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A Novel Transcriptional Repressor, Rhit, Is Involved in Heat-Inducible and Age-Dependent Expression of Mpv17-Like Protein, a Participant in Reactive Oxygen Species Metabolism▽

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Mpv17-like protein (M-LP) is a protein that has been suggested to be involved in the metabolism of reactive oxygen species. The two M-LP isoforms in mouse, M-LP$_S$ and M-LP$_L$, are generated by the alternative usage of promoters. M-LP$_S$ is expressed exclusively in kidneys after the age of 6 weeks, whereas M-LP$_L$ is expressed ubiquitously. To elucidate the molecular basis of M-LP$_S$ expression, we searched for *cis*-regulatory elements in the promoter region of M-LP$_S$ and identified heat shock element half-sites as positive elements and a Tramtrack 69K (Ttk 69K) binding site as a negative element. Furthermore, we isolated a novel transcription repressor, Rhit (regulator of heat-induced transcription), that binds to the Ttk 69K binding site within the M-LP$_S$ promoter by DNA affinity chromatography and confirmed its participation in the transcriptional regulation of M-LP$_S$ by RNA interference (RNAi). Sequence analysis revealed that Rhit contains a KRAB (Krüppel-associated box) domain and a DNA-binding domain composed of eight C_2H_2-type zinc fingers. Interestingly, exposure to heat shock stress resulted in the upregulation of M-LP$_S$ expression concurrent with the downregulation of Rhit expression. Moreover, the age-dependent expression of M-LP$_S$ was inversely correlated with that of Rhit. These observations strongly suggest that Rhit acts as a repressor in the heat-induced and age-dependent transcriptional regulation of M-LP$_S$.

The Mpv17-like protein (M-LP) gene was originally identified on the basis of an expressed sequence tag obtained by differential display screening of age-dependently expressed genes in mouse kidneys (15, 16). Subsequent analysis revealed that the *M-LP* gene encodes a protein closely similar to the Mpv17 protein and to the 22-kDa peroxisomal membrane protein (6, 38). The *Mpv17* gene was found to be a recessive kidney disease gene in mouse, and Mpv17 gene-inactivated mice show symptoms that resemble human glomerulosclerosis or Alport syndrome. The molecular functions of the Mpv17 protein have yet to be elucidated, but it is believed that it participates in the metabolism of reactive oxygen species (ROS) and that glomerular damage is due to the overproduction of ROS (5, 36, 39). Moreover, it was recently reported that mutations in the human *Mpv17* gene cause infantile hepatic mitochondrial DNA depletion syndrome (32). The mouse *M-LP* gene is composed of five exons, extends over a 19-kb region on chromosome 16, and is expressed as two distinct splicing transcripts generated by the alternative usage of two distinct promoters localized upstream of exon 1 and of exon 2 of the *M-LP* gene (19). M-LP$_L$, encoded by exons 2, 3, 4, and 5, is composed of 194 amino acid residues and is integrated into the peroxisome membrane (18), whereas M-LPs, encoded by exons 1, 3, 4, and 5, is composed of 90 amino acid residues lacking the N-terminal half of M-LP$_L$ and is localized to the cytosol (19). A comparison of the tissue distribution and age-dependent expression patterns of the two isoforms revealed that M-LP$_L$ expression has features characteristic of a housekeeping gene, while M-LP$_S$ has those of a luxury gene. M-LP$_L$ mRNA is expressed ubiquitously, whereas M-LP$_S$ mRNA is expressed specifically in the kidney. M-LP$_L$ is expressed over a wide age range, although its amount changes age dependently, whereas M-LP$_S$ expression commences at the sixth week of life, increases steadily, reaches its highest level in the ninth month of life, and decreases with aging. The molecular function of the M-LP protein has yet to be elucidated, but both isoforms are considered to participate in the enzymatic antioxidant defense system in a manner similar to that of the Mpv17 protein. In previous studies, it was demonstrated that transfection with M-LP$_L$ upregulates the expression of the mitochondrial manganese superoxide dismutase (*SOD2*) gene, whereas transfection with M-LP$_S$ upregulates the expression of the *SOD2* gene and downregulates the expression of the cellular glutathione peroxidase (*Gpx1*) and plasma glutathione peroxidase (*Gpx3*) genes (18, 19). These observations strongly suggest that M-LP participates in ROS metabolism and that these two isoforms, which show different expression patterns and activities, have different roles. Additionally, a recent study by Krick et al. revealed that M-LP interacts with serine protease HtrA2 and that, as a result, HtrA2 is prevented from translocation into the cytosol, where it exerts a proapoptotic function (21).

To clarify the molecular basis of the age-dependent change in M-LP$_S$ expression, we searched for and characterized the specific positive and negative regulatory elements in the upstream region of the *M-LP* gene, and by means of DNA affinity chromatography, we isolated a novel transcription factor that binds to the negative regulatory element. Because of its participation in the heat-induced upregulation of M-LP$_S$, we have

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TABLE 1. Oligonucleotides used as primers and probes

Expt	Oligonucleotide	Orientation	Positions	Sequence (5′–3′)[a]
Reporter construction	Hind-LAAS1	Reverse	314–295	CCCaagcttGCTGTGTCCCTTGAGTTGTG
	Xho-LAS1	Forward	−1924–−1905	CCGctcgagCCTGAACCTGGCTTTCCCTAA
	Xho-LAS2	Forward	−1346–−1327	CCGctcgagAATCTGGGTCCATGGAGCTT
	Xho-LAS3	Forward	−854–−835	CCGctcgagACGTCAGCTCAACAGACTGA
	Xho-LAS4	Forward	−375–−356	CCGctcgagCTGCCTGCTGATCTAGGTGA
	Xho-LAS5	Forward	−285–−267	CCGctcgagTCATGCAGCGCTCAGGTTC
	Xho-LAS6	Forward	−241–−223	CCGctcgagAAATCGTGGCCAGCTACTG
	Xho-LAS7	Forward	−205–−187	CCGctcgagATTCGCCGTTGCAAGATGG
	Xho-LAS8	Forward	−155–−137	CCGctcgagAATAATCTGCGCATGTGCC
	Xho-LAS9	Forward	−107–−93	CCGctcgagCCTTCCCCGTGACGA
	Xho-LAS10	Forward	8–27	CCGctcgagGCTCTGGCTCTTGCTTCTTG
EMSA	Probe 1	Forward	−375–−346	CTGCCTGCTGATCTAGGTGATCAAATCCTA
		Reverse		TAGGATTTGATCACCTAGATCAGCAGGCAG
	Probe 2	Forward	−345–−316	ACCAAGATGCAACAATCCACTCACATGAGC
		Reverse		GCTCATGTGAGTGGATTGTTGCATCTTGGT
	Probe 3	Forward	−315–−286	ACCTGGAGAATGCAGGATCTGATTCGTCCG
		Reverse		CGGACGAATCAGATCCTGCATTCTCCAGGT
	Probe 4	Forward	−285–−242	TCATGCAGCGCTCAGGTTCCAAGATTCCACTTCCAAAACTGATA
		Reverse		TATCAGTTTTGGAAGTGGAATCTTGGAACCTGAGCGCTGC ATGA
	Probe 5	Forward	−205–−156	ATTCGCCGTTGCAAGATGGCGCTGACATCCTGTGTTCTAAGTG GTAAACA
		Reverse		TGTTTACCACTTAGAACACAGGATGTCAGCGCCATCTTGCAACG GCGAAT
	Probe 6	Forward	−155–−108	AATAATCTGCGCATGTGCCAAGGGTAGTTTTCCACCCCATGTGC TCTG
		Reverse		CAGAGCACATGGGGTGGAAAACTACCCTTGGCACATGCGCAG ATTATT
	Probe hsp70	Forward		CGGGGAAGCTCCACCAGACGCGAAACTGCTGGAAGATTCC
		Reverse		GGAATCTTCCAGCAGTTTCGCGTCTGGTGGAGCTTCCCCG
ChIP assay	Chip-S1	Forward	−406–−383	AAGGTGGCAGTCAGTGGAAGGACA
	Chip-A1	Reverse	−188–−211	CATCTTGCAACGGCGAATGTGAGG
Site-directed mutagenesis	M-Ttk69K-S	Forward	−317–−280	GCACCTGGAGAAT**T**CAGG**GTGT**GTGATTCGTCCGTCATGC
	M-Ttk69K-A	Reverse	−280–−317	GCATGACGGACGAATCA**C**A**C**CCTG**A**ATTCTCCAGGTGC
	M-HSE285-S1	Forward	−271–−230	GG**GCT**CAAGA**GCT**CAC**GCT**CAAAACTGATAAAATCGTGGCCA
	M-HSE285-A1	Reverse		TGAGCGCTGCATGACTCGAG
	M-HSE241-S1	Forward	−206–−146	CA**GCT**GCCGTTGCAAGATGGCGCTGACATCCTGTG**GCT**TAAGT GGTAAACAAATAATCTGC
	M-HSE241-A1	Reverse		TGAGGGCGGCTCCCCACA
	M-HSE155-S1	Forward	−128–−104	TT**GCT**CACCCCATGTGCTCTGCCTT
	M-HSE155-A1	Reverse		CTACCCTTGGCACATGCGCA
	KRAB-del-S1	Forward		AAGTGTGGTCAGCACGTGGCTA
	KRAB-del-A1	Reverse		CGGCATCTGGGACCAGGCAG
DNA affinity chromatography	P3S$(GT)_5$	Forward		ACCTGGAGAATGCAGGATCTGATTCGTCCGGTGTGTGTGT
	P3A$(GT)_5$	Reverse		CGGACGAATCAGATCCTGCATTCTCCAGGTGTGTGTGTGT
Expression vector construction	Rhit-EXP-S1	Forward		CGCggatccTTCTCCATCCGTTCTGTTCTGGC
	Rhit-EXP-A1	Reverse		CCGgaattcACAGCTTGAAATCCAGGCCTTTAAT
	HSF1-EXP-S1	Forward		CCCCaagcttGAGATGGATCTGGCCGTGGG
	HSF1-EXP-A1	Reverse		CGgaattcCTAGGAGACAGTGGGGTCCT
siRNA	si50	Forward		CCCAUGAGGUCCCAGGUAATT
		Reverse		UUACCUGGGACCUCAUGGGTT
	si1085	Forward		GCUCGGACCUAGUCACACATT
		Reverse		UGUGUGACUAGGUCCGAGCTT
	si1267	Forward		GCACAUAAUCGCACCCACATT
		Reverse		UGUGGGUGCGAUUAUGUGCTT

[a] HindIII, XhoI, BamHI, and EcoRI sites are shown in lowercase type, and nucleotides altered to abolish the Ttk 69K site and the predicted HSEs are shown in boldface type.

named the new transcriptional repressor Rhit (regulator of heat-induced transcription). In the present study, we report the structural features and age-dependent expression pattern of Rhit and provide evidences that M-LP_S is a heat-inducible protein that is transcriptionally regulated by Rhit and heat shock factor (HSF) and that the age-dependent expression of Rhit underlies that of M-LP_S.

MATERIALS AND METHODS

Cells. The mouse kidney cell line TCMK-1 was obtained from the American Type Culture Collection (ATCC, Manassas, VA). The cells were maintained in modified Eagle's minimum essential medium (ATCC 30-2003) containing 10% (vol/vol) fetal calf serum (Invitrogen, Carlsbad, CA) and 0.1% (wt/vol) lactalbumin hydrolysate (Sigma-Aldrich, St. Louis, MO) in a humidified incubator at 37°C with an atmosphere of 5% CO_2.

Generation of luciferase reporter constructs and dual-luciferase assays. DNA segments containing different portions of the 5′-upstream sequences of the *M-LP* gene were PCR amplified by using the sets of primers shown in Table 1 and genomic DNA extracted from C57BL/6 mouse kidneys as the template and were cloned into the XhoI/HindIII site of the pGL3-Basic vector (Promega, Madison, WI). XhoI sites were added to the 5′ terminus of all the forward primers, and a HindIII site was added to the reverse primer. The first nucleotide of exon 1 is defined here as position +1. TCMK-1 cells were grown in 12-well plates to 20 to 30% confluency and transiently cotransfected with 1 μg of firefly luciferase reporter constructs and 1 μg of pRL-TK *Renilla* luciferase reporter (Promega) by use of 3 μl of Lipofectamine 2000 reagent (Invitrogen) according to the manu-

facturer's instructions. At 48 h after transfection, luciferase activities were assayed by using the Dual-Luciferase reporter assay system (Promega) according to the manufacturer's protocol. Light emission was measured with a Lumat LB9506 (EG&G Berthold, Bad Wildbad, Germany). The pGL3-Control vector (Promega) containing the simian virus 40 (SV40) promoter and the pGL3-Basic vector without the promoter sequence were used as positive and negative controls, respectively. Values in relative light units were obtained, and the activities of the experimental reporter (firefly luciferase) were normalized to the activities of the internal control (*Renilla* luciferase). Assays were performed in triplicate.

EMSA. Nuclear extracts were prepared from TCMK-1 cells with a nuclear extract kit (Active Motif, Carlsbad, CA) and stored at −80°C until use. Six sets of complementary oligonucleotides corresponding to the putative protein-binding sites within the 5′-upstream sequences of the *M-LP* gene and one set corresponding to the HSF-binding site within the promoter region of the mouse *Hsp70* gene (14) were synthesized as shown in Table 1. To prepare the biotin-labeled double-stranded DNA probe, both complementary oligonucleotides were end labeled separately with a biotin 3′-end DNA labeling kit (Pierce, Rockford, IL) and then annealed before use. Electrophoretic mobility shift assay (EMSA) was performed by using the LightShift chemiluminescent EMSA kit (Pierce) in accordance with the manufacturer's protocol. Briefly, EMSA reaction mixtures containing 2 to 20 μg of nuclear extract, 2 μl of binding buffer (100 mM Tris-HCl, 500 mM KCl, 10 mM dithiothreitol [DTT] [pH 7.5]), and 1 μg of poly(dI-dC) · poly(dI-dC) in a total volume of 16 μl were preincubated at room temperature for 15 min, 4 μl of biotin-labeled probe (5 nM) was then added, and incubation was continued for 20 min. For competition experiments, excess unlabeled competitor oligonucleotides were incubated with the EMSA reaction mixtures before the addition of the biotin-labeled probe. The binding reaction mixtures were separated on 6% polyacrylamide gels with 0.5× Tris-borate-EDTA (TBE). After electrophoresis, the binding reaction mixtures were transferred onto a nylon membrane (Hybond-N^+; GE Healthcare, Uppsala, Sweden) with an electroblotting apparatus (KS-8453; Oriental Instruments, Tokyo, Japan), and the membrane was cross-linked at 120 mJ/cm^2 with a UV light cross-linker (CX-2000; BM Equipment, Tokyo, Japan). The detection of biotin-labeled DNA was performed with a LightShift chemiluminescent EMSA kit (Pierce).

ChIP assay. Chromatin immunoprecipitation (ChIP) assays were performed by using the EZ ChIP kit (Upstate, Lake Placid, NY) and anti-HSF1 and anti-GAPDH (glyceraldehyde-3-phosphate dehydrogenase) (Santa Cruz Biotechnology, Santa Cruz, CA) antibodies according to the manufacturers' instructions. The DNA sequence isolated via HSF1-specific immunoselection was detected by PCR using primers described in Table 1. Cycling conditions were 94°C for 15 min, followed by 32 cycles of 94°C for 20 s, 59°C for 30 s, 72°C for 30 s, and then 72°C for 2 min. The PCR products were subjected to 2% agarose gel electrophoresis and stained with ethidium bromide.

Site-directed mutagenesis. Mutations in the Ttk 69K motif were introduced into the luciferase reporter construct pGL3-375 by using a QuikChange II site-directed mutagenesis kit (Stratagene, La Jolla, CA), and mutations in the predicted heat shock elements (HSEs) were introduced into pGL3-285, pGL3-241, and pGL3-155 with a KOD-Plus mutagenesis kit (Toyobo, Osaka, Japan), each in accordance with the manufacturer's instructions. The primers used for mutant strand synthesis are listed in Table 1. Plasmid DNA with the targeted mutations was isolated and sequenced to verify the mutated sequences.

DNA affinity chromatography. Purification of Rhit by DNA affinity chromatography was performed according to the oligonucleotide trapping method reported previously by Gadgil and Jarrett and Mitra et al. (9, 25) by using a batch method. Briefly, the trapping resin was prepared by the coupling of $(AC)_5$ oligonucleotides (5′-NH_2-ACACACACAC-3′; 200 nmol) to CNBr-activated Sepharose 4B (1.2 g, dry weight; Sigma) according to the manufacturer's instructions. Approximately 43 nmol $(AC)_5$ oligonucleotides was coupled per ml of Sepharose. To prepare a probe, oligonucleotides corresponding to each strand of probe 3 plus $(GT)_5$ tails at the 3′ termini [P3S$(GT)_5$ and P3A$(GT)_5$] were synthesized as described in Table 1 and annealed just prior to use. A reaction mixture containing 600 μg of nuclear extract from TCMK-1 cells, 120 μl of binding buffer (100 mM Tris-HCl, 500 mM KCl, 10 mM DTT, 10 mM EDTA [pH 7.5]), 120 μl of 1% (vol/vol) Nonidet P-40, and 60 μg of poly(dI-dC) · poly(dI-dC) in a total volume of 1,188 μl was added to a 1.5-ml Eppendorf tube and preincubated at room temperature for 15 min, followed by incubation for an additional 20 min with 12 μl of probe (500 nM). Next, 120 μl of $(AC)_5$-Sepharose (50% slurry) was added, and the suspension was mixed in a cold room (4°C) for 1 h on a tube rotator (NRC-20D; Nissin, Tokyo, Japan). The trapping resin was washed five times with 250 μl of TE 0.4 buffer (10 mM Tris-HCl, 1 mM EDTA, 0.4 M NaCl [pH 7.5]), 600 μl of TE buffer (10 mM Tris-HCl, 1 mM EDTA [pH 7.5]) prewarmed to 37°C was then added to the resin, and the protein-DNA complex was eluted by incubation for 5 min in a water bath (37°C). The batch purification procedure was carried out 40 times, and all the protein-DNA complex solutions prepared were pooled, concentrated, and subjected to EMSA and 10% SDS-PAGE according the method of Laemmli (23). After SDS-PAGE, the protein was transferred onto a Sequi-Blot polyvinylidene difluoride (PVDF) membrane (Bio-Rad, Hercules, CA) by electroblotting using an electroblotting apparatus and cyclohexylaminopropanesulfonic acid (CAPS) buffer at 250 mA for 1 h. The protein bands were visualized by staining with 0.2% (wt/vol) Coomassie brilliant blue R250 in 40% (vol/vol) methanol and 10% (vol/vol) acetic acid followed by destaining with 60% (vol/vol) methanol, excised from the membrane, and subjected to automatic Edman degradation by use of a Procise 494 cLC protein sequencing system (Applied Biosystems, Foster City, CA).

Construction of Rhit cDNA. The full-length cDNA of Rhit was constructed by a rapid amplification of cDNA ends (RACE) method using the Smart RACE cDNA amplification kit (BD Biosciences, Palo Alto, CA). Total RNA was extracted from TCMK-1 cells with an RNeasy minikit (Qiagen). Each first-strand cDNA for the 5′- and 3′-RACE reaction was synthesized from 1 μg of total RNA with Superscript II reverse transcriptase (Invitrogen). The following gene-specific primers for 5′- and 3′-RACE were designed on the basis of the mouse chromosome 17 genomic contig sequence (GenBank accession no. NT_039649.7) so that each RACE product would overlap: antisense primers Zfp13-5RA1 (5′-GGCACAGC GGTCGCACACGTAGG-3′; positions 90301781 to 90301803 of accession no. NT_039649.7) and Zfp13-5RA2 (5′-CCACACTGCTCGCACTTGTATGT-3′; positions 90302034 to 90302056 of accession no. NT_039649.7) for 5′-RACE and sense primer Zfp13-3RS1 (5′-CCTTGGCCGTCGCAACCCTAGAG-3′; positions 90302334 to 90302312 of accession no. NT_039649.7) and Zfp13-3RS2 (5′-GTGACACGGGCAAGAAGACATACA-3′; positions 90302073 to 90302050 of accession no. NT_039649.7) for 3′-RACE. The RACE products were subcloned into TA cloning vector pCR2.1 (Invitrogen) and sequenced.

Overexpression of Rhit and HSF1. DNA fragments encoding full-length Rhit and HSF1 were created by PCR amplification using the primers listed in Table 1 and the first-strand cDNA synthesized using total RNA extracted from TCMK-1 cells as the template. The PCR products were cloned into the BamHI/EcoRI site of pcDNA3.1 (the resulting vector was designated pcDNA3.1/Rhit) or the HindIII/EcoRI site of pcDNA3.1 (the resulting vector was designated pcDNA3.1/HSF1), respectively. An expression vector for Rhit lacking the Krüppel-associated box (KRAB) domain was created by introducing a deletion into pcDNA3.1/Rhit with a KOD-Plus mutagenesis kit (the resulting vector was designated pcDNA3.1/RhitdelKRAB). The primers used for mutant strand synthesis are listed in Table 1. TCMK-1 cells were transiently transfected by use of Lipofectamine 2000 reagent in accordance with the manufacturer's instructions. Two days after transfection, nuclear extracts were prepared from the transfected cells for EMSA analysis, or cells were processed by use of the method described above for Western blotting.

siRNA. For the RNA interference (RNAi)-mediated knockdown of Rhit, small interfering RNA (siRNA) duplexes of 21 bp with a 2-base deoxynucleotide overhang were synthesized by B-Bridge KK (Tokyo, Japan), as described in Table 1. The control siRNA was purchased from B-Bridge KK (S5C-0600 [5′-ATCCGCGCGATAGTACGTA-3′]). siRNA duplexes for the knockdown of HSF1 were purchased from Darmacon (Lafayette, CO) (siGENOME SMARTpool). TCMK-1 cells were transfected with siRNA duplexes by use of Lipofectamine 2000 reagent according to the manufacturer's instructions.

Measurement of mRNA levels. mRNA levels of M-LP_S and Rhit were analyzed by reverse transcription (RT)-PCR and quantitative real-time PCR. The gene-specific primers used for the measurement of M-LP_S and Rhit were as follows: sense primer 93F-S (5′-CTTGCTCTGGCTCTTGCTTC-3′; positions 10408363 to 10408382 of GenBank accession no. NT_039624) and antisense primer 9A-3 (5′-CGCAGCAAACCGGAATCTTC-3′; positions 10414002 to 10413983 of accession no. NT_039624) and sense primer Rhit-S2 (5′-AAGCCC TACCACTGCCTCGAC-3′; positions 90301535 to 90301555 of accession no. NT_039649.7) and antisense primer Rhit-A1 (5′-CTGAAGCTCTTGCCGCAA AGT-3′; positions 90301434 to 90301454 of accession no. NT_039649.7). The extraction of total RNA from C57BL/6 mouse kidneys (3 days, 1 and 8 weeks, and 4, 6, and 15 months after birth) was performed by use of an RNeasy minikit (Qiagen) as described in our previous reports (17, 33). Quantitative real-time PCR analysis was performed by using an ABI Prism 7700 sequence detection system (Applied Biosystems) and SYBR green PCR master mix (Applied Biosystems) according to the manufacturer's instructions. The amount of mRNA was normalized to the internal control GAPDH. All PCR assays were performed in triplicate.

Western blotting. To assess the effect of heat treatment on M-LP_S expression, TCMK-1 cells grown to 90% confluence in 60-mm dishes were incubated at 43°C for 10, 20, 30, and 60 min. Immediately after incubation, cells were washed with

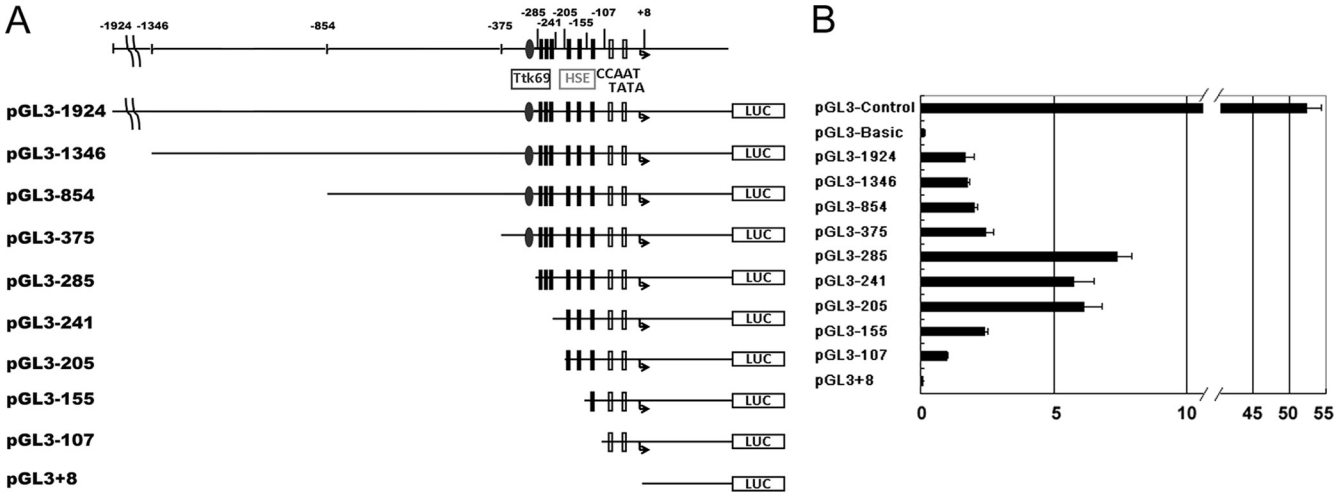

FIG. 1. Luciferase reporter assay of *M-LP* promoter activity for identification of *cis*-regulatory elements. (A) Schematic representation of potential *cis* elements within the promoter region of *M-LP*. +1 indicates the position of the transcription start site of exon 1. The shaded oval and the solid rectangles indicate the putative binding sites for Ttk 69K and HSF, respectively. (B) Relative luciferase activities of the reporter constructs containing different portions of the 5′-upstream sequences of the *M-LP* gene. The results are expressed as ratios relative to the value for pGL3-107, which has basal *M-LP* promoter activity. The bars represent the means ± standard deviations (SD) of the results from three independent experiments.

ice-cold phosphate-buffered saline, harvested by scraping into 1 ml of the same buffer, and collected by centrifugation. The precipitated cells were mixed with 100 μl of 0.35 M Tris-HCl (pH 6.8) containing 10% (wt/vol) SDS, 35% (vol/vol) glycerol, 0.6 M DTT, and 0.01% (wt/vol) bromophenol blue; heated at 100°C for 5 min; and subjected to 15% SDS-PAGE. After electrophoresis, the proteins were transferred onto an Immobilon-P membrane (Millipore, Bedford, MA). The membrane was blocked with 0.1% nonfat dried milk in phosphate-buffered saline–Tween (PBS-T) (80 mM Na_2HPO_4, 20 mM NaH_2PO_4, 100 mM NaCl, and 0.1% Tween 20), treated with anti-M-LP (20) as the primary antibody, and then treated with horseradish peroxidase-conjugated goat antibody against rabbit IgG (Bio-Rad) as a secondary antibody. The signals were visualized by using an ECL Plus Western blotting detection system (Amersham Biosciences).

Nucleotide sequence accession number. The Rhit cDNA sequence has been deposited in the GenBank database under accession no. FJ593171.

RESULTS

Search for *cis* elements in the region upstream of exon 1 of the *M-LP* gene. We previously reported that TATA and CCAAT boxes exist 22 to 31 and 64 to 75 bp upstream of exon 1 of the *M-LP* gene, respectively, and that a GC box exists 103 to 116 bp upstream of exon 2. These results suggested that $M\text{-}LP_L$ and $M\text{-}LP_S$ are generated by the alternative use of two distinct promoters and that the tissue-specific and age-dependent expression of $M\text{-}LP_S$ is regulated by the promoter located upstream of exon 1 (19). As a first step to identify the *cis*-regulatory elements within the putative promoter region for $M\text{-}LP_S$, a series of truncation constructs was created for luciferase reporter assays using the 2-kb upstream fragment of exon 1 (Fig. 1A). A transient transfection assay of TCMK-1 cells revealed multiple possible *cis* elements. The DNA fragment comprising nucleotides −375 to −286 was found to reduce promoter activity by 67%, implying that a negative regulatory element is present in this sequence. In contrast, DNA fragments comprising nucleotides −285 to −242, −205 to −156, and −155 to −108 enhanced the activity by 1.3-, 2.5-, and 2.4-fold, respectively, implying that positive regulatory elements are involved in these sequences (Fig. 1B). Next, we performed EMSA with TCMK-1 nuclear extracts to examine if these sequences within the promoter region bind transcription factors. For the 90-bp DNA sequence spanning positions −375 to −285, which contains a possible negative element, three consecutive 30-bp oligonucleotides were synthesized as probes (probes 1 to 3) (Fig. 2). Probe 1 (positions −375 to −346) and probe 2 (positions −345 to −316) did not form any significant bands, whereas a DNA-protein complex was formed with probe 3 (positions −315 to −286) and was competed out by unlabeled probe 3 (Fig. 3, lanes 1 to 6). These results demonstrate that the sequence from positions −315 to −286 contains

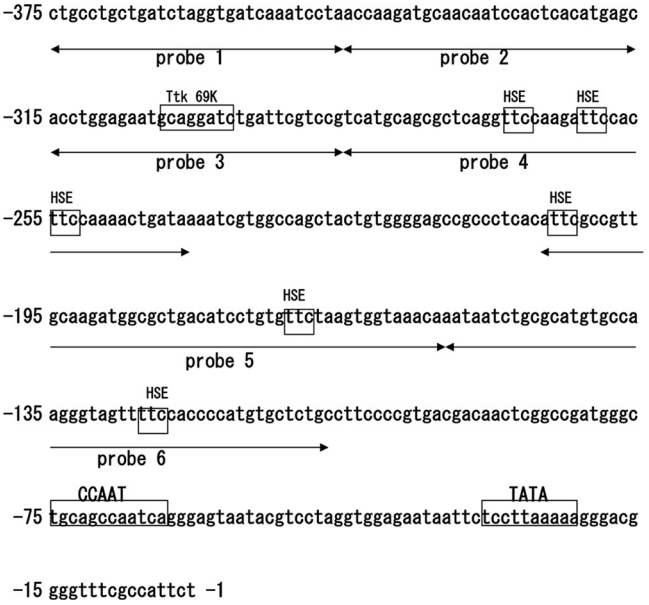

FIG. 2. Sequence of the 5′-flanking region of the *M-LP* gene and localization of putative *cis*-regulatory elements and EMSA probes.

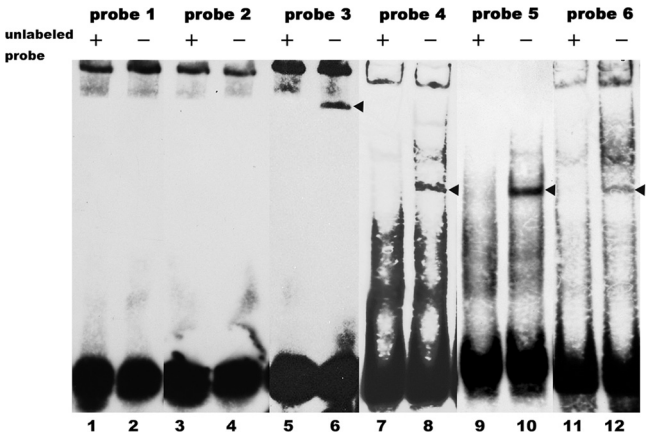

FIG. 3. EMSA using biotin-labeled DNA probes corresponding to the putative protein-binding sites within the 5′-upstream sequences of the *M-LP* gene and nuclear extracts from TCMK-1 cells. The sequences and the positions of the probes are shown in Table 1 and Fig. 2. For competition experiments, a 200-fold excess amount of unlabeled oligonucleotides was incubated with the EMSA reaction mixtures before the addition of the biotin-labeled probe.

a specific binding site for a transcription factor. Three probes corresponding to the DNA sequences from positions −285 to −242 (probe 4), −205 to −156 (probe 5), and −155 to −108 (probe 6), respectively, were then synthesized, each of which included a putative positive regulatory element. DNA-protein complexes were formed with all three probes (probes 4, 5, and 6), but the band intensities of probes 4 and 6 were lower than that of probe 5 (Fig. 3, lanes 7 to 12). All these bands disappeared upon competition with their corresponding unlabeled probes, suggesting that all these sequences bind specifically to a protein(s).

Identification of the binding site of the positive regulatory elements. A search for transcription factor binding sites with TFSEARCH (http://www.cbrc.jp/research/db/TFSEARCHJ.html) within the three sequences encompassing possible positive regulatory elements (positions −285 to −242, −205 to −156, and −155 to −108) revealed that they all contain heat shock element (HSE) half-sites (nGAAn). The canonical HSE is a minimum of two nGAAn elements arranged as an inverted dyad repeat, although a single nGAAn element or certain other variant sequences are also functional in transcription regulation (11, 22, 31, 37). The sequences spanning positions −285 to −242, −205 to −156, and −155 to −108 contain three, two, and one nGAAn element(s), respectively (Fig. 2), although none form a perfect inverted dyad repeat. To examine if these nGAAn elements have a positive regulatory role, we abolished the nGAAn element(s) involved in three luciferase reporter constructs and evaluated their promoter activities: three TTCs of pGL3-285 (positions −269 to −267, −261 to −259, and −255 to −253), two TTCs of pGL3-241 (positions −204 to −202 and −171 to −169), and one TTC of pGL3-155 (positions −126 to −124) were replaced with a GCT(s), which was chosen by considering the scoring matrix for HSE (TRANSFAC matrix record M00146). The resulting three vectors were designated pGL3-285mut, pGL3-241mut, and pGL3-155mut, respectively. As shown in Fig. 4A, the activity of pGL3-285mut was reduced by 35% compared with that of pGL3-285, whereas those of pGL3-241mut and pGL3-155mut were unchanged relative to their original constructs. It follows from these results that at least the three TTCs abolished in pGL3-285 are involved in positive transcriptional regulation, presumably as a site for the binding of HSF. Mammals possess three genes encoding HSF isoforms, HSF1, HSF2, and HSF4, and HSF1 is the primary HSF activated during heat stress (29). Previous extensive studies of HSF1 have shown that upon heat treatment, HSF1 trimerizes, translocates to the nucleus, undergoes phosphorylation, and binds the HSE, whereas under normal (unheated) conditions, most HSF is present in the

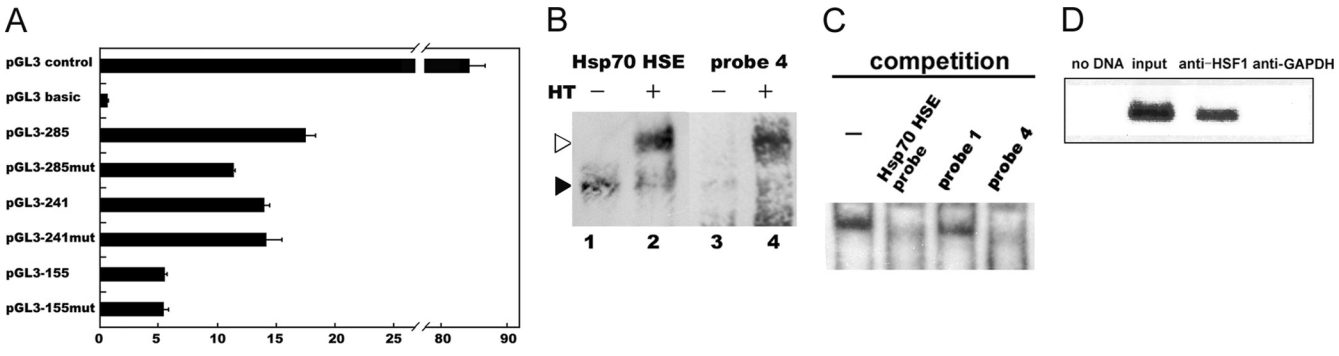

FIG. 4. Identification of the HSF1-binding sequence in the *M-LP* gene. (A) Relative luciferase activities of the wild-type (pGL-285, pGL-241, and pGL-155) and mutant (pGL-285mut, pGL-241mut, and pGL-155mut) constructs. The results are expressed as ratios relative to the value for pGL3-basic. The bars represent the means ± SD of the results from three independent experiments. (B) EMSA of nuclear extracts from unstressed and heat-treated (43°C for 1 h) TCMK-1 cells. Hsp70 HSE and HT indicate a probe comprising the HSE sequence from the mouse *Hsp70* promoter and heat treatment, respectively. The sequences and the locations of the probes are shown in Table 1 and Fig. 2. The solid and clear triangles represent faster-migrating and more slowly migrating bands, respectively. (C) Competition assay for binding to the HSE sequence from the mouse *Hsp70* promoter. A 200-fold excess amount of unlabeled oligonucleotides—HSE sequence probe, probe 4, and an unrelated oligonucleotide (probe 1)—was incubated with the EMSA reaction mixtures containing nuclear extracts from heat-treated (43°C for 1 h) TCMK-1 cells before the addition of the biotin-labeled probe. (D) ChIP assay using anti-HSF1 antibody. Anti-GAPDH antibody was used as the negative control. The input and precipitated DNAs with anti-HSF1 antibody or anti-GAPDH antibody as the negative control were PCR amplified with specific primers for the putative HSE region in the M-LP$_S$ promoter. The PCR products (219 bp) were subjected to 2% agarose gel electrophoresis and stained with ethidium bromide.

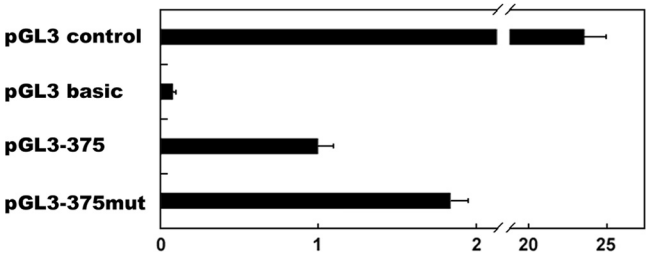

FIG. 5. Relative luciferase activities of the wild-type (pGL-375) and mutant (pGL-375mut) constructs. The results are expressed as ratios relative to the value for pGL3-375, which has an intact Ttk 69K binding site. The bars represent the means ± SD of the results from three independent experiments.

cytosol as a monomer, and only little binding activity is present in the nucleus (3, 29). Therefore, we examined the effect of heat treatment on DNA-protein complex formation with the sequence involving the three TCCs (positions −269 to −267, −261 to −259, and −255 to −253) that were shown to constitute a potential binding site for HSE. EMSA of nuclear extracts using a probe comprising the HSE sequence from the mouse *Hsp70* promoter showed that heat treatment at 43°C for 1 h induced detectable HSF activation, characterized by the appearance of a more slowly migrating high-intensity band (presumably a phosphorylated form) (Fig. 4B, lanes 1 and 2). A similar effect was observed for complex formation with probe 4, corresponding to the DNA sequences from positions −285 to −242 (Fig. 4B, lanes 3 and 4). Next, we adopted an EMSA-based competition assay to examine whether HSF binds to this sequence. As shown in Fig. 4C, a 200-fold excess of unlabeled probe 4 completely blocked HSF binding to the labeled HSE sequence probe, and competition was as complete as that of the unlabeled HSE sequence probe, strongly suggesting that HSF binds to the sequence span from positions −285 to −242. Finally, to confirm the *in vivo* interaction of HSF with the promoter region for M-LP_S, we performed a ChIP assay using anti-HSF1 antibody. Anti-GAPDH antibody was used as the negative control. The precipitated DNA was subjected to PCR with specific primers for the putative HSE region in the M-LP_S promoter. DNA fragments with the expected size were clearly detected by using anti-HSF1 antibody, while no band was detected when anti-GAPDH antibody was used (Fig. 4D). Together, these results strongly suggest that the M-LP_S promoter located upstream of exon 1 contains an HSF1-binding site.

Identification of the binding site of the negative regulatory element. A search for transcription factor-binding sites with TFSEARCH within the sequence from positions −315 to −286, which encompasses the possible negative regulatory element, revealed that it contains a binding site for Ttk 69K at positions −304 to −297. To examine if the Ttk 69K binding site is the negative regulatory element in this region, we abolished the Ttk 69K binding site of the pGL3-375 luciferase reporter construct by introducing three point mutations (the resulting vector was designated pGL3-375mut) and evaluated promoter activity. The activity of pGL3-375mut was 1.8-fold higher than that of pGL3-375, indicating that the binding site for Ttk 69K (positions −304 to −297) is involved in negative transcriptional regulation (Fig. 5).

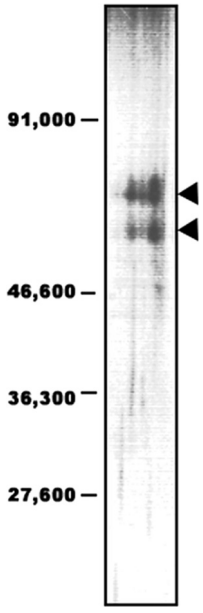

FIG. 6. DNA affinity purification of Rhit. DNA affinity chromatography was performed according to the oligonucleotide trapping method by a batch method. The batch purification was repeated 40 times, and all the protein-DNA complex solutions prepared were pooled, concentrated, and subjected to 10% SDS-PAGE.

Purification of Rhit by DNA affinity chromatography. Ttk 69K is a zinc finger transcription factor of *Drosophila melanogaster* that was previously identified as a repressor of the pair-rule genes *even-skipped* and *fushi tarazu* (12). Since a mammalian homolog of Ttk 69K has not been identified so far, we performed a BLASTP search (NCBI) of the mouse protein database using the Ttk 69K protein sequence (Swiss Prot accession no. P17789) as a query. However, no protein corresponding to a putative mouse homolog could be identified, although some sequences were found to have homology with the BTB (broad-complex, Tramtrack, and bric-a-brac) domain in the N-terminal region or the zinc finger domain in the C-terminal region of Ttk 69K. Accordingly, we attempted to use DNA affinity chromatography to purify the DNA-binding protein that interacts with the Ttk 69K-binding site of the M-LP_S promoter. To assess the DNA-binding activity of the purified fraction eluted from the DNA-protein complex trapping resin, we performed EMSA and confirmed the formation of a DNA-protein complex that migrates to the expected position. SDS-PAGE analysis of the purified protein revealed the presence of two bands with molecular masses of around 60 kDa (Fig. 6). The proteins were electroblotted onto a membrane, and the major (slowly migrating) band was excised and subjected to N-terminal-amino-acid analysis by Edman degradation. The N-terminal sequence was determined to be "QSIAA." A BLASTP search of the mouse protein database revealed that 35 sequences matched the query sequence "QSIAA." We searched for a sequence that appeared in both sets of BLASTP matches (with the Ttk 69K sequence and with "QSIAA") and identified zinc finger protein 13 (Zfp13) as the

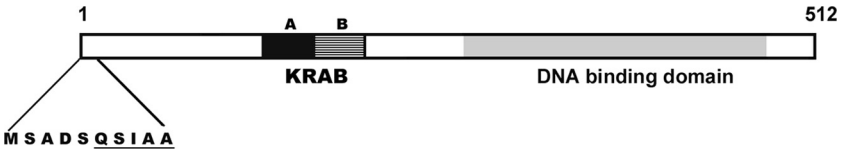

FIG. 7. Schematic representation of the structure of Rhit. The DNA-binding domain consists of eight C_2H_2-type zinc fingers.

only candidate. Since the expression of Zfp13 at the mRNA level was confirmed by the amplification of a corresponding cDNA by RT-PCR, the construction of the full-length cDNA was carried out by a RACE method using RNA from TCMK-1 cells as a template. The full-length cDNA constructed from the combination of the 5′- and 3′-RACE products comprised 2,103 bp, including an open reading frame of 1,539 nucleotides (512 amino acids). The obtained sequence has been deposited in the GenBank database as Rhit mRNA (accession no. FJ593171). Zfp13 is a predicted protein identified only at the mRNA level but is presumed to be expressed as two alternatively spliced transcripts, each comprising 6 exons that generate open reading frames encoding two distinct isoforms composed of 512 amino acids (isoform 1) and 505 amino acids (isoform 2), respectively. The difference between the two isoforms is the lack of the last 12 nucleotides of exon 3 in transcript 2. A comparison of the cDNA sequences of Rhit and Zfp13 (GenBank accession no. NM_011747) and the corresponding genomic sequence showed that the first exon of Rhit is completely different from that of Zfp13, whereas the other exons are consistent with those of Zfp13 transcript 1. Accordingly, the predicted protein from the Rhit cDNA sequence was identical to Zfp13 isoform 1, because the start codon is located in exon 2. The putative amino acid sequence of Rhit contained the sequence "QSIAA" in the N terminus (positions 5 to 10), a Krüppel-associated box (KRAB) domain (positions 124 to 196) consisting of KRAB-A and KRAB-B boxes, and a DNA-binding domain composed of eight C_2H_2-type zinc fingers in the C terminus (Fig. 7) and had an expected mass of 56,054 Da, which is compatible with the result from the electrophoretic separation of the DNA affinity-purified protein. N-terminal sequence analysis suggested that the N-terminal 5 amino acids of the translated protein are removed by posttranslational proteolytic cleavage. Homology with Ttk 69K was observed in the zinc finger domains, especially between the first to second domains of Ttk 69K and the seventh to eighth domains of Rhit (38% identity and 52% similarity).

Identification of Rhit as a negative regulatory element. To examine the ability of Rhit to bind to the Ttk 69K-binding site, a Rhit expression vector (pcDNA3.1/Rhit) was transfected into TCMK-1 cells, and the nuclear extract was analyzed by EMSA using probe 3, which includes the binding site for Ttk 69K. TMCK-1 cells transfected with pcDNA3.1/Rhit possessed significantly stronger binding activity than did control cells, indicating that Rhit has the ability to bind to probe 3, which contains the binding site for Ttk 69K (Fig. 8A). Next, we performed an RNAi experiment to verify that Rhit functions as a negative regulatory element in M-LP_S expression (Fig. 8B). Compared with a control siRNA duplex, transfection with three different siRNA duplexes targeting Rhit (si50, si1085, and si1267) led to 21.0, 47.1, and 52.7% reductions in Rhit expression at the mRNA level, respectively, as assessed by real-time PCR analysis. The RNAi-mediated knockdown of Rhit resulted in an increase in M-LP_S expression, and the increases in M-LP_S expression inversely correlated with reductions in Rhit expression. Furthermore, TCMK-1 cells were transfected with pcDNA3.1/Rhit or an expression vector for Rhit lacking the KRAB domain (pcDNA3.1/RhitdelKRAB), which is considered to be involved in transcriptional repression, and the alteration of the quantity of the M-LP_S protein was evaluated (Fig. 8C). Transfection with pcDNA3.1/Rhit led to a suppression of the M-LP_S protein in comparison with control cells, whereas transfection with pcDNA3.1/RhitdelKRAB had no effect. These results strongly suggest that Rhit functions as a negative regulatory element in M-LP_S expression and that the KRAB domain is essential for transcriptional repression.

Heat-induced and age-related alterations in M-LP_S expression. Since HSF was demonstrated to be involved in M-LP gene expression, we examined the effect of heat treatment on M-LP_S expression at the protein and mRNA levels. In the

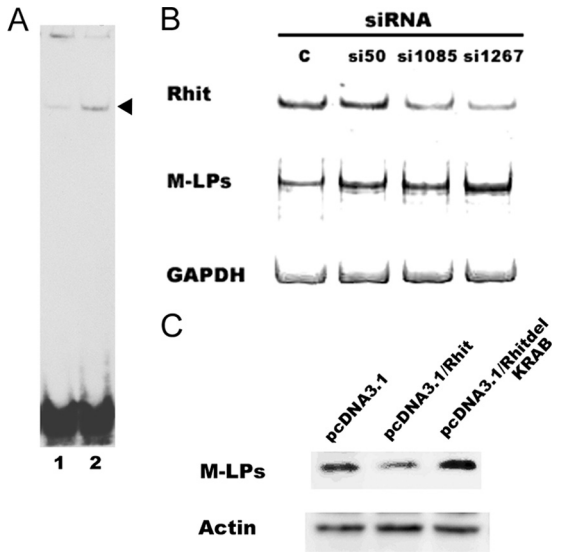

FIG. 8. Identification of Rhit as a negative regulatory element. (A) EMSA of nuclear extracts from Rhit expression cells. TCMK-1 cells were transiently transfected with pcDNA3.1 as a control (lane 1) or pcDNA3.1/Rhit (lane 2). Thirty micrograms of nuclear extracts was subjected to analysis. (B) RNAi-mediated knockdown of Rhit. TCMK-1 cells were transfected with control or Rhit siRNAs (si50, si1085, and si1267), and expressions of Rhit and M-LP_S were detected by RT-PCR with GAPDH as an internal control. (C) Alterations of M-LP_S protein expression by transfection with the Rhit expression vector (pcDNA3.1/Rhit) or an expression vector for Rhit lacking the KRAB domain (pcDNA3.1/RhitdelKRAB).

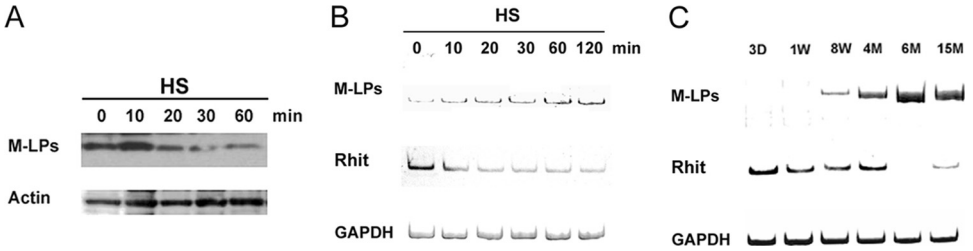

FIG. 9. Heat-induced and age-related alterations in M-LP$_S$ expression. (A) Western blot analysis of M-LP$_S$ during the time course of heat treatment. TCMK-1 cells were incubated at 43°C for 10, 20, 30, and 60 min. Cell extracts were subjected to 15% SDS-PAGE. Immunoblot analysis was performed with anti-M-LP as the primary antibody and then with secondary antibody against rabbit IgG. Expression of actin is shown at the bottom as an internal control. (B) RT-PCR analyses of M-LP$_S$ and Rhit during the time course of heat treatment. TCMK-1 cells were incubated at 43°C for 10, 20, 30, 60, and 120 min. Expression of GAPDH is shown at the bottom as an internal control. (C) Age-dependent expressions of M-LP$_S$ and Rhit mRNAs in mouse kidney. The expression of GAPDH is shown at the bottom as an internal control.

Western blot experiment, the quantity of the M-LP$_S$ protein increased at 10 min after the onset of heat treatment and then decreased gradually (Fig. 9A). After 20 min of heat treatment, the quantity of the M-LP$_S$ protein was reduced to a lower level than that at 0 min, suggesting that heat treatment may lead to increased protein synthesis and degradation. On the other hand, in the RT-PCR analysis, the quantity of M-LP$_S$ mRNA increased steadily over a period of 1 h (Fig. 9B). Previous studies reported that heat treatment induces a rapid translocation of HSF1 into the nucleus and activates transcription within minutes and that the transcriptional activation of heat shock genes is maintained until the 60-min time point and then diminishes (1, 3). The alterations in the quantity of M-LP$_S$ mRNA during heat shock in this study are consistent with previous observations. Moreover, we analyzed the effect of heat treatment on Rhit expression at the mRNA level. Interestingly, the quantity of Rhit mRNA decreased steadily over a period of 1 h, the opposite pattern to that of M-LP$_S$ (Fig. 9B).

Next, we examined the effects of HSF1 depletion, HSF1 overexpression, and Rhit overexpression on changes in M-LP$_S$ expression: TCMK-1 cells were transfected with siRNA duplexes targeting HSF1 (siRNA-HSF1), an HSF expression vector (pcDNA3.1/HSF1), or pcDNA3.1/Rhit, respectively, and the quantity of the M-LP$_S$ protein was evaluated before and after heat treatment. Transfection with siRNA-HSF1 led to a 43.0% reduction of HSF1 expression at the mRNA level relative to that of a control siRNA, as assessed by real-time PCR. Under conditions without heat treatment, transfection with siRNA-HSF1 suppressed the expression of the M-LP$_S$ protein relative to a control siRNA (Fig. 10A), whereas transfection with pcDNA3.1/HSF1 induced expression in comparison with a control pcDNA3.1 (Fig. 10B). Heat treatment had no effect on M-LP$_S$ protein expression in HSF1-reduced cells, whereas it had some effect in HSF1-overexpressing cells. On the other hand, transfection with pcDNA3.1/Rhit suppressed the expression of the M-LP$_S$ protein under conditions without heat treatment, and heat treatment induced M-LP$_S$ protein expression in Rhit-overexpressing cells (Fig. 10C). In this connection, to make sure that HSF1 and Rhit do not mutually regulate the expression of their genes, we confirmed by real-time PCR that transfection with pcDNA3.1/HSF1 did not change the amount of Rhit mRNA and that transfection with pcDNA3.1/Rhit did not change the amount of HSF1 mRNA (data not shown). Taken together, these results strongly suggested that HSF and Rhit act cooperatively to regulate the heat-induced expression of M-LP$_S$.

M-LP$_S$ was originally identified as an age-dependently expressed gene (15). To investigate the possibility that Rhit is involved in age-dependent transcriptional regulation, we measured Rhit mRNA levels in the kidneys of 3-day-old to 15-month-old mice. As shown in Fig. 9C, the amount of Rhit expressed decreased steadily until the age of 6 months and then increased slightly, showing a striking inverse relationship between M-LP$_S$ and Rhit. Since the level of HSF1 does not change with age (13, 30), our observations strongly suggest that Rhit is involved in the age-dependent expression of M-LP$_S$.

DISCUSSION

In this study, we isolated a novel transcription factor, Rhit, and found that Rhit belongs to the KRAB-containing protein family, which is characterized by the presence of a KRAB domain, an evolutionarily highly conserved region of about 50 to 75 amino acids, and multiple (4 to over 30) zinc fingers (4, 34). KRAB-containing proteins exist only in tetrapod vertebrates and make up one-third of the zinc finger proteins en-

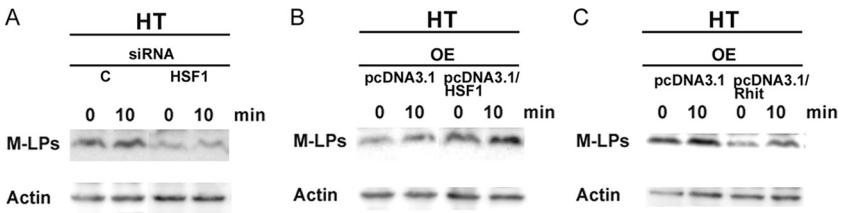

FIG. 10. Alterations of M-LP$_S$ protein expression by transfection with siRNA targeting HSF1 (A), an HSF1 expression vector (pcDNA3.1/HSF1) (B), and a Rhit expression vector (pcDNA3.1/Rhit) (C). HT and OE indicate heat treatment and overexpression, respectively.

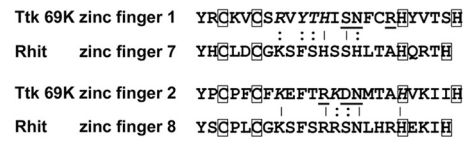

FIG. 11. Amino acid sequences of the first and the second zinc fingers of Ttk 69K compared with the seventh and the eighth zinc fingers of Rhit. Boxes indicate conserved cysteine and histidine residues, which coordinate an atom of zinc. The amino acids used for base contacts are underlined, and those for phosphate contacts are shown in italic type. Identical amino acids are indicated by vertical strokes, and similar residues are indicated by dots.

coded in mammalian genomes. The KRAB domain located near the N terminus is composed of one or both of the KRAB-A and KRAB-B boxes; the A box is involved in repression by binding to corepressors, whereas the B box is considered to enhance repression (35). Rhit contains both the A and B boxes as well as eight C_2H_2-type zinc fingers and was demonstrated to have transcriptional repressor activity. Although most KRAB-containing protein genes are expressed ubiquitously, some of them have been reported to be expressed preferentially in specific tissues or restricted developmental stages and so are thought to be involved in cell differentiation, development, apoptosis, and neoplastic transformation (28, 34). One feature of Rhit, the age-dependent change in expression, suggests its participation in development and/or aging. In spite of extensive evidence that KRAB-containing proteins function as transcriptional repressors, little is known about the mechanisms of the transcriptional repression and the binding to DNA. Gebelein and Urrutia (10) used a random oligonucleotide binding assay to identify a 27-bp sequence containing a central core sequence (TACCA[A/G]CCCTACAG) as the binding element for the KRAB-containing protein KS1 (KRAB suppressor of transformation 1) and showed that the KRAB domain and nine zinc fingers are required for transcriptional repression. On the other hand, biochemical and structural studies have been performed on non-KRAB C_2H_2-type zinc finger proteins. Those studies suggested that each zinc finger motif makes contact with 3 to 4 nucleotides and that three amino acids within the α-helical region of each finger play a critical role in determining the DNA-binding specificity (7, 8, 26, 27). Among these studies, a structural analysis of Ttk 69K by Fairall et al. (8) is quite informative with respect to the binding mechanism of Rhit, because Rhit was purified by DNA affinity chromatography based on direct interactions with the oligonucleotides containing the Ttk 69K-binding motif. Those authors showed that within the first and the second zinc fingers of Ttk 69K, 6 and 7 residues make contact with bases and phosphates, respectively (Fig. 11). A comparison of the zinc fingers of Ttk 69K with the seventh and eighth zinc fingers of Rhit revealed that the residues at these positions correspond quite well (46.2% identity and 92.3% similarity), suggesting that these zinc fingers are at least partly responsible for binding to DNA. Collectively, the finding that Rhit contains both the KRAB domain, which functions as a repressor, and the zinc fingers, which have high homology with those of Ttk 69K, is compatible with our observation that Rhit acts as a transcriptional repressor of the *M-LP* gene.

To clarify the function of M-LP from the viewpoint of transcriptional regulation, we characterized the promoter region of the *M-LP* gene. A search for *cis*-regulatory elements in the promoter region led us to identify HSE-like sequences containing several TTC motifs. It was reported previously that a TTC-rich sequence can be bound by HSF in a heat-inducible manner or in response to other stresses (11). The observations that M-LP_S expression is upregulated by heat treatment at the protein and mRNA levels, together with the results of EMSA, strongly suggest that M-LP_S transcription is in part under the control of HSF. HSFs are considered to be implicated in the transcriptional responses to various environmental, chemical, and pathophysiological stresses and certain developmental and differentiation signals (24). Mammals possess three genes encoding the HSF isoforms HSF1, HSF2, and HSF4, and HSF1 is the primary HSF activated during heat stress (29). The molecular mechanism for HSF1 activation was poorly understood until recently, but there is now compelling evidence that HSF1 senses both heat and hydrogen peroxide (H_2O_2) directly and assembles into a homotrimer in a redox-regulated manner. Ahn and Thiele (2) previously demonstrated that two cysteine residues within the DNA-binding motif of HSF1 are required for disulfide bond formation in response to heat or H_2O_2 stress, which leads to the formation of a homotrimer, DNA binding, nuclear translocation, and target gene activation. Such redox-regulated behavior is likely to have implications for development, aging, and pathophysiological conditions. In our previous study, we showed that transfection with M-LP_S upregulates the expression of the *SOD2* gene and downregulates the expression of the *Gpx1* and *Gpx3* genes. Since SOD is an enzyme involved in H_2O_2 synthesis and Gpx is considered to be a major enzyme involved in H_2O_2 catabolism, it seems reasonable to conclude that alterations in the expression levels of these enzymes caused by M-LP_S in turn cause an increase in the intracellular concentration of H_2O_2. Therefore, it is possible to envisage a regulatory cycle in which H_2O_2 stress activates HSF, which upregulates M-LP_S expression, which leads to the up- and downregulation of the *SOD* and *Gpx* genes, and which increases the H_2O_2 concentration. Here we demonstrated that both HSF and Rhit are involved in M-LP_S induction by heat treatment, whereas probably only Rhit is involved in the age-dependent expression of M-LP_S. Therefore, it seems reasonable that the these transcription factors are used as the situation demands—in a stringent response to heat or oxidative stress or in age-dependent changes over a broad range of ages.

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