site of Mitochondrial Research Society). However we have not observed such recovery of blindness in the members of this family. Though the pedigree is so small and additional biochemical date is not available in this study, we suggest that an A9016G sequence variant is specific for this family and may increase the penetration of LHON with the combination of secondary mutations (Mackey, 1994). Above information will continue to provide us with new insights into the pathophysiology of mitochondrial disease.

Acknowledgements

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Web site of Mitochondrial Research Society.

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Table 1. PCR primers used for amplification of the entire mtDNA of Russian LHON patients.

Fragment	Forward primer	Reverse primer	Length(bp)
1	Gn549F:5'-caa cca aac ccc aaa gac ac-3'	Gn3424B:5'-aac gtt ggg gcc ttt gcg ta-3'	2876
2	Gn3205F:5'-cca cac cca ccc aag aac ag-3'	Gn5910B:5'-cga aca tca gtg ggg gtg ag-3'	2706
3	Gn5662F:5'-cca atg gga ctt aaa ccc ac-3'	Gn8028B:5'-gct tca atc ggg agt act ac-3'	2367
4	Gn7794F:5'-cca tca tcc tag tcc tca tc-3'	Gn10515B:5'-gtg aga tgg taa atg cta g-3'	2722
5	Gn10289F:5'-agc cct aca aac aac taa cc-3'	Gn12360B:5'-ggt tat agt agt gtg cat gg-3'	2072
6	Gn12144F:5'-aca tca tta ccg ggt ttt cc-3'	Gn14762B:5'-tgc gta ttg ggg tca ttg g-3'	2619
7	Gn14641F:5'-acc cac act caa cag aaa c-3'	Gn706B:5'-gga tgc ttg cat gtg taa tc-3'	2635

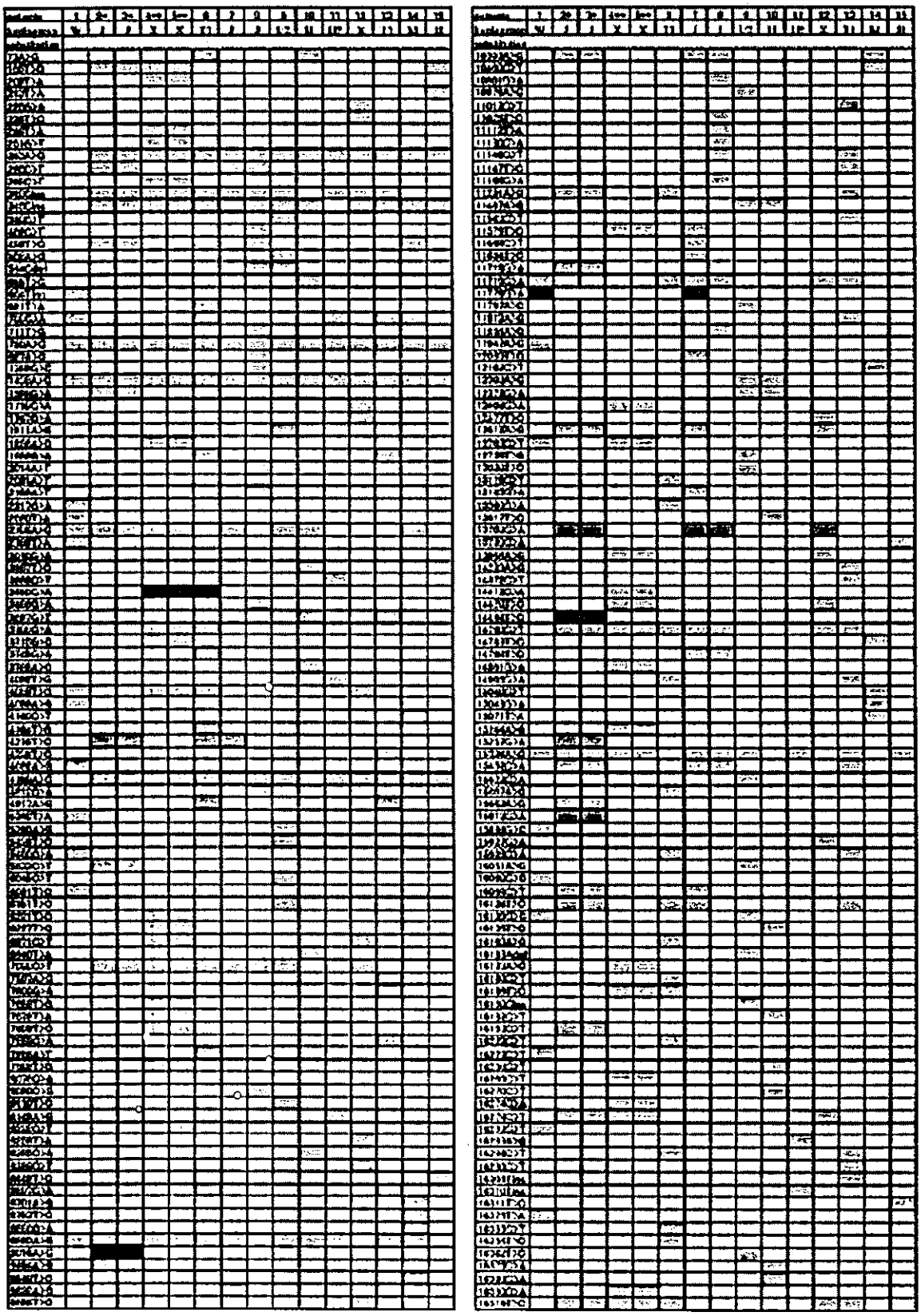
Table 2. Forward primers used for direct sequence.

Number	Primer	5'-position
1.	CQ549:5'-caa cca aac ccc aaa gac acc	549
2.	CQ611:5'-gaa aat gtt tag acg ggc tc	611
3.	CQ 1161:5'-aac tca aag gac ctg gcg gt	1161
4.	CQ1690:5'-cca ctc cac ctt act acc aga c	1690
5.	CQ2164:5'-ccc ata gta ggc cta aaa gca gcc	2164
6.	CQ2629:5'-atg aat ggc tcc acg agg gtt c	2629
7.	CQ3116:5'-cct ccc tgt acg aaa gga c	3116
8.	CQ3205:5'-cca cac cca ccc aag aac agg g	3205
9.	CQ3712:5'-gta gcc caa aca atc tca ta	3712
10.	CQ4240:5'-tcc agc att ccc cct caa ac	4240
11.	CQ4621:5'-gtt cca cag aag ctg cca tc	4621
12.	CQ5151:5'-cta cta cta tct cgc acc tg	5151
13.	CQ5662:5'-cca atg gga ctt aaa ccc ac	5662
14.	CQ6251:5'-tat agt gga ggc cgg agc ag	6251

15.	CQ6801:5'-gac aca cga gca tat ttc ac	6801
16.	CQ7321:5'-gag aag cct tcg ctt cga ag	7321
17.	CQ7794:5'-cca tca tcc tag tcc tca tc	7794
18.	CQ8239:5'-ctt tga aat agg gcc cgt att tac c	8239
19.	CQ8634:5'-tct cat caa caa ccg act aa	8634
20.	CQ9091:5'-aca ctt atc atc ttc aca at	9091
21.	CQ9621:5'-gca tca gga gta tca atc ac	9621
22.	CQ10133:5'-aca act caa cgg cta cat ag	10133
23.	CQ10289:5'-agc cct aca aac aac taa cc	10289
24.	CQ10616:5'-caa cac cca ctc cct ctt ag	10616
25.	CQ11183:5'-cgc ctg aac gca ggc aca ta	11183
26.	CQ11671:5'-aac ccc ctg aag ctt cac cg	11671
27.	CQ11900:5'-gtg cta gta acc acg ttc tcc	11900
28.	CQ12144:5'-aca tca tta ccg ggt ttt cc	12144
30.	CQ12602:5'-tca tcc ctg tag cat tgt tc	12602
31.	CQ13101:5'-agg aat ctt ctt act cat cc	13101
32.	CQ13490:5'-cct cac agg ttt cta ctc c	13491
33.	CQ13949:5'-cct atc tag gcc ttc tta cga gcc	13949
34.	CQ14511:5'-cta tta aac cca tat aac ct	14511

35.	CQ14641:5'-acc cac act caa cag aaa c	14641
36.	CQ15121:5'-aac agc ctt cat agg cta tg	15121
37.	CQ15535:5'-cac ccc tcc cca cat caa gcc	15535
38.	CQ15838:5'-cct aat acc aac tat ctc cc	15838
39.	CQ16200:5'-aca agc aag tac agc aat ca	16200
40.	CQ132:5'-ctt tga ttc ctg cct cat cc °	132

Table 3. Sequence variation in the mtDNA of the 15 LHON patients.



* and ** : showed the members of the same family.

Gray boxes showed nucleotide change in this study.

Black boxes showed primary mutation of LHON.

Green boxes showed secondary mutation of LHON.

Purple box showed novel polymorphism of LHON in our family.

Figure 1. Pedigree of the family.

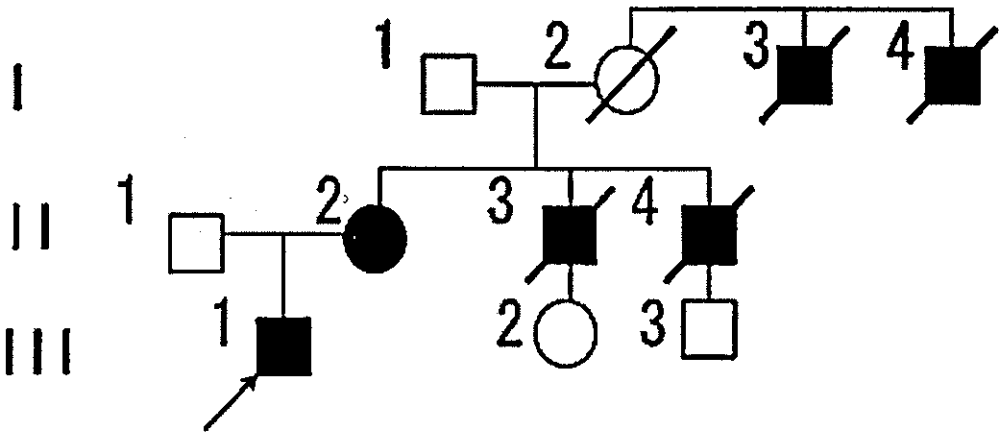


Figure 2. PCR-RFLP analysis of an A9016G mutation

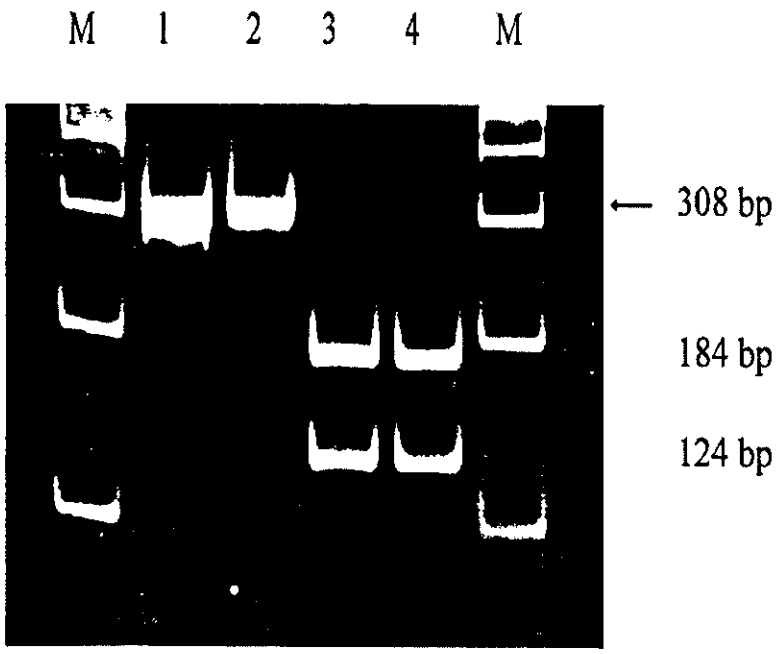


Figure 3. ATPase 6 polypeptide through evolution

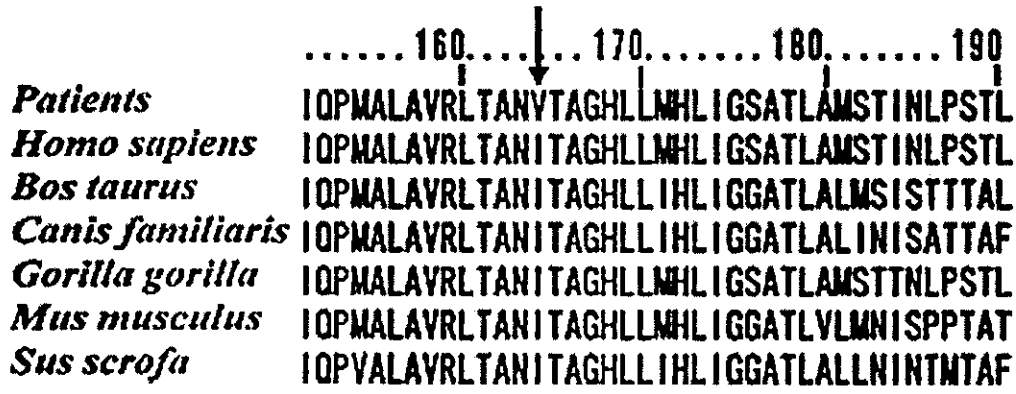


Figure legends

Figure 1.

Pedigree of the family. *Arrow* shows the Proband (III.1), and filled symbols show affected members for LHON (subjects I.3, I.4, II.2, II.3, and II.4). Five persons out of 5 men in the maternal relatives in this family are LHON indicated 100% penetrance in men.

Figure 2.

HpyCH4 IV restriction endonuclease digestion pattern for the A9016G shows in the 12% PAGE. The full length of PCR product (308bp) was cut into two fragments in the presence of the A9016G. Both patients showed heteroplasmic condition in the 93 %of mutant. Lane designations: 1 = undigested PCR product, 2 = control after digestion, 3 = PCR from mother after digestion, 4 = PCR from proband after digestion.

Figure 3.

Comparison of mitochondrial ATPase 6 polypeptide among different species through evolution. The A9016G changes a conserved amino acid from isoleucine into valine at the aminoacid number of 164.

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A NOVEL MYC-TARGET GENE, *MIMITIN*, THAT IS INVOLVED IN CELL PROLIFERATION OF ESOPHAGEAL SQUAMOUS CELL CARCINOMA

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Running Title: A novel Myc target gene *mimitin*

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Myc is a ubiquitous mediator of cell proliferation that transactivates the expression of various genes through E-box sites. Here we report a novel gene, *mimitin* (Myc-induced mitochondrial protein), that encodes a mitochondrial protein with a MW of 20 kd. We demonstrated that the transcription of *mimitin* is directly stimulated by c-Myc. To investigate the role of Mimitin, its expression was suppressed by the RNA interference (RNAi) technique. While specific inhibition of *mimitin* expression did not affect cell proliferation in human cervical carcinoma, colon adenocarcinoma, and hepatocarcinoma cell lines, it did suppress cell proliferation in human glioblastoma, esophageal squamous cell carcinoma (ESCC), and embryonic lung fibroblastic cells, with the greatest suppression efficiency in ESCC cells. To investigate whether *mimitin* is related to tumorigenesis in ESCC *in vivo*, the expression of Mimitin protein in ESCC tissues was studied. Mimitin was highly expressed in 80% (28 out of 35) of ESCC tumors, suggesting that high expression of Mimitin is a characteristic feature of ESCC. The expression level of Mimitin was found to be correlated with that of c-Myc and cell proliferation, but not with the histopathological grade, stage of cancer, or age of patients. Taken together, these results suggest that the novel gene *mimitin* is a direct transcriptional target of c-Myc, and is involved in Myc-dependent cell proliferation at least in ESCC cells.

INTRODUCTION

The *myc* family of proto-oncogenes consists of three main genes: c-*myc*, N-*myc*, and L-*myc* (1-5). Deregulated expression of *myc* family genes through gene amplification, viral promoter insertion, chromosomal translocation, or promoter mutation has long been known to be associated with neoplastic diseases in a wide range of vertebrates including humans. Embryonic mice with c-*myc* or N-*myc* deleted develop multi-organ hypoplasia and die during mid-embryogenesis (6-8). These results indicate that the *myc* family genes are central regulators of cell growth (4, 5, 9).

c-*myc* is one of the most widely studied proto-oncogenes, and it is the best characterized member of the *myc* gene family. In general, c-*myc* expression is associated with cell proliferation and is down-regulated in quiescent and differentiated cells. The protein encoded by c-*myc* is a member of the basic helix-loop-helix leucine zipper transcription factors (5, 9). c-Myc protein has been shown to directly transactivate the expression of a number of genes (10), including *ornithine decarboxylase* (*ODC*) (11), *RCC1* (12), *nucleolin* (13), *cyclin D2* (14), and *mina53* (15), through E-box sites (CACGTG elements) on their genomic DNA. However, c-*myc* remains enigmatic, and information about additional genes controlled by c-*myc* may help elucidate its function.

Esophageal squamous cell carcinoma (ESCC) is a highly aggressive disease with a poor prognosis (16). It was suggested that genetic changes associated with the development of ESCC involve activation of proto-oncogenes (17), including cyclin D1 (18), c-*erb*B2, and c-*myc*, and inactivation of several tumor suppressor genes, including p53, Rb, and p16 (19-22). However, the precise mechanisms of the development of ESCC remain unclear.

Here we report a novel gene, *mimitin*, whose expression is directly induced by c-Myc. The *mimitin* gene encodes a protein with a MW of 20 kd, which localizes in mitochondria. Our results suggest that *mimitin* contributes to cell proliferation in ESCC cells.

EXPERIMENTAL PROCEDURES

Cells and cell culture - The human cells, cervical carcinoma HeLa, promyelocytic leukemia HL60, colon adenocarcinoma SW620, glioblastoma T98G, T98G-expressing c-MycER chimeric protein (T98Gmycer-2 cells) (15), ESCC TE-11 (23, 24), hepatocellular carcinoma Kyn2 (25), and embryonic lung HEL (26) were described previously. T98G cells were cultured in Eagle's minimum essential medium supplemented with non-essential amino acids and 10% fetal calf serum (FCS). The other cells were cultured in Dulbecco's modified Eagle's medium containing 10% FCS.

Polymerase chain reaction (PCR), rapid amplification of 5' cDNA ends (5'-RACE) and 3' cDNA ends (3'-RACE) - PCR amplifications were performed using Ex Taq polymerase (Takara, Shiga, Japan) or KOD polymerase (Toyobo, Osaka, Japan) according to their manufacturer's instructions. 5'-RACE reactions were performed as described previously (15, 27). The first PCR for 5'-RACE was performed using the primer, mimitin-RACE-1 (5'-GCTGCTGGGAGCCACTGAGGGTTCT-3', a sequence in the EST clone AI803230) and the AP1 primer provided by the supplier. Nested PCR was performed using the mimitin-RACE-2 primer (5'-GCATGGCCTTTAATTTGAGTTTGAAC-3'), which corresponds to a sequence in the upstream region of mimitin-RACE-1, and the AP2 primer provided by the supplier. The amplified 0.5 kb fragment was cloned into a pGEM-T vector (Promega, Madison, WI) and sequenced.

The 3'-RACE was performed with the same protocol as the 5'-RACE using primers, mimitin-3'RACE-1 (5'-GCGGCTGGAGCATTACCCCTACTGC-3', a sequence that was found in the 5'-RACE product) for the first PCR, and mimitin-3'RACE-2 primer (5'-AAACTGGGTGGACGGCATGGGTTGG-3'), which corresponds to a sequence in the downstream region of mimitin-3'RACE-1 for nested PCR. The amplified 0.6 kb fragment was cloned into a pGEM-T vector (Promega) to produce pT/mimitin, and sequenced. It was found that pT/mimitin contains a DNA sequence encoding the whole 169 amino acid protein.

Plasmids - cDNA for human *mimitin* was amplified by PCR with 5'-CCGGAATTCTGCCATGGGTTGGTCTCAGGATTTGTTCC-3' (5'-mimitinEco), adding an *Eco*RI site just before the initiation methionine, and 5'-GGAAGATCTGAATTCATTGATTGTGGCTCTTGCCATCTCG-3', adding an *Eco*RI site just after the stop codon, as primers from pT/mimitin. The 0.5 kb fragment was cleaved with *Eco*RI, and inserted into fragments of a pCAGGS mammalian expression vector (28), an *E. coli* expression vector pGEX-3X (Amersham Biosciences, Piscataway, NJ), and an *E. coli* expression vector pET28b(+) (Novagen, Madison, WI), which were cleaved with *Eco*RI and dephosphorylated with *E. coli* alkaline phosphatase, to produce pCAGGS/mimitin, pGEX/mimitin for GST-Mimitin fusion protein, and pET28/mimitin for His x 6 tagged-Mimitin protein, respectively. cDNA for human *mimitin* was amplified by PCR with 5'- mimitinEco, and 5'-GCATGGTACCTGATTGTGGCTCTTGCCATC-3', deleting the stop codon and adding a *Kpn*I site, as primers from pT/mimitin. The 0.5 kb fragment was cleaved with *Eco*RI and *Kpn*I, and inserted into a fragment of a pcDNA3.1/myc-His(-)B mammalian expression vector (Invitrogen, Carlsbad, CA) to produce pcDNA/mimitin-mychis. Reporter plasmids containing a *mimitin* genomic DNA fragment were constructed as described below. A 0.9 kb genomic DNA fragment of the human *mimitin* gene, which extends from the promoter region to intron 1, was amplified by PCR with 5'-GTGGAGGGCAGTTATTCTTTGGAG-3' and 5'-GGGCTCTCACTTTTCCTTCCTTTA-3', and cloned into a pGEM-T vector (Promega) to produce pT/mimitin(promoter). The DNA fragment was amplified from pT/mimitin(promoter) with 5'-GTGGAGGGCAGTTATTCTTTGGAG-3' and 5'-GGGGCTCCCATGGTTCCTTCCTTTACTGACCTC-3', adding a *Nco*I site. The 0.9 kb amplified fragment was cleaved with *Bam*HI and *Nco*I to produce a 0.7 kb fragment, and inserted into the

4.8 kb fragment of a pGL3-Basic vector that contained firefly *luciferase* cDNA (Promega), which was cleaved with *Bgl*II and *Nco*I to produce pmimitin(W)luci. The *mimitin* genomic DNA fragment contains a CACGTG element in the coding region of exon 1. Mutations were introduced at the CACGTG element of pmimitin(W)luci as described previously (15), to produce pmimitin(mE)luci, in which CACGTG was mutated to CACCCG. pRL-CMV containing the *Renilla reniformis luciferase* gene under the control of a CMV promoter was purchased from Promega.

RNA preparation, differential display using a DNA chip (cDNA micro array), Northern blot analysis, and preparation of probe DNAs - Total RNA was isolated from cells as described previously (15). Poly(A^+) RNA aliquots from T98Gmycer-2 cells untreated or treated with 4-hydroxytamoxifen (OHT) were subjected to differential display using a DNA chip (UniGEM Human V Ver. 2, Incyte Genomics, Palo Alto, CA) as described previously (15). Northern blot analysis was performed as described previously (15). The DNA probes for c-*myc* (12) and *glyceraldehyde-3-phosphate dehydrogenase (GAPDH)* (29) were described previously. The DNA probe for *mimitin* was a 0.5 kb *Eco*RI fragment of pCAGGS/mimitin.

Transient expression assay - A transient expression assay was performed basically as previously described (15). In brief, T98Gmycer-2 cells were plated into a dish and cultured in medium supplemented with 0.25% FCS for 20-24 h. One μg of the reporter plasmids and 20 ng of pRL-CMV as an internal transfection marker were transfected. Ten to 12 hours later, OHT was added to activate MycER chimeric protein, and cells were further cultured for 9-12 h. Cells were then collected and analyzed for luciferase activity.

Chromatin immunoprecipitation - Chromatin immunoprecipitation assay was performed as described previously (15), basically according to the methods described by Boyd et al. (30). Immunoprecipitated DNA fragments were detected by PCR, using the primers 5'-GTGGAGGGCAGTTATTCTTTGGAG-3' and 5'-GGGCTCTCACTTTTCCTTCCTTTA-3', which amplified a 0.9 kb fragment containing the sequence of the promoter, exon 1 and part of intron 1 of the human *mimitin* gene. A DNA fragment of the human gene encoding the trifunctional enzyme carbamoyl-phosphate synthase/aspartate carbamoyltransferase/dihydroorotase (*cad*) was also amplified by PCR, using the primers described previously (31).

Antibodies - The GST fusion protein GST-Mimitin was expressed using pGEX/mimitin in *E. coli* JM109 and isolated by a glutathione Sepharose column (Amersham Biosciences), and further purified by SDS-PAGE. Rabbits were immunized with the recombinant polypeptide. Polyclonal anti-Mimitin antibody was purified from rabbit serum using Sepharose 4B conjugated with recombinant His x 6-tagged Mimitin polypeptide isolated by Nickel Column as described previously (15). Antibody-2 against c-Myc (12) and the antibody against mitochondrial protein (Tom22) (32) were described previously. Anti-c-Myc antibody-1 (N262) (Santa Cruz Biotechnology, Santa Cruz, CA), anti-MAPK/ERK-1 antibody (K-23) (Santa Cruz Biotechnology), anti-Tom20 mouse antibody (F-10) (Santa Cruz Biotechnology), goat anti-rabbit IgG-HRP antibody (Santa Cruz Biotechnology, Cat# sc-2054), Alexa 488-conjugated anti-rabbit IgG (Molecular Probes, Eugene, OR), Alexa 568-conjugated anti-mouse IgG (Molecular Probes), and anti-β-actin monoclonal antibody (AC-15) (Sigma-Aldrich Fine Chemicals, St. Louis, MO) were purchased.

Western blot analysis and indirect immunofluorescence staining - Cell lysates were extracted and analyzed by Western blotting as described previously (33). HeLa cells grown on glass coverslips were fixed in 4% paraformaldehyde in PBS for 30 min at room temperature, permeabilized with 1% Triton X-100 in PBS, stained with an indirect immunofluoresence staining technique and observed as described previously (15, 34).

T98G cells were fractionated into enriched mitochondrial and cytosolic fractions, using a mitochondria/cytosol fractionation kit (BioVision Research Products, Mountain View, CA, USA, Cat# K256-100).

Introduction of siRNA into cells - A small interference RNA molecule (siRNA) targeting human c-*myc* and a control siRNA (Ambion Austin, TX, cat# 4604) were purchased. The siRNA sequences targeting human *mimitin*, which are positions 72-90 (si-mimitin-1) and 330-348 (si-mimitin-2) relative to the first nucleotide of the start codon and the 2-nucleotide 3' overhang of deoxythymidine, were chemically synthesized (Hokkaido System Science, Sapporo, Japan). Transfection was carried out with 200 pmol siRNA per well using Oligofectamine (Invitrogen) as described previously (15).

Tissues and immunostaining - Routinely processed formalin-fixed and paraffin-embedded specimens from 35 Japanese patients with ESCC operated on from 1997 to 1998 at the Department of Surgery of Kurume University Hospital were used. One block containing a marginal region of tumor was selected from each patient. The specimens were assigned a TNM stage based on the UICC criteria for tumor classification (35). Haematoxylin and eosin (HE)-stained sections were also classified according to the histopathological grading system as well (G1), moderately (G2), or poorly (G3) differentiated. Histopathological grades and stagings were determined by two pathologists. The characteristics of the tissues are outlined in Supplemental information, Table S1. Immunostaining of tissues was performed essentially as described before (33). This study was approved by the institutional ethics review board of Kurume University.

For evaluation of the level of staining by antibodies, the most highly stained area of each section was scored on a scale from 0 to 4 by visual observation. To estimate the percentage of stained cells, the numbers of positive and negative cells in a field were counted and expressed as the percentage of cells stained. More than 500 cells in each section were counted. A staining index was calculated as staining intensity x average percentage of cells stained. Tissues were scored by two independent observers [N. A. and M. T.] without prior knowledge of the patients' other data with a very high correlation between scores ($P < 0.0001$).

Statistical analysis - The Mimitin staining index was compared with the cell proliferation index (the staining index for Ki-67), the staining index for c-Myc, histopathological grade, stage of cancer, and age of patient by the Spearman rank correlation coefficient and statistical test of independence, using the statistical analysis program StatView ver. 5 (SAS Institute, Cary, NC).

RESULTS

Identification of mimitin as a Myc-controlled gene - To conditionally induce c-Myc activity, the estrogen-inducible Myc system (12, 36, 37) was used. The chimeric protein c-MycER consists of c-Myc and the estrogen-binding domain of the estrogen receptor. c-MycER anchors to cytoskeletal components of cells in the absence of estrogen. When estrogen or its analogous molecule 4-hydroxytamoxifen (OHT) binds to the chimeric protein, it becomes free to function as c-Myc. The human glioblastoma cell line T98G expressing c-MycER protein (T98Gmycer-2 cells) was established previously (15). Total RNAs from T98Gmycer-2 cells in an exponentially growing phase in the presence or absence of OHT for 20 h were processed by cDNA micro array analysis.

To shed light on a new facet of c-Myc functions, we focused on human EST sequences among genes stimulated by c-MycER activation. The signal for EST clone AI803230 was stimulated 2.2-fold with c-Myc activation. This stimulation rate was similar to those of the Myc target genes, *ODC* (2.6-fold), *nucleolin* (1.6-fold), and *mina53* (1.9-fold), measured in this study. cDNAs encoding the 5' upstream and 3' downstream parts of the EST clone AI803230 were isolated using the 5'-RACE and 3'-RACE protocols from a library of human erythroid leukemia cells. It was found that EST AI803230 includes part of the cDNA that encodes a protein of 169 amino acids with a predicted molecular weight of 19.9 kd (Fig. 1A).

When an antibody raised against this protein was used in Western blotting, a band of about 20 kd, which has a similar mobility to that predicted from the amino acid sequence, was detected in T98G cells (Fig. 1B left panel). When T98G cells were transfected with mammalian expression plasmids coding this protein or the c-myc-tagged

protein, this antibody recognized a 20 kd band with increased intensity and a slowly migrating band (Fig. 1B, right panel) in the cell lysates. As described later (Fig. 3), the treatment of cells with siRNAs specific for this gene specifically reduced the intensity of the band. These results indicate that this antibody specifically recognized the protein.

When c-MycER was activated in T98Gmycer-2 cells by OHT, the intensity of the band was increased, while that for β-actin was not (Fig. 1B). The c-MycER activation in T98Gmycer-2 cells had little effect on cell proliferation (data not shown). These results suggest that the expression of this protein can be induced by c-Myc without necessarily stimulating cell proliferation.

The subcellular localization of this protein was visualized by indirect immunofluorescence staining. As shown in Fig. 1C, the protein was present in the cytoplasmic region of cells with a maze-like localization. Double staining with antibodies against this protein and Tom20 (a component of the preprotein translocase of the outer membrane of mitochondria) (Fig. 1C) or cytochrome c (data not shown) indicated that the staining for this protein was mostly consistent with mitochondria. We also detected mitochondrial localization of this protein when the c-myc-tagged construct was transfected and the tagged-protein was detected by anti-Tag antibody (9E10) (data not shown). Therefore, we refer to this gene and the protein, respectively, as *mimitin* and Mimitin (Myc-induced mitochondrial protein).

We also used a biochemical approach to study Mimitin subcellular localization. Cells were fractionated into enriched mitochondria and cytosolic fractions, and analyzed by Western blotting using antibodies against Mimitin, a cytosolic protein (MAPK/ERK), and a mitochondrial protein (Tom22) (32). The results in Fig. 1D show that Mimitin was fractionated into the mitochondrial fraction but not into the cytosolic fraction, further supporting our observation that Mimitin is a mitochondrial protein.

Mimitin is conserved in human and mouse - Mouse cDNA that appears to be a homologue of human *mimitin* was found by a Blast search (GenBank accession No. AK007894). The predicted amino acid sequence for mouse Mimitin was aligned with that of human Mimitin (Fig. 1A). The open reading frame (ORF) of mouse *mimitin* encodes a 168 amino acid protein. The human and mouse amino acid sequences of Mimitin proteins are 80% identical to each other, suggesting that *mimitin* is conserved in mammals. There is an ATP/GTP binding motif (P-loop within amino acid positions 146-153 in human Mimitin) in human and mouse Mimitin (Fig. 1A). A domain called Complex1_17_2kD (Fig. 1A, surrounded by a dotted line), was detected using the SMART program (38). The nucleotide 3 bp upstream of the methionine initiation codon is G in *mimitin* of both species, conforming to a Kozak consensus sequence (39).

Expression of mimitin mRNA - The expression profile of *mimitin* mRNA was examined. As shown in Fig. 2A, serum stimulation of serum-starved T98G cells resulted in a detectable increase at 6 h and a 3-fold increase in the expression of *mimitin* mRNA at 12-15 h. An increase of the c-*myc* mRNA level was detected at 3 h after serum addition. The expression of *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*), which is widely used as a control, is high in cells overexpressing c-Myc, but is not directly controlled by c-Myc (40). By 9 h, the expression of *GAPDH* mRNA was almost unchanged, but a 2-fold increase was detectable at 12-15 h. These results suggest that up-regulation of *mimitin* mRNA followed the increase of c-*myc* mRNA, and preceded the increase of *GAPDH* mRNA.

In human promyelocytic leukemia HL60 cells, cell proliferation is arrested and the c-*myc* expression level is reduced by phorbol 12-myristate 13-acetate (TPA) (41, 42). This experimental system was used to investigate whether Myc-targeted genes are affected while Myc is shut off (15, 43). As shown in Fig. 2B, when HL60 cells were cultured with 10 nM TPA for 3 hours, the level of c-*myc* mRNA started to decline and reached one fifth at 7 h. Down-regulation of *mimitin* mRNA followed the decrease in c-*myc* mRNA, and the level of *mimitin* mRNA had decreased to one half and one third at 12 h and 24 h, respectively. The level of *GAPDH* mRNA had not changed at 12 h and had decreased to 60% only at 24 h. These experiments indicate that the down-regulation of *mimitin* expression

followed that of c-*myc* and preceded the decrease of *GAPDH* mRNA.

Next, the effect of c-Myc activation in c-MycER on *mimitin* mRNA was investigated (Fig. 2C). The *mimitin* mRNA level in OHT-treated T98Gmycer-2 cells rose steadily for 9 h, showing nearly 2-fold induction, although OHT-treatment of T98G parent cells did not stimulate the *mimitin* mRNA level. Induction of *mimitin* mRNA by OHT in T98Gmycer-2 cells was maintained in the presence of the protein-synthesis inhibitor cycloheximide (CHX), while treatment with CHX had little effect on the *mimitin* mRNA level in T98G parent cells. The *GAPDH* mRNA level did not change in this experiment. These results indicate that the *mimitin* gene is a direct target of Myc.

c-Myc stimulates the gene expression of mimitin through a mimitin genomic DNA fragment - We found the sequence for exon 1 of human *mimitin* in a genome data-base (AC073546; chromosome 5 clone RP11-406P13). More recently, human genomic DNA sequences, which include full-length *mimitin* cDNA sequences, were found by the UCSC Genome Browser, showing that the human *mimitin* gene maps to chromosome 5 (5q12.1). To investigate the promoter activity of the *mimitin* gene, a 0.7 kb human *mimitin* genomic DNA fragment containing upstream of exon 1, exon 1 and part of intron 1 was joined to firefly *luciferase* cDNA to construct a reporter plasmid, pmimitin(W)luci. Transient expression assays in T98Gmycer-2 cells indicate that the DNA fragment has promoter activity. After c-MycER activation by OHT, the luciferase activity was increased up to 1.8-fold (Fig. 2D, a bar for wild). This stimulation is comparable to that observed for an increase in *mimitin* mRNA level by OHT in T98Gmycer-2 cells (Fig. 2C).

Exon 1 has one E-box site (CACGTG element). When the CACGTG element was mutated to CACCCG, luciferase activity was not increased by the activation of c-MycER as a pGL3-Basic empty vector (Fig. 2D, bars for mutant and pBasic). These results indicate that c-Myc stimulates the expression of pmimitin(W)luci through the CACGTG element.

c-Myc binds to the mimitin gene in vivo - To examine c-Myc protein binding to the endogenous *mimitin* gene during proliferation of HL60 cells, chromatin immunoprecipitation was performed as described previously (30, 44). After immunoprecipitation, enrichment of the endogenous *mimitin* gene fragment in each sample was monitored by PCR using primers that specifically amplify a *mimitin* DNA fragment, which containing the CACGTG element. As shown in Fig. 2E, two different anti-c-Myc antibodies immunoprecipitated the *mimitin* DNA fragment from HL60 cells in the proliferating phase, while the same antibodies did not immunoprecipitate detectable levels of DNA fragment from cells treated with TPA. Enrichment of the *mimitin* genomic DNA fragment is dependent on c-Myc binding to the *mimitin* gene, because a nonspecific antibody did not immunoprecipitate the *mimitin* DNA fragment. Additionally, binding of c-Myc detected in the *mimitin* gene is specific, because antibodies against c-Myc enriched the well-established Myc-target gene, *cad* (Fig. 2F), but did not enrich the genomic DNA fragment containing an E-box that is located in a chromosomal region without any detectable gene on chromosome 22 (Fig. 2E). These results demonstrate that the *mimitin* gene is bound by c-Myc specifically at the proliferating phase in HL60 cells.

Mimitin is involved in proliferation of some kinds of cultured cells - To gain insight into the biological function of *mimitin*, the expression of *mimitin* was specifically suppressed by the RNAi technique. As shown in Fig. 3A, the expression of Mimitin protein was reduced by siRNA specific for *mimitin* in colon carcinoma SW620, cervical carcinoma HeLa, ESCC TE-11, glioblastoma T98G, embryonic lung HEL, and hepatocellular carcinoma Kyn2 cells, while the expression of β-actin was not reduced in these cells. These results suggest that the siRNA specifically reduced the expression of Mimitin. However, the effect of the suppression of *mimitin* expression differed between cell lines. While specific inhibition of *mimitin* expression reduced cell proliferation of TE-11, T98G and HEL cells, it hardly affected cell proliferation of SW620, HeLa, and Kyn2 cells (Fig. 3B).

The levels of c-Myc protein in cell lines used here were compared by Western blotting. As shown in Fig. 3C, the two cell lines SW620 and