues in each HMG-box domain (Fig. 1, arrowhead), which are shown to be involved in stabilizing protein–nucleic acid complexes (*19*). Of these, three tryptophan residues (W88, W189 and W218 in human TFAM) were entirely conserved in HMG-5 (Fig. 1). Thus, we suppose that HMG-5 is a *C. elegans* orthologue of TFAM. In addition, by predicting its mitochondrial target sequence, HMG-5 was shown to have a high probability of localizing to the mitochondria: Target P1.1 (probability score: 0.804) (*20*) and Mitoprot (probability score: 0.8533) (*21*) (Table I). These programs also predicted high probability scores for TFAM from flies to mammals and yeast Abf-2, a functional orthologue of TFAM, localizing to the mitochondria (Table I). Thus, it is likely that HMG-5 localizes and functions in the mitochondria, similar to TFAM.

To clarify the role of HMG-5 in mtDNA maintenance, HMG-5 was knocked down by RNAi in

Table I. Prediction of mitochondrial targeting sequence by TargetP 1.1 and Mitoprot.

Protein name	TargetP 1.1	Mitoprot
Hs-TFAM	0.836	0.9399
Mm-TFAM	0.612	0.7713
Dm-TFAM	0.6	0.6762
Ce-HMG-5	0.804	0.8533
Sc-ABF-2	0.822	0.9308

The amino acid sequences of TFAM from humans (Hs, *Homo sapiens*), mice (Mm, *Mus musculus*) and flies (Dm, *Drosophila melanogaster*); HMG-5 (Ce, *Caenorhabditis elegans*); and ABF-2 (Sc, *Saccharomyces cerevisiae*) were applied to the prediction programs TargetP1.1 and Mitoprot.

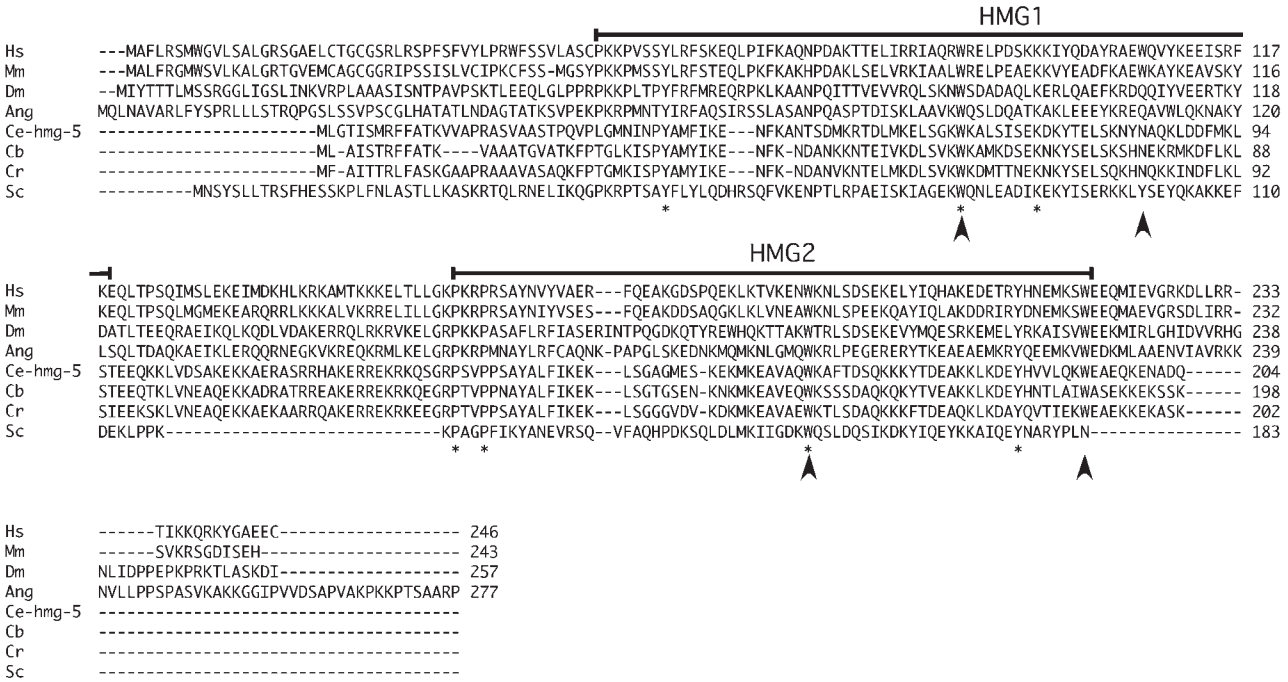

Fig. 1 Multiple alignments of TFAM protein sequences. Gap sequences are denoted by 'dashed lines'. Bold lines upper of the sequences indicates HMG-box domain. Asterisks indicate perfectly conserved amino acid residues. Arrowheads indicate conserved tryptophan residues in HMG-box domain (see text). Hs, *Homo sapiens;* Mm, *Mus musculus;* Dm, *Drosophila melanogaster;* Ang, *Anopheles gambiae;* Ce, *Caenorhabditis elegans;* Cb, *C. briggsae;* Cr, *C. remanei* and Sc, *Saccharomyces cerevisiae*.

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C. elegans. The coding region of HMG-5 was amplified by PCR from mammalian expression vector for HMG-5-Myc (described bellow), and used as template for *in vitro* transcription to produce dsRNA. L4 hermaphrodites were soaked in *hmg-5* dsRNA solution for 24 h and transferred to new plates every 12 h, and the progeny remaining on the plate were also scored. The *hmg-5 (RNAi)* P0 hermaphrodites showed almost no visible phenotype and contained a nearly equal number of germline cells in their gonads compared to the control, according to nuclear staining of the cells (Fig. 2A). There is little difference between the number of F1 progeny produced by *hmg-5 (RNAi)* and control P0 (data not shown), supporting the normal development of germline cells in the P0 worms. Then, we examined the copy number of mtDNA relative to nuclear DNA content in the P0 worms. By quantitative PCR analysis, we found that the *hmg-5 (RNAi)* P0 contained a significantly reduced amount of mtDNA compared to the control and *gfp (RNAi)* P0 (Fig. 2B). These results demonstrate that HMG-5 maintains the copy number of mtDNA in *C. elegans* similarly to mammalian TFAM.

Biochemical properties of HMG-5 in cultured mammalian cells

Functionally important proteins are well conserved from worm to human, as are their localizations. There are no immortal cell lines derived from *C. elegans*. Therefore, to clarify the biochemical properties of HMG-5, we used human HeLa cells, in which characterization of TFAM properties is well established. The coding region of HMG-5 (612 bp) was amplified by RT-PCR from total RNA of adult N2 strain (Fig. 3A), and subcloned into the mammalian expression vector pEF4/Myc-His. There were no detectable shorter splicing variants of HMG-5 during adult and developmental stages (Fig. 3A, Supplementary Fig. 1). We then expressed C-terminally Myc-tagged HMG-5 in HeLa cells and examined its localization. HMG-5-Myc was found to localize in the mitochondria (Fig. 3B and C), and its signals were spotted and sublocalized in the mitochondria (Fig. 3C). The sublocalization pattern of HMG-5-Myc is similar to those of mtDNA and TFAM signals (*15*, *22*, *23*), suggesting that HMG-5 is included in the mtDNA–protein complex (nucleoid) in HeLa cell mitochondria. Actually, a part of immunofluorescent signals of HMG-5-Myc overlapped with mtDNA signals stained with fluorescent *in situ* hybridization (FISH) (Fig. 3D).

Based on the hypothesis that *C. elegans* HMG-5 would be a functional orthologue of TFAM, we tested whether it associates with mitochondrial nucleoids in HeLa cells. The mitochondria purified from HeLa cells overexpressed exogenous HMG-5 gene were separated into NP-40-soluble and -insoluble fractions. mtDNA and human TFAM are known to be contained in the NP-40-insoluble fraction as mitochondrial nucleoids (*7*). In our experiment, it was confirmed that mtDNA and TFAM were mainly detected in the NP-40-insoluble fraction; whereas, p32, a mitochondrial matrix protein that is unrelated to the nucleoids, was only detected in the NP-40-soluble fraction (Fig. 4). Under these conditions, HMG-5 was also detected in the NP-40-insoluble fraction (Fig. 4), indicating that HMG-5 is included in the nucleoids in HeLa cells.

TFAM interacts directly with mtDNA in a sequence non-specific manner and packages it (*23*, *24*). Next, to investigate the interaction of exogenous HMG-5 with human mtDNA in HeLa cells, immunoprecipitation was performed with mitochondrial extracts expressing HMG-5-Myc gene. In the case of human TFAM, TFAM associated with mtDNA because it was co-immunoprecipitated with mtDNA by the analysis (*23*). By immunoprecipitation with anti-Myc antibody and quantitative PCR against mtDNA, we found that mtDNA was significantly co-precipitated with

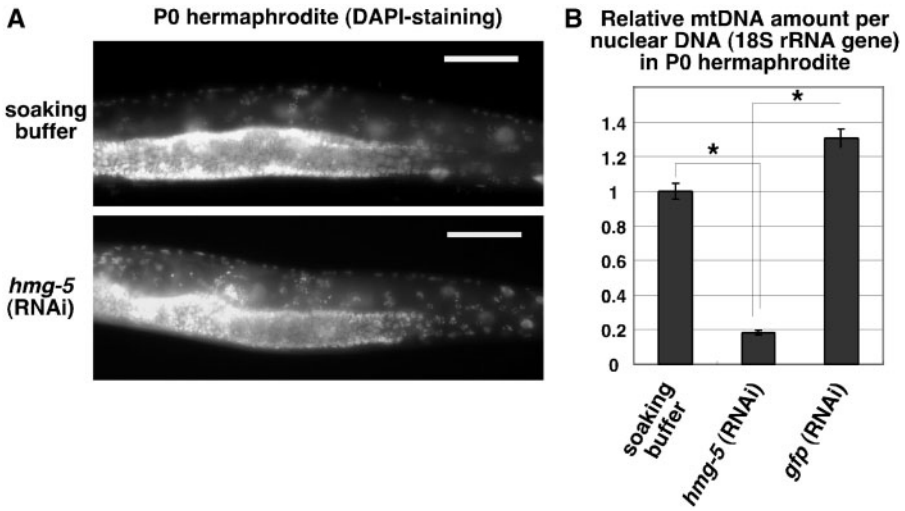

Fig. 2 Knockdown of HMG-5 reduces the mtDNA copy number. L4 worms were soaked in the dsRNA solution (*hmg-5 (RNAi)* or *gfp (RNAi)*) or not (soaking buffer) for 24 h and then recovered to a new plate. After 48 h incubation on the plate, the P0 worms were subjected to DAPI-staining (A) or quantitative PCR against mtDNA (B). (A) DAPI image of the gonads of the P0 worms. Scale bars; 50 μm. (B) The relative amount of *C. elegans* mtDNA per nuclear gene (18S rRNA) in the P0 worms was quantified in three PCR experiments. $*P < 0.01$, paired *t*-test.

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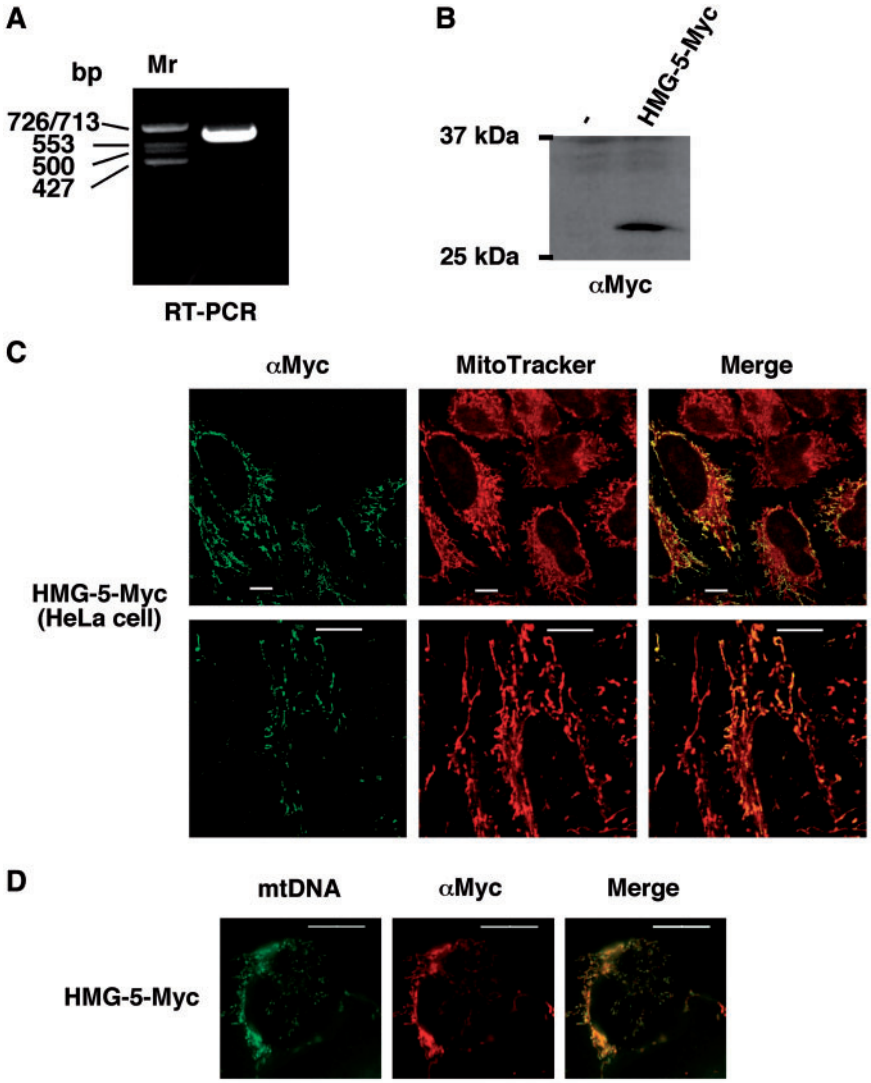

Fig. 3 Mitochondrial localization of HMG-5 in HeLa cells. (A) The coding region of HMG-5 (612 bp) was amplified by RT–PCR from total RNA of adult N2 strain. Molecular size marker (ϕX174/*Hin*f I) is shown on the left. (B) C-terminally Myc-tagged HMG-5 was expressed in HeLa cells. Whole cell extracts expressing HMG-5-Myc or not (−) were separated by SDS–PAGE and immunoblotted with anti-Myc antibody. (C) HeLa cells expressing HMG-5-Myc were stained with MitoTracker and immunostained with anti-Myc antibody. The images were then merged. (D) HeLa cells expressing HMG-5-Myc were probed with anti-Myc antibody and mtDNA probe, sequentially. Scale bars, 10 μm.

HMG-5-Myc protein (Fig. 5A and B). Using another primer set, it was confirmed that mtDNA fragments were only amplified in the fraction containing HMG5-Myc (Supplementary Fig. 2). Therefore, these results indicate that HMG-5 interacts with human mtDNA in HeLa cells.

HMG-5 interacts with mtDNA non-specifically and itself

The above results strongly suggest that HMG-5 is a functional orthologue of TFAM and maintains mtDNA in *C. elegans*. To detect direct interactions between HMG-5 and *C. elegans* mtDNA, a GST pull-down experiment was performed. The full-length of *C. elegans* HMG-5 was expressed as GST fusion protein in bacteria and purified. Membrane fractions containing mitochondria were prepared from the *C. elegans* wild-type strain (*17*), and the extracts were mixed with GST or GST-HMG-5. After the pull-down assay, *C. elegans* mtDNA was amplified and quantified by quantitative PCR. This experiment showed that *C. elegans* mtDNA was significantly precipitated with GST-HMG-5 but not with GST (Fig. 5C), demonstrating that HMG-5 is able to bind to *C. elegans* mtDNA. The binding of HMG-5 to both of human and *C. elegans* mtDNA suggests its feature as non-specific DNA-binding protein. Supporting this, a GST pull-down assay showed that GST-HMG-5 interacts directly with pUC19 plasmid DNA (Supplementary Fig. 3).

Mammalian TFAM can form homodimer through its interaction (*25*). We then tested whether HMG-5 is able to associate with itself. *In vitro* translated HMG-5-Myc was mixed with GST or GST-HMG-5, and GST pull-down was performed. The HMG-5-Myc was specifically co-precipitated with GST-HMG-5 (Fig. 5D), showing that HMG-5 can interact with itself.

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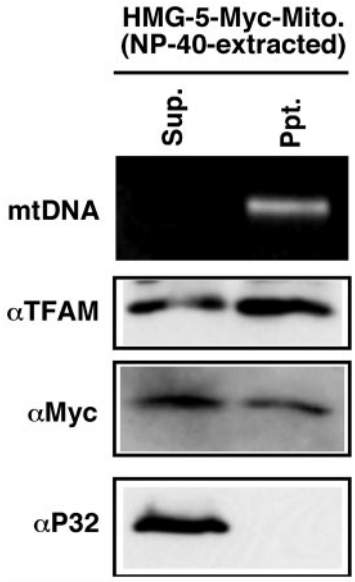

Fig. 4 HMG-5 was contained in the nucleoid fraction. Mitochondria were purified from HeLa cells expressing HMG-5-Myc. The mitochondria were separated into NP-40-soluble (Sup.) and insoluble fractions (Ppt.). The mtDNA extracted from each fraction was subjected to electrophoresis on agarose gels as described previously (*34*). The proteins extracted from each fraction were subjected to immunoblot analysis with the indicated antibodies.

Discussion

Here, we show that HMG-5 is a functional orthologue of the mitochondrial protein TFAM from its biochemical properties and physiological function *in vivo*.

Several mitochondrial nucleoid proteins, such as mitochondrial single-stranded DNA-binding protein (mtSSB, known as par2.1 in *C. elegans*) and mtDNA polymerase gamma (POLG), are conserved between *C. elegans* and mammals (*12*, *13*, *26*). Knockdown or a deficiency of them reduces the copy number of mtDNA (*12*, *13*), indicating their role in mtDNA maintenance. Recently, comprehensive knockdown of putative mitochondrial nucleoid proteins was performed in *C. elegans* (*27*). In the feeding RNAi system, knockdown of HMG-5 was shown to reduce the copy number of mtDNA in F1 progeny as like as our soaking system in P0 worms. Therefore, HMG-5 seems to be functional orthologue of mammalian TFAM, however, its biochemical properties have been largely unknown. HMG-5 was found to contain a possible mitochondrial targeting sequence by prediction programs (Table I), and recent proteomic analysis revealed that peptides produced by F45E4.9 (HMG-5) were detected in a purified mitochondrial fraction from *C. elegans* by mass spectrum analysis (*28*). HMG-5 was also selected as a possible mitochondrial protein, and its knockdown induces fragmentation of mitochondria (*29*). These lines of evidence strongly support the mitochondrial localization of HMG-5 in *C. elegans*. Mitochondrial localization of HMG-5 in *C. elegans* should be proven by immunodetection using specific antibody to HMG-5 or transgene technology in future study.

In our biochemical and immunohistological study, HMG-5 was found to localize to the mitochondria of mammalian cultured cells (Fig. 3). HMG-5 was contained in the nucleoid fraction and was found to associate with mtDNA in mammalian cells (Figs 4 and 5). HMG-5 was originally identified as double-stranded telomeric DNA-binding protein (*30*). HMG-5 binds not only to *C. elegans* telomeric DNA composed of TTAGGC repeats, but also to human and plant telomeric sequences, which are composed of TTAGGG and TTTAGGG repeats, respectively (*30*). Thus, they suggested that HMG-5 is not highly specific to telomeric sequences. In our experiment, HMG-5 was able to associate with mtDNA of human and *C. elegans* (Fig. 5), and pUC19 plasmid DNA (Supplementary Fig. 2), supporting its non-specific DNA-binding activity. If HMG binds preferentially to mtDNA or telomeric sequences *in vivo*, the specific structure within them including D-loop might be important for the recognition. Furthermore, HMG-5 can also associate with itself (Fig. 5D), as human TFAM cooperatively binds to DNA as a homodimer (*19*, *25*). Although the role of self-interaction of HMG-5 has not been clear, the interaction might facilitate their cooperative wrapping of mtDNA with high affinity. Considering above, HMG-5 shares several same biochemical properties with mammalian TFAM.

Which function is conserved between HMG-5 and mammalian TFAM? The maintenance of mtDNA copy number is also conserved in *C. elegans*. In addition to the regulation of mtDNA copy number, human TFAM is essential for the activation of mtDNA transcription, and its basic C-terminal tail is required for this function (*31*). Considering that the C-terminal tail is not conserved in yeast Abf2 or HMG-5 and that Abf2 is not required for the activation of mtDNA transcription (*32*), HMG-5 might play a reduced role in mtDNA transcriptional activation. Further investigation is required to examine whether HMG-5 regulates the transcription, replication or segregation of mtDNA in the organisms. From these observations, the maintenance of mtDNA copy number is a conserved function among TFAM family proteins from yeast to humans.

Caenorhabditis elegans is a good model for studying mitochondrial metabolism and aging. In addition, *C. elegans* provides a useful tool for studying mtDNA maintenance because heteroplasmy of mtDNA variants exists as well as human (*33*). There seems to be a close relationship between the amount of mtDNA and lifespan (*11*, *13*). Given that HMG-5 is a functional orthologue of TFAM and directly regulates mitochondrial genome content, we suppose that HMG-5 is a good target for investigating the relationship between the amount of mtDNA and lifespan.

Taken together, we conclude that HMG5 is a functional orthologue of TFAM; *i.e.* it acts as a mitochondrial nucleoid factor. Furthermore, we suggest that HMG-5 is a good candidate for investigating mtDNA metabolism, such as its replication and segregation, in multicellular organisms. Future studies aimed at identifying the mechanisms underlying the regulation of mtDNA metabolism during the worm's

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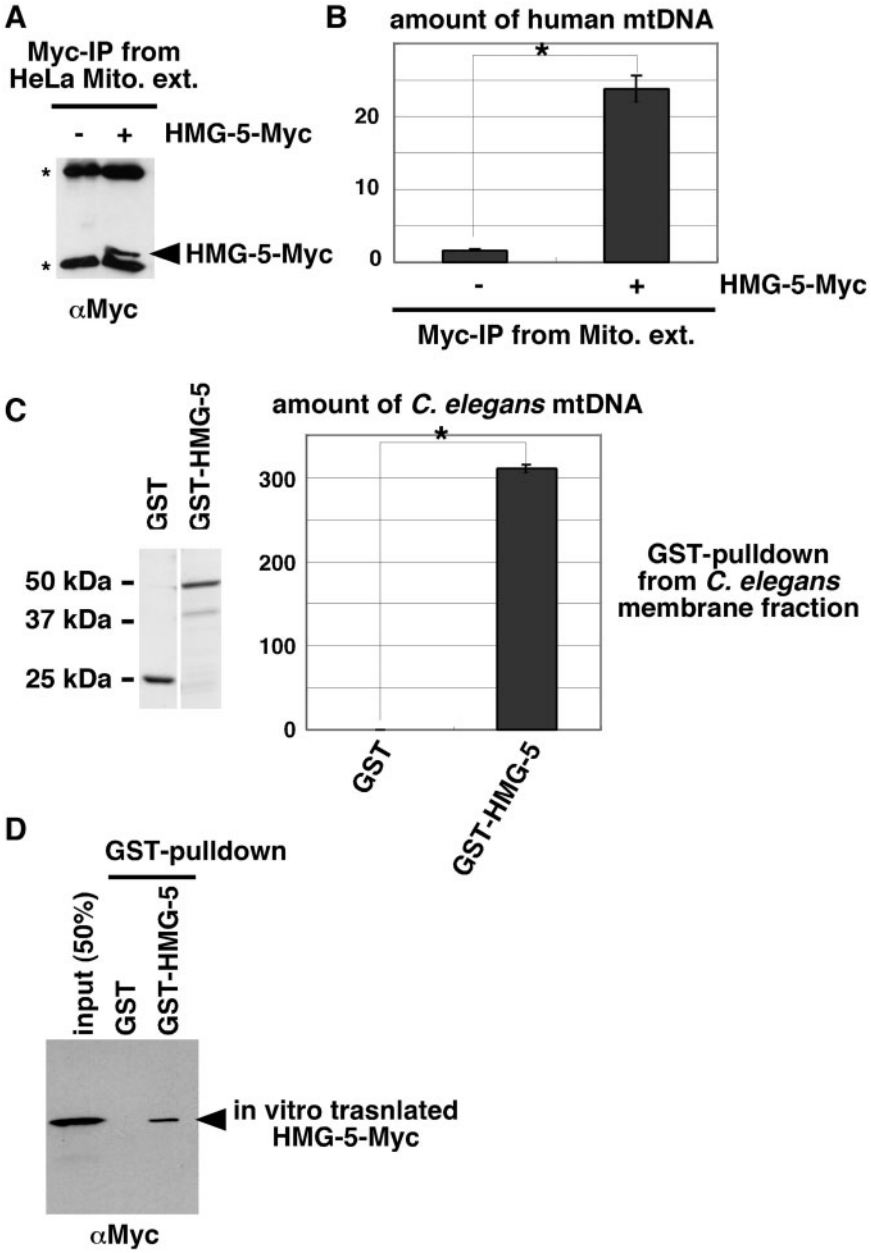

Fig. 5 Interaction between HMG-5 and mtDNA. (A) Mitochondrial extracts expressing HMG-5-Myc or not (−) were immunoprecipitated with anti-Myc antibody. The precipitants were separated by SDS–PAGE and immunoblotted with anti-Myc antibody. The asterisks represent the heavy and light chains of the antibody. (B) DNA was extracted from the co-precipitants with HMG-5-Myc or not (−) and subjected to quantitative PCR against human mtDNA. $*P<0.01$, paired *t*-test. (C). Left panel; GST and GST-HMG-5 used for the pull-down assay were separated by SDS–PAGE and stained with Coomassie Brilliant Blue R-250. Right panel; Mitochondrial membrane extracts prepared from *C. elegans* were pulled down with GST or GST-HMG-5, and the precipitants were subjected to quantitative PCR against *C. elegans* mtDNA. $*P<0.001$, paired *t*-test. (D) *In vitro* binding assay between GST-HMG-5 and *in vitro*-translated HMG-5-Myc. Fifty percent of the input HMG-5-Myc is also shown on the left. A GST pull-down assay showed that GST-HMG-5 interacts directly with HMG-5-Myc.

development will provide important insights into the human diseases caused by mtDNA abnormalities.

Supplementary Data

Supplementary Data are available at *JB* Online.

Acknowledgements

We are grateful to Dr Teruaki Takasaki for his technical advice.

Funding

N2 nematode strain used in this work was provided by the Caenorhabditis Genetics Center, which is funded by the National Institutes of Health National Center for Research Resources (NCRR); Ministry of Education, Culture, Sports, Science and Technology of Japan, Grant-in-Aid for Young Scientists (B) to M.S.; this publication was also subsidized by JKA through its promotion funds from Keirin race.

Conflict of Interest

None declared.

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