A Novel Myc Target Gene, mina53, That Is Involved in Cell Proliferation*

Received for publication, May 7, 2002, and in revised form, June 24, 2002 Published, JBC Papers in Press, June 28, 2002, DOI 10.1074/jbc.M204458200

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Myc is a ubiquitous mediator of cell proliferation and can transactivate the expression of various genes through E-box sites. Here we report a novel gene, mina53 (Myc-induced nuclear antigen with a molecular mass of 53 kDa). The mina53 gene encodes a protein with a molecular weight of 53 kDa, which is localized in the nucleus and with part of the protein concentrated in the nucleolus. When serum-starved cells were activated by serum, the level of c-myc mRNA was elevated, and an increase in mina53 mRNA followed the elevation of c-myc mRNA. When expression of c-myc was reduced in human promyelocytic leukemia HL60 cells by phorbol 12-myristate 13-acetate, the expression of mina53 mRNA and protein was reduced. The expression of mina53 mRNA and Mina53 protein was induced by ectopic introduction of wild type c-Myc but not by a mutant c-Myc lacking the transactivation domain. When c-Myc in the c-MycER chimeric protein was activated, mina53 mRNA was increased, even in the presence of an inhibitor for protein synthesis. E-box sites are present in a region proximal to the transcription initiation sites of the mina53 gene. The gene expression from the mina53 promoter was elevated by c-Myc through E-box sites. c-Myc protein bound to the mina53 promoter region in vivo in HL60 cells in the proliferating phase but not after treatment of cells with phorbol 12-myristate 13-acetate. Specific inhibition of mina53 expression by an RNA interference method severely suppressed cell proliferation. Taken together, these results indicate that mina53 is a direct target gene of Myc, suggesting that mina53 is involved in mammalian cell proliferation.

The myc family of proto-oncogenes consists mainly of three genes, c-myc, N-myc, and L-myc (1–5). Although the three genes exhibit distinct patterns of expression with respect to cell types and developmental stages of cells, they can substitute for 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 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). Dimerization of c-Myc protein with its obligate partner Max results in the formation of a heterodimer that binds to E-box sites (mainly CACGTG elements). Besides the basic helix-loop-helix leucine zipper domain (Max binding and DNA binding sites), c-Myc has another domain, the transactivation domain (TAD), and the c-Myc-Max heterodimer activates transcription of various genes by activities of these domains. c-Myc has been shown to directly transactivate the expression of a number of genes (10), including ornithine decarboxylase (11), cdc25A (12), RCC1 (13), cyclin D2 (14), and Id2 (15). But the transactivation of these genes by c-Myc is generally weak, between two to severalfold. Attempts to identify genes capable of re-establishing normal proliferative rates in c-myc–/– cells have resulted in the repeated identification of c-myc and N-myc (16, 17). These results suggest that myc controls not a gene but genes to regulate cell proliferation. Although much effort has been made to investigate c-Myc, c-myc still remains enigmatic, and information about additional genes controlled by c-myc can help elucidate the function of c-Myc.

Here we report a novel gene, mina53, whose expression is directly induced by c-Myc. The mina53 gene encodes a protein with a molecular mass of 53 kDa, which localizes in the nucleus and some of which is concentrated in the nucleolus. Specific inhibition of mina53 expression by an RNA interference method severely suppressed cell proliferation, suggesting that the mina53 contributes to cell growth induced by c-myc.

EXPERIMENTAL PROCEDURES

Cell Culture—Human glioblastoma cell line T98G cells were cultured in Eagle's medium supplemented with nonessential amino acids and 10% fetal calf serum (FCS). Human cervical carcinoma HeLa cells and rat fibroblast cell line 3Y1 and its derivatives were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Gibco BRL, Paisley, UK) supplemented with 10% FCS, nonessential amino acids, and antibiotics. Human embryonic kidney 293 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% FCS and nonessential amino acids. Human thyroid carcinoma FTC133 cells were cultured in Eagle's minimum essential medium (Gibco BRL, Paisley, UK) supplemented with 10% FCS. Human colon carcinoma HT-29 cells were cultured in McCoy's 5A modified medium (Gibco BRL, Paisley, UK) supplemented with 10% FCS. Human primary non-small cell lung carcinoma cells were cultured in RPMI 1640 medium (Gibco BRL, Paisley, UK) supplemented with 10% FCS. Mouse fibroblast cell line 3T3 was cultured in Dulbecco's modified Eagle's medium supplemented with 10% FCS and nonessential amino acids. All the cell lines were cultured at 37 °C in a humidified atmosphere of 5% CO2.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AB083198 (human Mina53 with 465 amino acids), AB083190 (human Mina53 with 464 amino acids), AB083191 (human Mina53 with 280 amino acids), AB083192 (mina53 gene promoter), AB083193 (5′-terminal end of mina53 mRNA (containing exon 1a), AB083194 (5′-terminal end of mina53 mRNA (containing exon 1b), and AB083195 (rat Mina53).

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1 The abbreviations used are: TAD, transactivation domain; RT, reverse transcriptase; RACE, rapid amplification of 5′ cDNA ends; CMV, cytomegalovirus; kb, kilobase(s); siRNA, small interference RNA; OHT, 4-hydroxytamoxifen; FCS, fetal calf serum; EST, expressed sequence tag; TPA, phorbol 12-myristate 13-acetate; HEL, human erythroid leukemia.
co’s modified Eagle’s medium supplemented with 10% FCS. Human promyelocytic leukemia HL60 cells were cultured in RPMI 1640 medium supplemented with 20% FCS. Rat colon cancer cell line RCN-9 (Riken Cell Bank, Saitama, Japan) was cultured in RPMI 1640 medium supplemented with 10% FCS. Human cancer cell lines, human erythroid cell line (HEL), MCFAS (ovarian cancer cell line) were obtained from Health Science Research Resources Bank, Osaka, Japan. These cells were grown in appropriate media.

PCR—PCR amplifications were performed in 50 μL of EX Taq buffer (Takara, Shiga, Japan) containing 10 pmol of each primer, 1.2 units of EX Taq DNA polymerase, and 200 μM dNTPs. Reverse transcriptase—RT-PCR primers were synthesized of single-strand cDNA of human erythrolymphoma HEL or rat colon cancer RCN-9 cells was performed on total RNA (1 μg) using a Superscript First-strand Synthesis system (Invitrogen). One μl (total 20 μl) of resultant single-strand cDNA was used as the template for PCR. The RT-PCR primers for the amplification were mina53 RT-F (5’-ACACCGGTGACGCGCocaccccggcgggtgga-3’), a sequence in the 5’-untranslated region) and mina53 RT-R (5’-GCTCTCTCTAGGGAAGATATTT-3’, a sequence in the 3’-untranslated region) for human and mina53 RT-F (5’-TTTCTTCCCTACTTTGGAACATTCG-3’, a sequence in the 5’-untranslated region and the first four coding nucleotides) and rat mina53 RT-R (5’-AATGATGCTTCTTTAAAAAGGATTGTTTT-3’, a sequence in the 3’-untranslated region) for rat. The temperature profile was 35 cycles of denaturing at 98°C for 15 s, annealing at 65°C for 1 min, and extension at 72°C for 2.5 min.

**Rapid Amplification of 5’ cDNA Ends (5’-RACE) Analysis—Reverse transcriptase reaction, double-strand cDNA synthesis, and adapter ligation from poly(A)+ RNA (1 μg) of HEL cells were performed using a Marathon cDNA amplification kit (CLONTECH, Palo Alto, CA) as described previously (18). The first PCR was performed using primers, mina53-RACE-1 (5’-GGGCTCTCTCCTGGAAGATATTT-3’, a sequence in the 5’-terminal region of expressed sequence tag (EST) clone W27666) and the API primer provided by the supplier. The temperature profile was initial denaturing at 94°C for 1 min followed by 25 cycles of denaturing at 98°C for 15 s and annealing and extension at 65°C for 3 min. One μl of the first RACE-PCR product diluted 1000 times was used as the template for nest RACE-PCR. Nest PCR was performed using mina53-RACE-2 primer (5’-CTGACTACGACTCTCTAGCTAGGCGATCTTAAGTTAATAATC-3’, a sequence in the middle part of mina53 cDNA) and the AP2 primer provided by the supplier. The temperature profile was initial denaturing at 94°C for 1 min followed by 25 cycles of denaturing at 96°C for 15 s and annealing and extension at 68°C for 3 min.

**Plasmids Encoding c-myc—Pe-myc/CDM5, in which human c-myc is driven by a CMV promoter, was described previously (13). Pe-myc/CDM8 was digested with PstI and self-ligated to produce pe-myc(d41-178)/CDM5, which encodes a mutant c-myc protein lacking the TAD. The 2-kb BstXI-PstI fragment, blunt-ended with Klenow, and cloned into a pCAGGS mammalian expression vector (19) to produce plasmid CAGGS4c-myc.

**Plasmids Encoding mina53—cDNA for human mina53 was amplified by PCR with 5’-GAAGCTTATGCCAAAGAAAGCAAAGCCTACAG-3’ and 3’-GAAGCTTATGCCAAAGAAAGCAAAGCCTACAG-3’ using a pCAGGS mammalian expression vector (19) to produce pCAGGS4c-myc.

**Ligation—A genomic DNA fragment of the human mina53 gene, which extends from the promoter region to intron 1, was amplified by PCR with 5’-CAGCAGCTGCTGCAAACAGGGTTAATC-3’, encoding a BamHI site at 5’-terminal region of expressed sequence tag (EST) clone W27666) and the AP1 primer provided by the supplier. The temperature profile was initial denaturing at 94°C for 1 min followed by 15 cycles of denaturing at 98°C for 15 s, annealing at 65°C for 1 min, and extension at 72°C for 2.5 min.

**Transcriptional Assay—** 3Y1 cells highly expressing c-Myc or mutant c-Myc lacking a large part of TAD, cells were transfected with 20 μg of pc-myc/CDM8 or pc-myc(d41-178)/CDM8 by a calcium phosphate method (20). Cells were cultured for 2 weeks in the medium containing 200 μg/ml hygromycin. Individual clones were isolated, and the expression of c-Myc or mutant c-Myc protein was detected by Western blot analysis using anti-c-Myc antibody. Clones 3Y1MycA and 3Y1MycB (expressing c-Myc protein) and 3Y1Myc(dTAD)A and 3Y1Myc(dTAD)B (expressing a mutant c-Myc protein lacking the transactivation domain) were established. To establish human glioblastoma T98G cells expressing c-Myc or mutant c-Myc lacking a large part of TAD, cells were transfected with 20 μg of pc-myc/CDM8 and 0.4 μg of pAcHyg, and the expression of c-MycER in individual clones was detected as described above.

**RNA Preparation—** Total RNA was isolated from cells by the acid guanidinium thiocyanate-phenol-chloroform extraction method using a diethyl pyrocarbonate-treated RNA preparation solution set (Nakalai, Kyoto, Japan).

**Differential Display Using DNA Chip (cDNA Micro Array)—** Total RNA from untreated T98Gmycer-2 cells and from cells treated by 4-hydroxytamoxifen (OHT) for 20 h was isolated. Poly(A)+ RNA was recovered and subjected to differential display using a DNA chip (Incyte Genomics, Palo Alto, CA). About 9000 kinds of cDNAs including EST clones were plated on the chip (Unigem Human V Version 2).

**Reporter Assay—** To establish rat fibroblast 3Y1 cells highly expressing c-Myc or mutant c-Myc lacking a large part of TAD, cells were transfected with 20 ng of pAcHyg and 20 μg of pc-myc/CDM8 or pc-myc(d41-178)/CDM8 by a calcium phosphate method (20). Cells were cultured for 2 weeks in the medium containing 200 μg/ml hygromycin. Individual clones were cloned, and the expression of c-Myc or mutant c-Myc protein was detected by Western blot analysis using anti-c-Myc antibody. Clones 3Y1MycA and 3Y1MycB (expressing c-Myc protein) and 3Y1Myc(dTAD)A and 3Y1Myc(dTAD)B (expressing a mutant c-Myc protein lacking the transactivation domain) were established. To establish human glioblastoma T98G cells expressing c-Myc or mutant c-Myc lacking a large part of TAD, cells were transfected with 20 μg of pc-myc/CDM8 and 0.4 μg of pAcHyg, and the expression of c-MycER in individual clones was detected as described above.
ization of *R. reniformis* luciferase activities, firefly luciferase activities with activated c-MycER were expressed as the ratio of activity without of the MycER activation. To normalize the effect of OHT not through MycER activation, the values from T98Gmycer-2 cells were divided by the values from T98G parent cells, which were treated by exactly the same conditions of T98Gmycer-2 cells described above.

**Chromatin Immunoprecipitation**—Chromatin immunoprecipitation assay was performed basically as described by Boyd et al. (21, 22). Immune complexes were recovered by adding 20 μl of protein A beads blocked by DNA and BSA (50% volume/volume). The beads were washed, the DNA fragments were eluted, and the eluted solutions were phenol/chloroform-extracted and ethanol-precipitated. Then, immunoprecipitated DNA fragments were detected by PCR. PCR primers were 5'-GCCC GGCCGCTGTTGGTGGCCACCTG-3' and 5'-TCTCTTTCTTCC-CCGCTTCTTCC-3’, which amplified a 483-bp fragment containing E-boxes near the two transcription start sites of the human *mina53* gene, and 5’-TTACAGTTAGCCTCTCAATGGC-3’ and 5’-GCAAGGCTTCAATTAGGACC-3’, which amplified the genomic sequence of a region containing an E-box in chromosome 22. This E-box is located in a chromosomal region without any detectable genes (14).

**Antibodies**—Human *mina53* (from the third amino acid Lys to the carboxyl-terminal end) was expressed using pET/aha53 in *E. coli* BL21(DE3) (Novagen) and isolated by SDS-PAGE. Rabbits were immunized with the recombinant polypeptide. The glutathione S-transferase fusion *mina53* was expressed using pGEX-hmina53 in *E. coli* JM109 and isolated by a glutathione-Sepharose column (Amersham Biosciences). Polyclonal antibody against *mina53* was purified from rabbit serum using Sepharose 4B conjugated with recombinant glutathione S-transferase *mina53* polypeptide as described previously (23). Antibody-1 against c-Myc was described previously (13). Anti-c-Myc antibody-2 (N262) (Santa Cruz Biotechnology, Santa Cruz, CA), mouse anti-nucleolin monoclonal antibody (C23) (Santa Cruz Biotechnology, Santa Cruz, CA), mouse anti-53 kDa nuclear antigen with a molecular mass of 52,600 Da (MA/ALdrich) were purchased.

**Western Blot Analysis and Indirect Immunofluorescence Staining**—Cells were transfected and extracted in 3% SDS sotruted containing 100 mM Tris, pH 6.8, 0.1% diethiothreitol, and 20% glycerol. Cell extracts were separated on 4–20% polyacrylamide gels and transferred to a polyvinylidene difluoro microporous membrane (Millipore, Bedford, MA). After treatment with antibodies, bands were detected using an enhanced chemiluminescence technique (Amersham Biosciences).

For indirect immunofluorescence staining, HeLa cells grown on glass coverslips in a 6-well plate were fixed in methanol for 10 min at −20°C. Anti-Mina53 rabbit antibody and mouse anti-nucleolin monoclonal antibody were added and incubated for 20 min at 37°C. After washing in 0.1% skim milk in phosphate-buffered saline three times, Alexa 488-conjugated anti-mouse IgG (Molecular Probes, Eugene, OR), Cy3-conjugated anti-rabbit IgG (Zymed Laboratories Inc., South San Francisco, CA), and anti-β-actin monoclonal antibody (AC-15) (Sigmaldrich) were purchased.

**Introduction of Small Interference RNA (siRNA)** into Cells—siRNAs were transfected into cDNA vectors to obtain siRNAs against each gene. These siRNAs were annealed and transfected essentially as described previously by Beilharz et al. (24). Twenty-four hours before transfection, cells in an exponentially growing phase were trypsinized and transferred to a 12-well plate. Transfection was carried out with 3Y1MycB cells were cultured for transfection without serum, and 3Y1MycB cells were cultured for transfection with 2% serum using Sepharose 4B conjugated with recombinant glutathione S-transferase-Mina53 polypeptide. The cDNA encoding the 5’ upstream part of EST clone W27666 was isolated using the 5’-RACE protocol from a library of HEL cells. We isolated two kinds of cDNAs with different sequences at the 5’ terminus. From the results of the nucleotide sequences from 5’-RACE experiments and ESTs, we predicted the whole sequence of the mRNA molecule. Using primers consistent with 5’- and 3’-part sequences of the predicted molecule, a 2.3-kb-length cDNA was amplified from total RNA of HEL cells by RT-PCR protocol. The amplified cDNA was cloned into a vector and sequenced. The majority of cDNA clones encode a protein of 465 amino acids with a predicted molecular mass of 52,800.28 Da (Fig. 1A). The subcellular localization of this protein was visualized by indirect immunofluorescence staining with the specific antibody. As shown in Fig. 1B, this protein is localized in the nucleus. For these reasons, we refer to this gene and protein as *mina53* and *Mina53* (Myc-induced nuclear antigen with a molecular mass of 53 kDa). In addition to the diffuse localization in the nucleus, strong dotted staining was observed within the nucleus and supposed to be nucleoli. Double staining with anti-nucleolin antibody indicated that these dots were consistent with nucleoli. Nuclear and nucleolar localization of *mina53* was also observed when green fluorescent protein-Mina53 fusion protein was expressed (data not shown). These results suggest that *mina53* is a nuclear protein, some of which is concentrated in nucleoli.

The cDNA encoding a 464-amino acid protein with a predicted molecular mass of 52,672.15 Da was also cloned. This protein lacks Gln-297 of the major protein. We also detected a cDNA that has 101 bp inserted in the middle part of the *mina53* mRNA. There is a stop codon in the inserted sequence, and the cDNA encodes 262 amino acids of *Mina53* protein from the initiation codon and 18 additional novel amino acids at the 3’ end. Although the amount of this mRNA was small, we detected this mRNA molecule in cDNA libraries made from a human ovary mucinous cystadenocarcinoma cell line (MCAS) and a human colon adenocarcinoma cell line (WiDr) (data not shown).

**RESULTS**

**A Method Used to Identify Genes Whose Expression Was Induced by c-Myc**—To conditionally activate c-Myc activity, the estrogen-inducible Myc system (25) was used. The chimeric protein c-MycER consists of human c-Myc and the estrogen binding domain of the human estrogen receptor. c-MycER anchors to cytoskeletal components of cells in the absence of estrogen. When estrogen or its derivative, OHT, binds to the chimeric protein, it becomes free to function as c-Myc. The human glioblastoma cell line T98G was used as the parental cells into which ectopic c-Myc activity was introduced. A T98G cell line expressing c-MycER protein (T98Gmycer-2 cells) was established. Total RNAs from T98Gmycer-2 cells in an exponentially growing phase in the presence or absence of OHT for 20 h were processed by a cDNA micro array analysis. Specific signals for Myc target genes, ornithine decarboxylase (11) and nucleolin (26), were increased by 2.6- and 1.6-fold, respectively, with c-MycER activation. These results suggest that Myc target genes could be detected in this experimental system.

**Identification of Human mina53**—To shed light on a new facet of Myc functions, we focused on human EST sequences among genes stimulated by c-MycER activation. The signal for EST clone W27666 was stimulated by 1.9-fold with c-Myc activation. This stimulation rate was similar to those of the Myc-targeted genes, ornithine decarboxylase and nucleolin, measured in this study.

**EST encoding the 5’ upstream part of EST clone W27666 was also found using the 5’-RACE protocol from a library of HEL cells. We isolated two kinds of cDNAs with different sequences at the 5’ terminus. From the results of the nucleotide sequences from 5’-RACE experiments and ESTs, we predicted the whole sequence of the mRNA molecule. Using primers consistent with 5’- and 3’-part sequences of the predicted molecule, a 2.3-kb-length cDNA was amplified from total RNA of HEL cells by RT-PCR protocol. The amplified cDNA was cloned into a vector and sequenced. The majority of cDNA clones encode a protein of 465 amino acids with a predicted molecular mass of 52,800.28 Da (Fig. 1A). The subcellular localization of this protein was visualized by indirect immunofluorescence staining with the specific antibody. As shown in Fig. 1B, this protein is localized in the nucleus. For these reasons, we refer to this gene and protein as *mina53* and *Mina53* (Myc-induced nuclear antigen with a molecular mass of 53 kDa). In addition to the diffuse localization in the nucleus, strong dotted staining was observed within the nucleus and supposed to be nucleoli. Double staining with anti-nucleolin antibody indicated that these dots were consistent with nucleoli. Nuclear and nucleolar localization of *mina53* was also observed when green fluorescent protein-Mina53 fusion protein was expressed (data not shown). These results suggest that *mina53* is a nuclear protein, some of which is concentrated in nucleoli.

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The open reading frames of rat and mouse mina53 encode 465 amino acid proteins, as did human mina53. Human and mouse amino acid sequences of Mina53 proteins were 72% identical to each other, those of human and rat were 75% identical, and those of rat and mouse were 86% identical, suggesting that the mina53 is conserved in mammals. A nucleotide 3 bp upstream of the methionine initiation codon is A in mina53 of all the three species, conforming to a Kozak consensus sequence (27).

Expression of mina53 mRNA—The expression profile of mina53 mRNA was examined by Northern blot analysis after T98G cells were stimulated by serum (Fig. 2A). Serum stimulation of serum-starved T98G cells resulted in a 5-fold increase in the expression of mina53 mRNA. The increase was detecta-
Human promyelocytic leukemia HL60 cells are terminally differentiated by phorbol 12-myristate 13-acetate (TPA) in which the c-myc expression level reduces (28, 29). This experimental system was used to investigate whether Myc-targeted genes are affected during the shut-off of Myc that accompanies hematopoietic differentiation (30). When HL60 cells were cultured with 10 nM TPA, the expression of a differentiation marker CD18 mRNA (31) increased, and a 3-fold-induction was observed at 24 h, confirming the differentiation in this system (Fig. 2B). At 3 h after the addition of TPA, the level of c-myc mRNA started to decline and reached \(\frac{1}{2}\) at 7 h. Down-regulation of mina53 mRNA followed the decrease in c-myc mRNA, and the level of mina53 mRNA decreased to \(\frac{1}{2}\) at 12 h. These experiments demonstrate that the expression pattern of mina53 correlated with c-myc expression.

Next, the effect of c-Myc activation on mina53 mRNA was investigated (Fig. 2C). The mina53 mRNA level rose steadily for 9 h in OHT-treated T98Gmcyer-2 cells, showing nearly 3-fold induction. The OHT treatment of T98G parent cells did not stimulate the mina53 mRNA level. Induction of mina53 mRNA by OHT in T98Gmcyer-2 cells was maintained in the presence of the protein synthesis inhibitor cycloheximide. Treatment with cycloheximide had little effect on the mina53 mRNA level in T98G parent cells. These results indicate that the mina53 gene is a direct target of Myc.

**Induction of Minna53 Protein by Myc Activation**—To examine the correlation between expression of Minna53 and c-Myc proteins, a specific antibody against Mini53 protein was used in Western blotting. Bands with similar mobility to that predicted from the amino acid sequence of Mina53 were detected in T98G and HL60 cells (Fig. 3A). When c-MycER was activated by OHT in T98Gmcyer-2 cells, the intensity of the band for Minna53 was increased, whereas that for \(\beta\)-actin was not. When HL60 cells were cultured with 10 nM TPA for 24 h, the expression of c-Myc and Minna53 proteins was decreased, whereas the expression of \(\beta\)-actin was not.

The control of Minna53 expression by c-Myc was also verified in rat fibroblast cell line 3Y1. Human c-Myc or mutant c-Myc that lacks TAD was stably expressed in 3Y1 cells. The expression of Minna53 protein was increased in the dose-dependent manner of wild type c-Myc expression (Fig. 3B, lanes for 3Y1MycA and 3Y1MycB). When the mutant Myc was expressed at a level comparable with that of wild type c-Myc (lanes for 3Y1MycA and 3Y1Myc(dTAD)A) or even at a much higher level than that of wild type Myc (lanes for 3Y1MycB and 3Y1Myc(dTAD)B), expression of Minna53 protein was not increased.

To confirm the results, mRNA for mina53 was detected by Northern blotting in these 3Y1 cell lines (Fig. 3C). The mina53 mRNA level was stimulated by 1.5- and 3-fold in 3Y1MycA and 3Y1MycB, respectively. On the other hand, mina53 mRNA level was not stimulated in 3Y1Myc(dTAD)A and 3Y1Myc(dTAD)B. These results suggest that c-Myc transactivates the expression of mina53.

**Structure of the Human mina53 Gene**—Human genomic DNA sequences, which include mina53 cDNA sequences, were found in the High Throughput Genomic Sequence (HTGS) database (GenBank™ AC026100, AC073245, and AC024892), and our cDNA sequences for mina53 were aligned with these genomic sequences from the GenBank™ data (Fig. 4A). The length of intron 2 was controversial between the GenBank™ data. To know the length of intron 2, the genomic DNA fragment was amplified by PCR using oligonucleotides correspond-
Fig. 2. Expression of mina53 mRNA. A, correlation of mina53 mRNA levels with c-myc expression levels in T98G cells. Serum-starved T98G cells were stimulated by the addition of serum final to 10%. RNA was isolated at the indicated time points and analyzed by Northern blotting to detect mina53 and c-myc mRNAs (left). 28 S and 18 S ribosomal RNAs are shown to indicate the amount of total RNA electrophoresed. The results were quantified and plotted (right). B, decrease in mina53 mRNA levels after c-myc mRNA disappearance in human promyelocytic leukemia HL60 cells. RNA was isolated at the indicated time points after exposure to 10 nM TPA and analyzed by Northern blotting to detect mina53, c-myc, and CD18 mRNAs (left). 28 S and 18 S ribosomal RNAs are shown. The results were quantified and plotted (right). C, increase of mina53 mRNA levels in T98G cells by activation of MycER protein (T98Gmycer-2 cells). After T98Gmycer-2 cells (mycer) and parental T98G cells (cont) were cultured in the medium supplemented with 0.25% serum for 40 h, cells were treated with 200 nM OHT, and RNA was isolated at the indicated time points. When indicated as +, 20 μg/ml cycloheximide (CHX) was added 20 min before the addition of OHT. RNA was analyzed by Northern blotting to detect mina53 mRNA (left). 28 S and 18 S ribosomal RNAs are also shown. The results were quantified and expressed as a bar graph (right).
that a 21-nucleotide siRNA duplex specifically suppresses gene expression in mammalian cell lines including human cervical carcinoma HeLa cells (24). To gain insight into the biological function of mina53, this new technique was applied to specifically inhibit the expression of Mina53 protein. As shown in Fig. 3 A, elevated expression of Mina53 protein by Myc activation. T98G and T98Gmycer-2 cells were cultured in the presence or absence of 0.2 μM OHT for 20 h. Cell extracts were subjected to Western blotting using a specific antibody against human Mina53 protein (left panel). HL60 cells were cultured in the presence or absence of 10 nM TPA for 20 h, and cell extracts were subjected to Western blotting using a specific antibody against human Mina53 protein and anti-c-Myc antibody-1 (right panel). The blotting papers detecting Mina53 were reprobed with anti-β-actin monoclonal antibody as loading controls. Profiles for protein staining are also shown (lower panel) to indicate the amount of protein electrophoresed. 

**FIG. 3. Western blot analysis of Mina53.** A, elevated expression of Mina53 protein by Myc activation. T98G and T98Gmycer-2 cells were cultured in the presence or absence of 0.2 μM OHT for 20 h. Cell extracts were subjected to Western blotting using a specific antibody against human Mina53 protein (left panel). HL60 cells were cultured in the presence or absence of 10 nM TPA for 20 h, and cell extracts were subjected to Western blotting using a specific antibody against human Mina53 protein and anti-c-Myc antibody-1 (right panel). The blotting papers detecting Mina53 were reprobed with anti-β-actin monoclonal antibody as loading controls. Profiles for protein staining are also shown (lower panel) to indicate the amount of protein electrophoresed. 

**FIG. 4. The gene structure of human mina53 and transient expression assay of mina53 promoter.** A, the genomic organization of human mina53. Boxes indicate exons; closed boxes mark the open reading frame, and the open boxes the 5′ and 3′ untranslated regions. The direction of transcription is from left to right. The transcription starts from exon 1a or exon 1b. The translation start site locates in exon 2. Exon 1b exists 0.25 kb downstream of exon 1a. A stop codon (TAG) lacks the first 3 bp of exon 7. Exon 5′ encodes the 101-bp sequence, which has rarely been identified in cDNAs (see the section structure of the human mina53 gene under “Results”). B, reporter plasmids. The reporter plasmid (pMina(W)Luci), for studies of promoter activity for mina53 expression, was constructed from a DNA fragment of mina53 (from upstream of exon 1a to part of intron 1) and firefly luciferase. The E-box-deleted or point-mutated reporter plasmids (pMina(dE)Luci, pMina(mE1/2)Luci, pMina(mE1)Luci, and pMina(mE2)Luci) are also shown. C, transient expression assay using T98Gmycer-2 cells. T98Gmycer-2 cells were transfected with the reporter plasmids and pRL-CMV (for standardization of transfection efficiency). One day later, OHT (final concentration 0.2 μM) was added to activate the MycER chimeric protein, and cells were further cultured for 17 h. Cell extracts were analyzed for activities of firefly luciferase. After normalization, firefly luciferase activities were expressed as the ratio to activities without MycER activation, as described under “Experimental Procedures.” Values are the means of four separate experiments. The bars indicate the S.E.
A Novel Myc Target Gene mina53

DNA that contains the E-box 2 in vivo in proliferating HL60 cells but not HL60 cells treated with TPA. Together, these results demonstrate that c-Myc directly induces the expression of mina53.

The high conservation of amino acid sequences of Mina53 protein between human and rodents suggest that the protein plays an important role in mammals. The expression of mina53 correlated with cell proliferation, suggesting a role of mina53 in cell proliferation. To gain insight into the biological function of Mina53, expression of mina53 was specifically inhibited in human cervical carcinoma HeLa cells and rat 3Y1 cells highly expressing c-myc. In HeLa cells, c-myc is highly expressed and plays an essential role in cell proliferation (32). A new technique, siRNA technique (24), was applied for the specific inhibition. RNA interference (RNAi) is the process of sequence-specific, post-transcriptional gene silencing, which is initiated by double-stranded RNA (dsRNA) that is homologous in sequence to the silenced gene due to sequence-specific dsRNA-mediated mRNA degradation. Recently it has been shown that 21-nucleotide siRNA duplexes specifically suppress the expression of endogenous genes in mammalian cells (24). Specific inhibition of Mina53 expression by siRNA duplex was clearly shown here because the expression of Mina53 protein in human and rat cells was specifically reduced by each cognate siRNA duplex for human and rat mina53, respectively, but not by a control siRNA (inverted sequence for human mina53) or a duplex directed against mina53 of different species. Five nucleotides of the 19-nucleotide sequence were different between human and rat mina53 siRNA duplexes. When the expression of Mina53 protein was reduced by siRNA, severe inhibition of cell proliferation was observed in both human and rat cells. These results indicate the importance of mina53 in proliferation of mammalian cells.

Recently, the role of c-myc in vivo has been extensively studied in mice in which c-myc expression is incrementally reduced to zero (33). These studies showed that reduction of c-Myc levels resulted in reduced body mass owing to multi-organ hypoplasia (reducing cell numbers). They stated that c-Myc activity determines the ratio of activated T cells that re-enter the cell cycle and the rate of cell division of fibroblasts. A recent in vitro study using time-lapse microscopy also suggested that c-myc regulates the decision of cells to enter or exit the cell cycle in rat cell culture systems (34). Therefore, it is possible that Mina53 may be one of factors directly involved in the cell cycle machinery, as other Myc target genes, including cyclin D2 (35, 36) and Id2 (15), are. It was shown that loss of Drosophila Myc (dMyc) retards cellular growth (accumulation of cell mass) and reduces cell size, whereas dMyc overproduction increases growth rate and cell size, suggesting that dMyc regulates the primary targets involved in cellular growth (37). Deregulated expression of Myc sometimes induces an increase of cell size in mammals also (38). For example, constitutive expression of a c-myc transgene under control of the Ig heavy-chain enhancer resulted in an increase in cell size of pretransformed B lymphocytes at all stages of B cell development independently of cell cycle phase. This increase correlated with an increase in protein synthesis (39). Recent reports also suggest that c-myc enhanced the expression of a large set of genes functioning in protein synthesis, including ribosome biogenesis (40, 41). Thus, mina53 may be one of those genes.

Recent studies suggest that the nucleolus might function as a "parasite" for certain proteins involved in eukaryotic cell cycle regulation. Sequestration of proteins into nucleoli prevents them from reaching their targets in other regions of the cell (42). Thus, Mina53 in nucleoli may be sequestered from the place where Mina53 functions, by which an activity of Mina53...
is controlled. The nucleolus has been considered to be a “ribosome factory” since the 1960s, and ribosome biogenesis is essential for cell proliferation. Thus, Mina53 in nucleoli may play a necessary role in ribosome biogenesis, which is consistent with the possibility we described above. Recent studies also suggest that the nucleolus participates in many other activities, including the processing or nuclear export of certain mRNAs, biogenesis of signal recognition particle RNA and telomerase RNA, and processing one of the spliceosomal small nuclear RNAs (43). These functions also appear to be essential for cell proliferation. Thus, it is possible that Mina53 plays a role in one of these functions.

It had been suggested that proliferation and differentiation represent alternative and mutually exclusive pathways for cells, and there is compelling evidence that Myc may function at a pivotal control point in the decision-making process (4). It had been reported that down-regulation of c-myc expression is correlated with differentiation in many cellular systems. It was also shown that constitutive expression of c-myc prevents cells from leaving the cell cycle and inhibits the differentiation, and suppression of c-myc alone is sufficient to leave the cell cycle and induce differentiation. However, down-regulation of c-myc is not always correlated with differentiation. Expression of c-myc is detectable in terminally differentiated cells such as keratinocytes. U937 cells can be differentiated without down-regulation of c-myc in certain conditions. Furthermore, transforming function of c-myc can be distinguished from the Myc-induced block of differentiation using a Myc mutant in primary quail myoblast. Therefore, relationship between c-myc, proliferation, and cellular differentiation still include controversial problems. One of the Myc target genes might be specifically involved in cell proliferation, differentiation, or both. Thus, studies of each Myc target gene on proliferation and differentiation will help elucidate the problems, and it is a next, important issue to determine whether mina53 is involved in cellular differentiation.

Although no domains whose functions have been experimentally demonstrated are found in Mina53, the amino acid sequence 128–271 of Mina53 represents a JmjC domain, a domain that was recently identified on the basis of significant sequence similarity among many genes (44). It was suggested that JmjC domains are present in some metalloenzymes that adopt the cupin fold, which is a flattened β-barrel structure containing two sheets of the five anti-parallel strands that form the walls of a zinc binding cleft. This domain has been often found together with DNA or chromatin binding domains, and JmjC domain-containing proteins are candidates for enzymes that regulate chromatin remodeling (44). Human and mouse Mina53 amino acid sequences in JmjC domains are 83.3% identical, whereas the sequences of the other portions are 66.6% identical between the two species, suggesting the functional importance of this domain. Therefore, Mina53 may regulate chromatin remodeling, which appears to largely affect patterns of gene expression.

Because of the clear relationship of mina53 with cell proliferation, mina53 appears to be an important target of c-myc.

![FIG. 6. Reduction of Mina53 protein expression by RNA interference and its effect on cell proliferation. A, reduction of Mina53 protein expression by RNA interference. siRNA duplexes specific for human mina53 and rat mina53 were synthesized. As a nonspecific control, a duplex with the inverted sequence of human mina53 siRNA was also synthesized. HeLa cells (left panel) and 3Y1MycB cells, which are rat 3Y1 cells that highly express human c-myc (right panel), were transfected with siRNA duplexes. Forty hours later, cell extracts were processed by Western blot analysis using anti-Mina53 antibody. B, cell proliferation of transfected cells. 2 × 10⁵ HeLa cells (left panel) and 1 × 10⁴ 3Y1MycB cells (right panel) were transfected with siRNA duplexes specific for human mina53 (●), rat mina53 (▲), and control siRNA duplex (○). At various days after transfection, cell numbers were counted and expressed on the y axis.](image-url)
Further studies will clarify the function of Mina53 protein and may contribute to solving the puzzle of Myc functions.

Acknowledgments—We thank Yasuko Noguchi for excellent technical assistance. We thank Dr. Yoshihiro Yoneda for critical reading of the manuscript. We thank Drs. Kathryn E. Boyd and Peggy J. Farnham for sending us their protocols for chromatin immunoprecipitation analysis.

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