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# Distinct Localizations and Repression Activities of MM-1 Isoforms Toward c-Myc

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**Abstract** MM-1 was identified as a c-Myc-binding protein and has been reported to repress the E-box-dependent transcription activity of c-Myc by recruiting HDAC1 complex via TIF1 β/KAP1. In this study, originally isolated MM-1 was found to be a fusion protein comprised of the N-terminal 13 amino acids from the sequence of chromosome 14 and of the rest of the amino acids from that of chromosome 12 and was found to be expressed ubiquitously in all human tissues. Four splicing isoforms of MM-1, MM-1α, MM-1β, MM-1γ, and MM-1δ, which are derived from the sequence of chromosome 12, were then identified. Of these isoforms, MM-1α, MM-1γ, and MM-1δ were found to be expressed in tissue-specific manners and MM-1β was found to be expressed ubiquitously. Although all of the isoforms potentially possessed c-Myc- and TIF1β-binding activities, MM-1β and MM-1δ were found to be mainly localized in the cytoplasm and MM-1α and MM-1γ were found to be localized in the nucleus together with both c-Myc and TIF1β. Furthermore, when repression activities of MM-1 isoforms toward c-Myc transcription activity were examined by reporter gene assays in HeLa cells, MM-1α, MM-1γ, and MM-1γ, but not MM-1β, were found to repress transcription activity of c-Myc, and the degrees of repression by MM-1γ and MM-1δ were smaller than those by MM-1 and MM-1α. These results suggest that each MM-1 isoform distinctly regulates c-Myc transcription activity in respective tissues. J. Cell. Biochem. 97: 145–155, 2006.


**Key words:** MM-1; c-Myc; transrepression; splicing isoform

## INTRODUCTION

The proto-oncogene product c-Myc as a transcription factor plays pivotal roles in cell proliferation, differentiation, and apoptosis induction, and its levels are tightly regulated at several steps, including transcription, translation, and protein stability [Dang, 1999; Obaya et al., 1999; Prendergast, 1999]. c-Myc contains two regions at the N-terminus and C-terminus, which are necessary for c-Myc to exert its full activities. Numbers of proteins that bind to either domain of c-Myc have been reported to modulate functions of c-Myc (see the homepage http://www.myc-cancer-gene.org/index.asp). We have reported that MM-1, a novel protein binding to the myc box II located in the N-proximal region of c-Myc, suppressed transcription and transformation activities of c-Myc and that A157R mutation of c-Myc, which is observed at high frequency in patients with lymphoma or leukemia, abrogated all of the functions of MM-1 toward c-Myc, indicating that MM-1 is a novel tumor suppressor [Mori et al., 1998; Fujioka et al., 2001]. As for the MM-1-dependent transrepression pathway of c-Myc, we have shown that MM-1 recruited HDAC complex to c-Myc via TIF1β, a corepressor [Satou et al., 2001]. We then identified the c-*fms* gene as a target gene to be repressed by the c-Myc-MM-1-TIF1β, complex, and abrogation of this pathway rendered the c-*fms* gene oncogenic

Abbreviations used: HDAC, histone deacetylase

Sequence data from this article have been deposited with the DDBJ/EMBL/GenBank Data Libraries under accession nos. AB055803, AB055804 and AB055805.

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in fibroblast cells [Satou et al., 2004]. MM-1 has been reported to bind to p73, a member of the p53 family of proteins, to modulate its function [Watanabe et al., 2002] and to be a component of the PFD/Gim complex, a chaperone that carries unfolded proteins to a chaperonin [Geissler et al., 1998; Vainberg et al., 1998]. In this study, we first identified that originally isolated MM-1 is a fusion protein derived from sequences of chromosomes 12 and 14. We then identified four splicing isoforms of MM-1 from sequences of chromosomes 12. The MM-1 isoforms were found to be distinctly expressed in tissues and to possess different degrees of repression activity toward c-Myc.

## MATERIALS AND METHODS

### Cells

Human 293T and HeLa cells were cultured in Dulbecco's modified Eagle's medium with 10% calf serum.

### Construction of Plasmids

Nucleotide sequences of the oligonucleotide used for PCR primers were as follows: MM-1Δ13 (EcoRI), 5′-GGGAATTCATGGCGCAGTCTATTAAC-3′; MM-1END (XhoI), 5′-GGCTCGAGCGGCCTTAGCAGTAGCCTG-3′. EST clones of accession numbers H03166 (Image clone 151640), AA287397 (Image clone 701099), and AK024094 (clone HEMBA004353), which correspond to cDNAs of MM-1β, -γ, and -δ, respectively, were obtained from Incyte Genome and Helix Research Institute. The regions of cDNAs of MM-1 isoforms from the ATG codon to the stop codon were amplified by PCR using pcDNA3-FLAG-MM-1 [Mori et al., 1998] or corresponding EST clones as templates with MM-1Δ13 (EcoRI) and MM-1END (XhoI) as primers. The resultant PCR products were digested with *Eco*RI and *Xho*I and inserted into *Eco*RI-*Xho*I sites of pcDNA3-FLAG, pEGFP-C1, or pCMV-GAL4. Other plasmids used in this study were described previously [Mori et al., 1998; Fujioka et al., 2001].

### Indirect Immunofluorescence

HeLa cells were transfected with 5 μg of pGFP-MM-1 and its splicing variants by the calcium phosphate precipitation method [Graham and Vander, 1973]. Forty-eight hours after transfection, cells were fixed with a solution containing 4% paraformaldehyde and reacted with a rabbit anti-c-Myc polyclonal antibody (N262, Santa Cruz). The cells were then reacted with a rhodamine-conjugated anti-rabbit IgG and observed under a confocal laser fluorescent microscope.

### Luciferase Assay

HeLa cells in a 6-cm dish were transfected with 0.5 μg of pCMV-β-gal, 0.5 μg of pEF-c-myc, and various amounts of pGFP-MM-1 or its isoforms together with 2 μg of p4xE-SVP-Luc by the calcium phosphate method [Graham and Vander, 1973]. Two days after transfection, whole cell extract was prepared by addition of the Triton X-100-containing solution from the Pica gene kit (Wako Pure Chemicals) to the cells. About a one-fifth volume of the extract was used for the β-galactosidase assay to normalize the transfection efficiencies, as described previously [Mori et al., 1998], and the luciferase activity due to the reporter plasmid was determined using a luminometer, Luminocounter Lumat LB 9507 (EG & G Berthold). The same experiments were repeated three times.

### RT-PCR

Nucleotide sequences of the oligonucleotide used for PCR primers were as follows: MM-1WT-S: 5′-GGTTGATGTCATGACTGTAG-3′; MM-1WT-AS: 5′-CTCTTGAAGAAGTCCTTGGC-3′; MM-1α-S: 5′-GAATTACTCGTCCCACTGAC-3′; MM-1α-AS: 5′-GCCCAGCTGGCACATTTATTGGCATTA-3′; MM-1β-S: 5′-GTGGAGTTCTTGTCCACGTCCATTG-3′; MM-1β-S2: 5′-GAACAAGAGCAACGAGGATGT-3′; MM-1γ-S2: 5′-GAACCAGCTGGACCAGATGTA-3′; MM-1δ-AS: 5′-CGCGTACCTAAAGTCCCAGCTA-3′; GAPDH up-S: 5′-GAAATCCCATCACCATCTTCCAGG-3′; GAPDH low-AS: 5′-CAGTAGAGGCAGGGATGATGTTC-3′. RT-PCRs to amplify cDNAs of human MM-1 and its splicing isoforms or human *GAPDH* were carried out using total RNA from human tissues (QIAGEN) with the above oligonucleotides as templates and primers, respectively. Conditions used for PCR were one cycle at 94°C for 5 min, 30–32 cycles at 94°C for 1 min, 60°C for 2 min, and 77°C for 3 min. PCR products were run on an agarose gel containing ethidium bromide and visualized under UV-light.

### Real-Time RT-PCR

Nucleotide sequences of the oligonucleotide used for Real-time RT-PCR primers were as

follows: MM-1WT-S: 5′-GGTTGATGTCATG-ACTGTAG-3′; MM-1WT-AS-SYBR: 5′-GAGCT-GAGCAATGGACGTGG-3′; ACTB-S: 5′-CCTG-GCATTGCCGACAGGAT-3′; ACTB-AS: 5′-CA-CACGGAGTACTTGCGCTC-3′. PCR fragments amplified using plasmid DNAs containing MM-1 cDNA and actin with the above oligonucleotides as templates and primers, respectively, were first inserted into pCR2.1-TOPO (Invitrogen) and 0.02–200 pg of them were used for making calibration curve. Real-time RT-PCRs to amplify cDNAs of human MM-1 were carried out using total RNA from human normal colon and colon cancer tissues (CLONTECH) with the above oligonucleotides as templates and primers, respectively. Reactions were carried out using Takara One Step SYBR RT-PCR Kit (Takara) and ABI PRISM 7700 under the conditions of one cycle at 42°C for 15 min, 95°C for 2 min, 40 cycles at 95°C for 5 s, 60°C for 1 min, and 95°C for 15 s.

### In Vivo Binding Assay

Two μg of pGFP-MM-1 or its mutants together with 2 μg of pcDNA3-Flag-c-Myc were transfected into human 293T cells 60% confluent in a 10-cm dish by the calcium phosphate precipitation technique [Graham and Vander, 1973]. Forty-eight hours after transfection, the whole cell extract was prepared by the procedure described previously [Mori et al., 1998; Fujioka et al., 2001]. Approximately 2 mg of 293T cell proteins was first immunoprecipitated with a mouse anti-FLAG antibody (M2, Sigma) or with non-specific mouse IgG under the same conditions as those described previously [Mori et al., 1998; Fujioka et al., 2001]. After washing with the same buffer, the precipitates were separated in a 12.5% polyacrylamide gel containing SDS, blotted onto a nitrocellulose filter, and reacted with a rabbit anti-GFP antibody (MBL) and with the mouse anti-FLAG antibody.

## RESULTS

### Identification of a Fusion Type of MM-1 in Human Cells

We previously reported that originally isolated cDNA of MM-1 from a human HeLa cDNA library is likely to be a fusion gene derived from the sequences of chromosomes 14 and 12 [Fujioka et al., 2001] (Fig. 1A). To confirm that mRNA corresponding to MM-1 cDNA is present in human cells, RT-PCR was carried out using sense and antisense primers whose sequences are derived from those of chromosomes 14 and 12 in MM-1 cDNA, respectively, and total RNAs from human tissues (Fig. 1B). To further verify the presence of MM-1 mRNA in cells, real-time RT-PCR was carried out using normal colon and colon cancer tissues (Fig. 1C). Reactions without RNA, reverse transcriptase or Taq DNA polymerase produced no cDNAs (Fig. 1C, lanes, 1–5). The results showed that MM-1 cDNAs were amplified from both normal colon and colon cancer tissues and that their expression levels were significantly not changed (Fig. 1C, lanes, 6 and 7). The results showed that mRNA corresponding to the fusion type of MM-1 was actually present and that this type of MM-1 was ubiquitously expressed in all of the tissues examined.

### Identification of Splicing Isoforms of MM-1

After a search for human EST clones that contain sequences homologous to that of MM-1, we identified four clones, whose clone numbers are Image 310729, Image 151640, Image 701099, and HEMBA004353. After nucleotide sequences of these clones, except for HEMBA004353, had been determined, we deposited the sequences of these cDNAs into the DDBJ/EMBL/GenBank database as MM-1α, MM-1β, and MM-1γ (accession numbers AB055803, AB055804, and AB055805), respectively. Since complete nucleotide sequences of HEMBA004353 clone (accession number, AK024094) had already been determined, we named this cDNA MM-1δ. The cDNAs for MM-1α, MM-1β, MM-1γ, and MM-1δ contain 1,029, 599,600, and 1,983 nucleotides and encode 154, 66, 109, and 94 amino acids, respectively (Fig. 2A). Sequences of these clones, MM-1α, MM-1β, MM-1γ, and MM-1δ, were compared to those of the human genome, and organization of the structure of the *MM-1* gene was clarified (Fig. 2A). The *MM-1* gene comprises six exons. Alignment showed that MM-1α cDNA is MM-1 cDNA lacking a nucleotide sequence derived from chromosome 14 and that MM-1β lacks exon 3 and MM-1γ lacks exons 2 and 3. MM-1δ was found to use a different frame on exon 4 from that of MM-1α, MM-1β, and MM-1γ, thereby encoding a small-sized protein compared to long-sized mRNA (Fig. 2B; Table I).

The expressions of MM-1α, MM-1β, MM-1γ, and MM-1δ mRNAs were examined by RT-PCR using various RNAs extracted from human

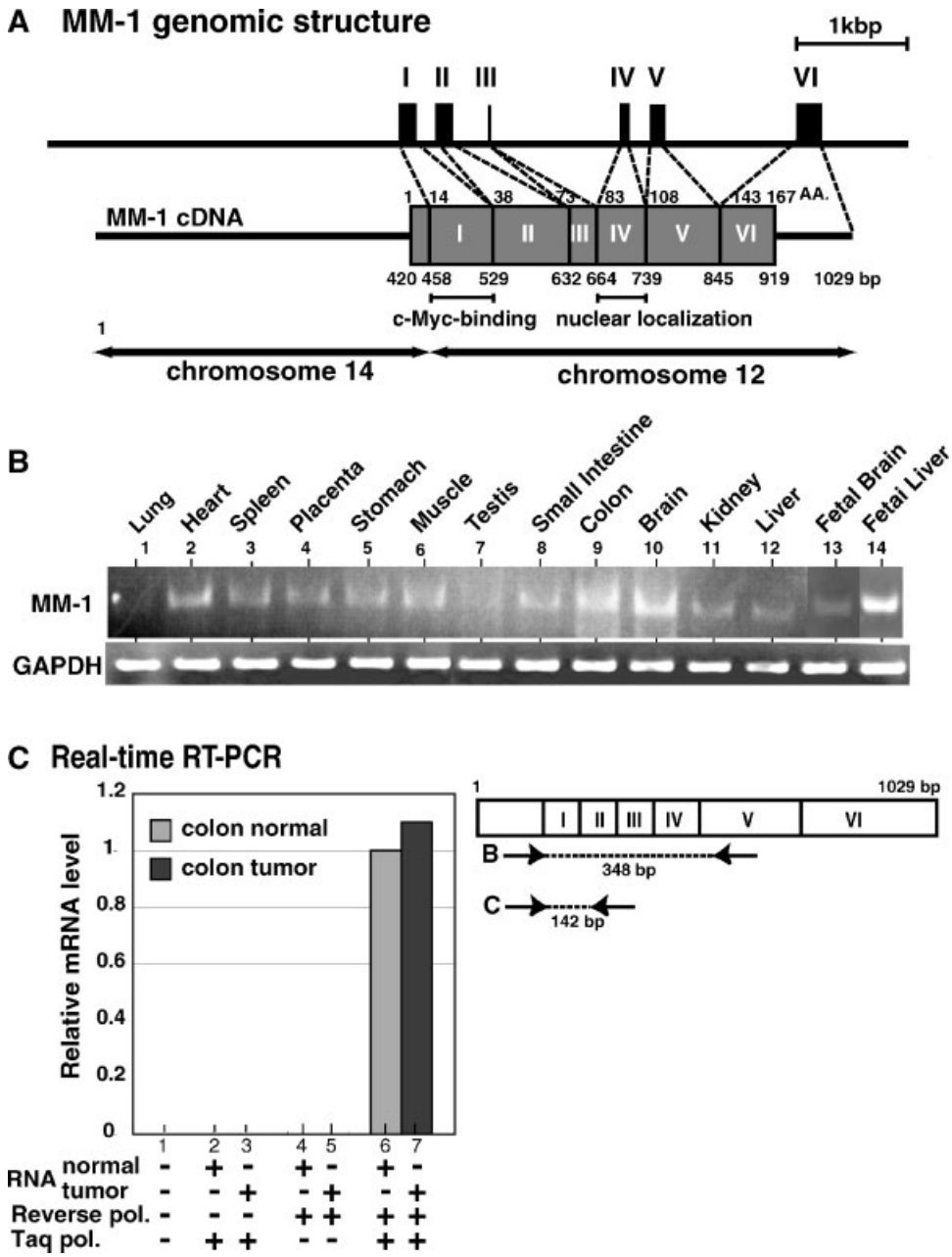


**Fig. 1.** Organization and expression of the human *MM-1* fusion gene. **A**: Physical map of the human *MM-1* fusion gene is shown. Exons are represented as squares and the numbers above the boxes representing genomic DNA indicate the exon numbers for the *MM-1* fusion gene transcripts. **B**: Human total RNAs were obtained from QIAGEN, and the expression of mRNA of the *MM-1* fusion gene was analyzed by RT-PCR with specific primers for the *MM-1* fusion gene on total RNA as substrates. The amplified DNAs were separated in 1.4% agarose gels and visualized under UV-light. Nucleotide numbers of DNA to be amplified by RT-PCR are also shown. **C**: Real-time RT-PCR was carried out using total RNAs from normal colon and colon cancer tissues and specific primers as described in Materials and Methods, and their relative expression levels are shown. Positions of primers used for PCR of B and C are indicated in the right of the figure.

tissues (Fig. 2C). The results showed that MM-1β was expressed ubiquitously in all of the tissues tested except for in the small intestine. MM-1α was found to be also expressed ubiquitously (data not shown) but to be strongly expressed in the muscle, fetal brain, and fetal liver after short cycles of PCR, MM-1γ was found to be expressed strongly in the fetal brain and fetal liver, and MM-1δ was found to be expressed strongly in the fetal brain, fetal liver, and small intestine (Fig. 2C). The expression pattern of MM-1 is therefore different from

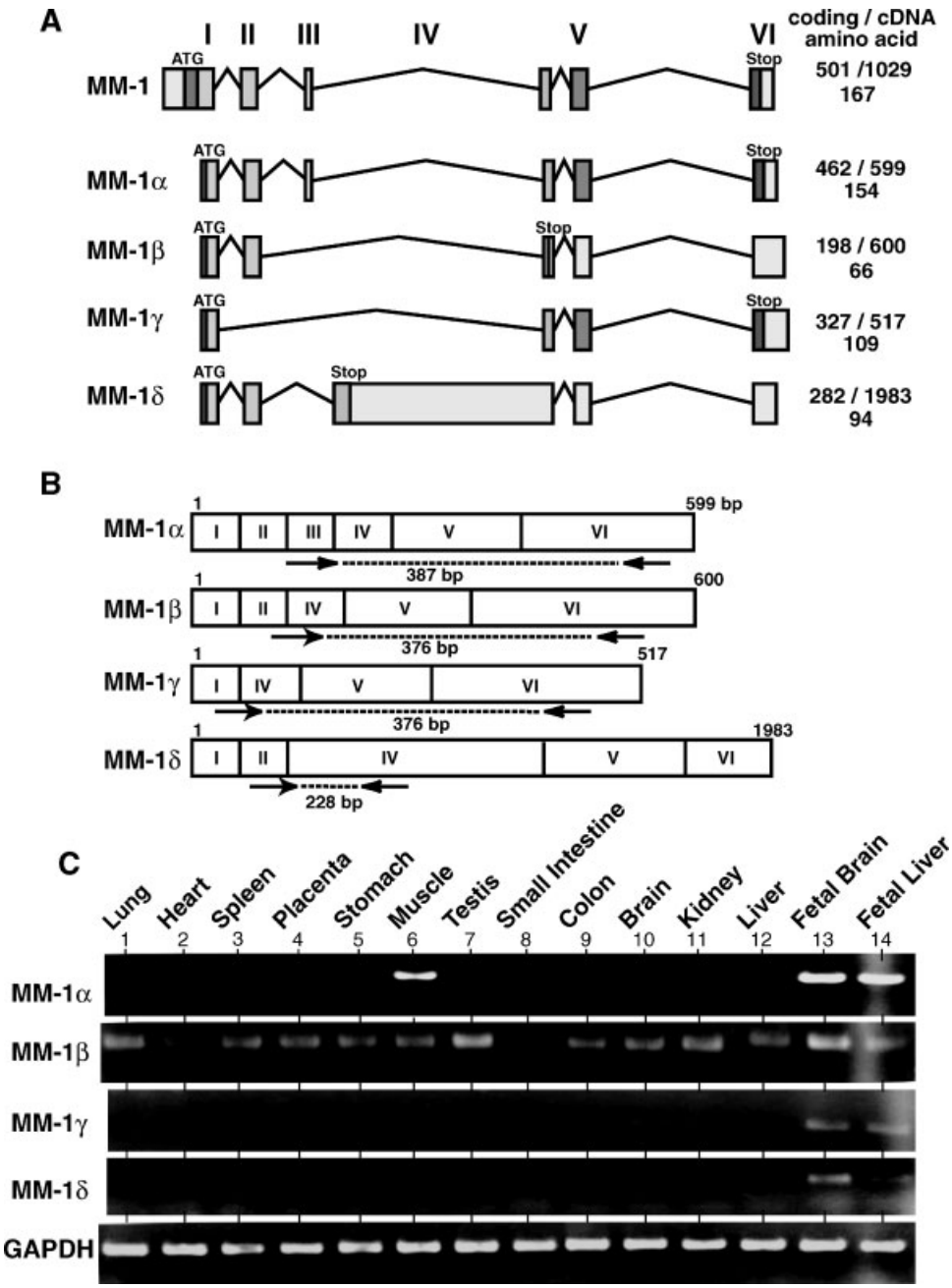


**Fig. 2.** Organization and expression of human MM-1 isoforms. **A**: Physical maps of exon and intron structures of human MM-1 isoforms are shown. Exons are represented as squares, in which the regions coding for proteins are represented as black or dark boxes, and the numbers above the boxes representing genomic DNA indicate the exon numbers for transcripts of the *MM-1* genes. **B**: Compositions of exons for MM-1 isoforms and positions of specific primers used for RT-PCR are shown. Nucleotide numbers of DNA to be amplified by RT-PCR are also shown. **C**: The expressions of mRNAs of *MM-1* isoforms were analyzed by RT-PCR with specific primers for the *MM-1* isoforms gene on total RNA as substrates. The amplified DNAs were separated in 1.4% agarose gels and visualized under UV-light.

those of MM-1γ and -1δ and is similar to those of MM-1α and MM-1β.

### Binding Activities of MM-1 Isoforms to c-Myc

To observe the complex formation of MM-1 splicing isoforms with c-Myc in vivo, expression vectors for GFP-tagged MM-1α, -1β, -1γ, and -1δ together with or without Flag-tagged c-Myc were transfected into human 293T cells. Forty-eight hours after transfection, cell extract was prepared and the proteins in the extract were first immunoprecipitated with an anti-FLAG

**TABLE I. Organization of the Human *MM-1* Gene**

| Exon | Acceptor | Length (bp) | Donner | Amino acid position | Intron | Length (bp) |
|---|---|---|---|---|---|---|
| I | — | 72 | CTG GAC CAG gtggggacgg<br>D Q E | 1–24 | 1 | 201 |
| II | tcctctgtag GAA GTG GAG<br>V E F | 104 | CAA CGA GG gtatgggtag<br>N E G | 25–59 | 2 | 301 |
| III | gctttcacag G GAA AGA<br>K E | 31 | ACG AGT TCT gtatcctttc<br>T S S | 60–69 | 3 | 1,577 |
| IV | tgcttctcag ATG TAT GTC<br>M Y V | 75 | TGT AGA GAA G gtgagtgaga<br>V E K | 70–94 | 4 | 121 |
| V | gatcttgtag AC AGC TGA<br>T A E | 106 | ATG AAA CAG G gtaagttttt<br>M K Q | 95–130 | 5 | 1,122 |
| VI | ttttccacag CC GTC ATG<br>A V M | 183 | — | 131–154 | 5 | 1,122 |
| Exon IV for MM-1δ | | | | | | |
| IV | ttttttgag A CGG AAT<br>R N | 1,418 | -gtgagtgaga<br>— | 60–94 | | |

Sequences of the exon–intron junctions of the human *MM-1* gene are shown. The intronic sequence is given in base pairs. Enclosed amino acids are indicated below the first base of the corresponding codon by single-letter codes.

antibody. The anti-FLAG antibody precipitated Flag-c-Myc (Fig. 3A, middle panel). GFP-MM-1 isoforms, on the other hand, were detected by Western blot analysis in the immunoprecipitates from FLAG-tagged c-Myc-transfected cells, but not from non-transfected cells, with an anti-GFP antibody (Fig. 3A, upper panel). Gal4 DNA-binding region (Gal4BD)-tagged MM-1 isoforms were then cotransfected with Flag-tagged c-Myc into 293T cells. The proteins in the extract were first immunoprecipitated with the anti-FLAG antibody or non-specific IgG and analyzed by Western blotting with an anti-Gal4BD antibody (Fig. 3B). The results again showed that all of the MM-1 isoforms bound to Flag-c-Myc. These results indicate that MM-1α, MM-1β, MM-1γ, or MM-1δ is associated with c-Myc in ectopic expressed 293T cells. In vitro binding assays using $^{35}$S-labeled c-Myc that had been synthesized in a reticulocyte lysate and GST-MM-1 isoforms that had been expressed in and purified from *E. coli* were also carried out. The results showed that all of the GST-MM-1 isoforms bound to c-Myc, suggesting that MM-1α, MM-1β, MM-1γ, and MM-1δ directly bind to c-Myc (data not shown).

### Cellular Localizations of MM-1 Isoforms

To determine the cellular localizations of MM-1α, MM-1β, MM-1γ, and MM-1δ, human HeLa cells were transfected with expression vectors for GFP-tagged MM-1α, MM-1β, MM-1γ, or MM-1δ alone or together with c-Myc. GFP-MM-1 was also transfected into HeLa cells. Forty-eight hours after transfection, HeLa cells were stained with an anti-c-Myc antibody, and c-Myc and GFP-MM isoforms were detected by a rhodamine-conjugated second antibody and by self-fluorescence, respectively. These were then visualized under a confocal laser microscope (Fig. 4). When HeLa cells were transfected with GFP-MM-1, -MM-1α, -MM-1β, -MM-1γ or -MM-1δ alone, MM-1, MM-1α and MM-1γ were found to be localized strongly in the nucleus and weakly in the cytoplasm, and MM-1β and MM-1δ were found to be localized strongly in the cytoplasm and faintly in the nucleus (Fig. 4A). In HeLa cells cotransfected with GFP-MM-1, -MM-1α, -MM-1β, -MM-1γ, or -MM-1δ and c-Myc, all of the MM-1 isoforms were found to be still localized in the nucleus or cytoplasm as observed in cells transfected with MM-1 isoforms alone (Fig. 4B). It was also found that MM-1, MM-1α, and MM-1γ were strongly colocalized with c-Myc in the nucleus as shown by the yellow color but that MM-1β and MM-1δ were marginally or hardly colocalized with c-Myc in the nucleus (Fig. 4B).

We have shown that MM-1 binds to and is colocalized with TIF1β in the nucleus to recruit HDAC complex to c-Myc [Satou et al., 2001]. We therefore examined binding activities of MM-1 isoforms to TIF1β. To do this, expression vectors for GFP-tagged MM-1α, -MM-1β, -MM-1γ, and -MM-1δ together with Flag-tagged TIF1β were transfected into human 293T cells. Forty-eight hours after transfection, cell extract was prepared and the proteins in the extract were first immunoprecipitated with an anti-FLAG

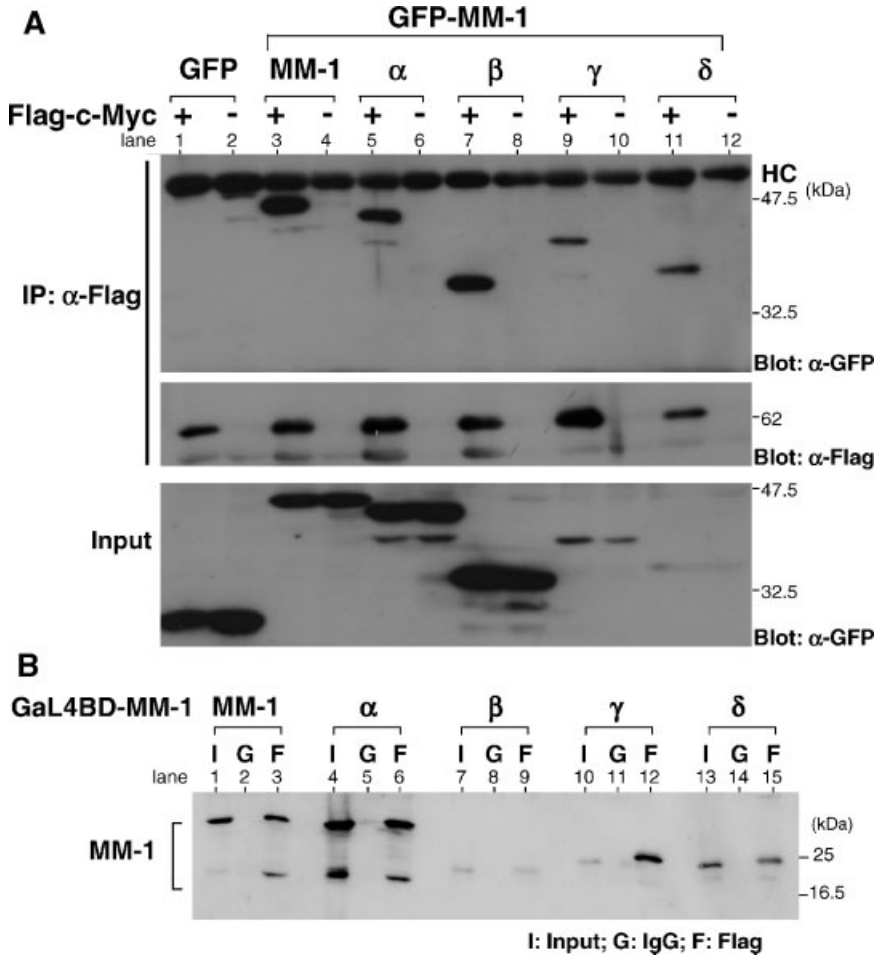


**Fig. 3.** Association of MM-1 isoforms with c-Myc in human cells. **A**: Expression vectors for GFP-MM-1α, -MM-1β,-MM-1γ, or -MM-1δ along with or without Flag-c-Myc were introduced into human 293T cells by the calcium phosphate precipitation technique [Graham and Vander, 1973]. Two days after transfection, cell extracts were prepared, and the proteins in the extracts were first immunoprecipitated (IP) with an anti-FLAG monoclonal antibody (M2, Sigma). The proteins in the precipitates were separated in a 10% or 12.5% polyacrylamide gel and analyzed by Western blotting with the anti-FLAG antibody (Fig. 3A, middle panel) or an anti-GFP polyclonal antibody (MBL) (Fig. 3, upper panel). The proteins used for immunoprecipitation were also analyzed by Western blotting with the anti-GFP antibody (Input, Fig. 3A, lower part). **B**: Expression vectors for GAL4BD-MM-1α, -MM-1β, -MM-1γ, or -MM-1δ along with Flag-c-Myc were introduced into human 293T cells by the calcium phosphate precipitation technique [Graham and Vander, 1973]. Two days after transfection, cell extracts were prepared, and the proteins in the extracts were first immunoprecipitated (IP) with an anti-FLAG monoclonal antibody or non-specific IgG. The proteins in the precipitates were separated in a 12.5% polyacrylamide gel and analyzed by Western blotting with an anti-GAL4BD antibody (N-19, Santa Cruz).

antibody or non-specific IgG. The anti-FLAG antibody precipitated Flag TIF1β (Fig. 5A, lower panel). The results showed that all of the MM-1 isoforms bound to Flag-TIF1β (Fig. 5A, upper panel).

Colocalization of MM-1 isoforms with TIF1β were then examined in HeLa cells, into which expression vectors for GFP-tagged MM-1 isoforms were transfected with Flag-tagged TIF1β. The results showed that, as in the case of c-Myc and MM-1 isoforms, MM-1, MM-1α, and MM-1γ were strongly colocalized with TIIF1β in the nucleus but that MM-1β and MM-1δ were marginally or hardly colocalized with TIF1β in HeLa cells (Fig. 5B).

### Repression Activities of MM-1 Isoforms Toward c-Myc

MM-1 represses transcription activity of c-Myc through the TIF1β-HDAC complex [Satou et al., 2001]. To verify the effect of MM-1 isoforms on the transcription activity of c-Myc, human HeLa cells were transfected with expression vectors for c-Myc and each MM-1

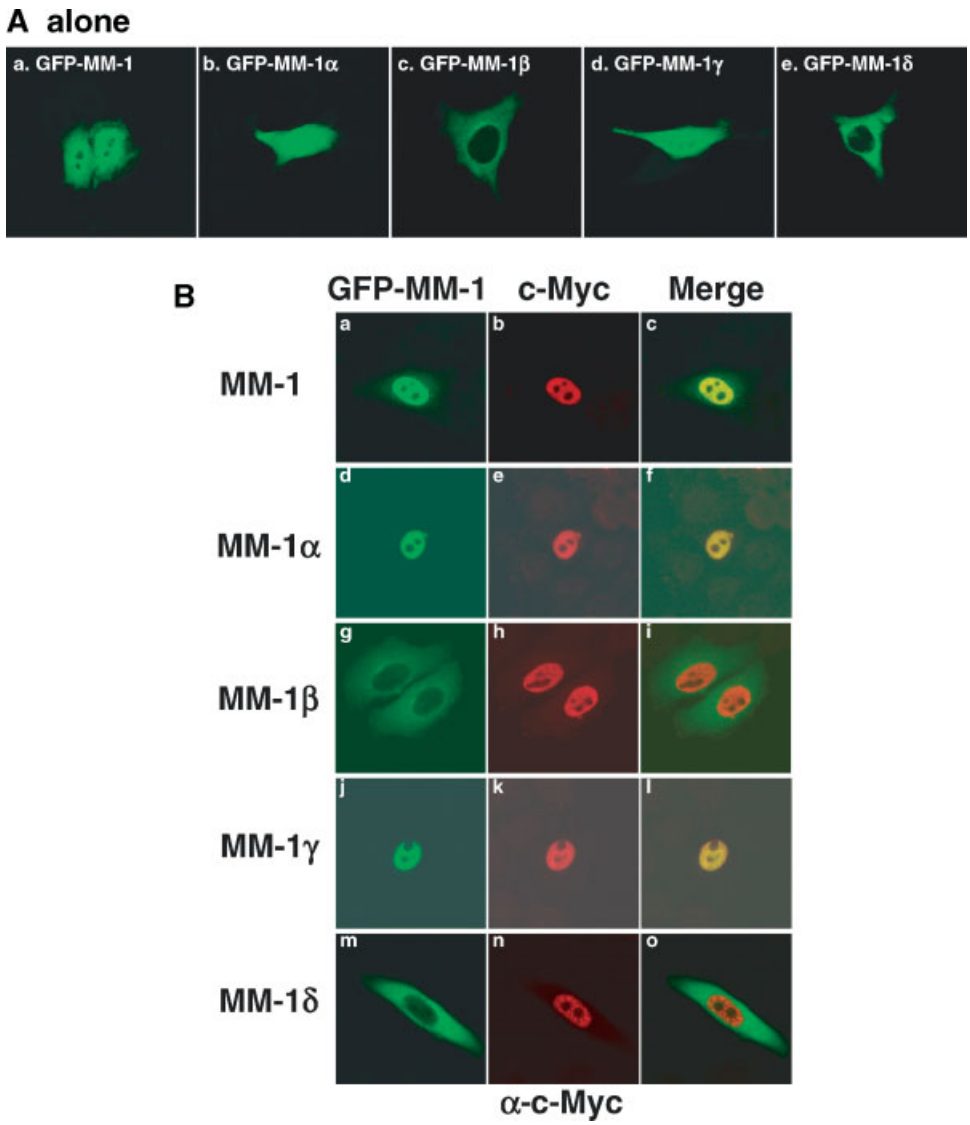


**Fig. 4.** Localization of MM-1 isoforms in human cells. **A**: Expression vectors for GFP-MM-1 isoforms were transfected into human HeLa cells by the calcium phosphate precipitation technique [Graham and Vander, 1973]. Two days after transfection, the cells were fixed, visualized by self-fluorescence of GFP, and observed under a confocal laser fluorescent microscope. **B**: Expression vectors for GFP- MM-1α, -MM-1β, -MM-1γ, or -MM-1δ were cotransfected with c-Myc. The cells were reacted with an anti-c-Myc polyclonal antibody and visualized with a rhodamine-conjugated anti-rabbit antibody and by self-fluorescence of GFP as described in A. The two figures were merged (Merge).

isoform together with the luciferase gene linked to the tetramerized E-box sequence (4xE) followed by the SV40 promoter. When HeLa cells were co-transfected with various amounts of pCMV-GFP-MM-1 isoforms and 0.5 μg of pEF-c-myc, the luciferase activities enhanced by c-Myc were strongly repressed by co-introduced pCMV-GFP-MM-1 or pCMV-GFP-MM-1α in a dose-dependent manner (Fig. 6). Weak repression of c-Myc transcription activities was also observed in cells transfected with MM-1γ and MM-1δ, while no repression activity was found in cells transfected with MM-1β (Fig. 6). The vector pCMV-GFP alone showed no effect on either the activity enhanced by pEF-c-myc or the basal activity in the presence of pEF as described previously [Mori et al., 1998] (data not shown). The results suggest that repression activities of MM-1 isoforms, except for MM-1δ, toward c-Myc are consistent with activities of colocalization with c-Myc in the nucleus.

## DISCUSSION

In this study, we first identified that originally isolated MM-1 is a fusion gene derived from

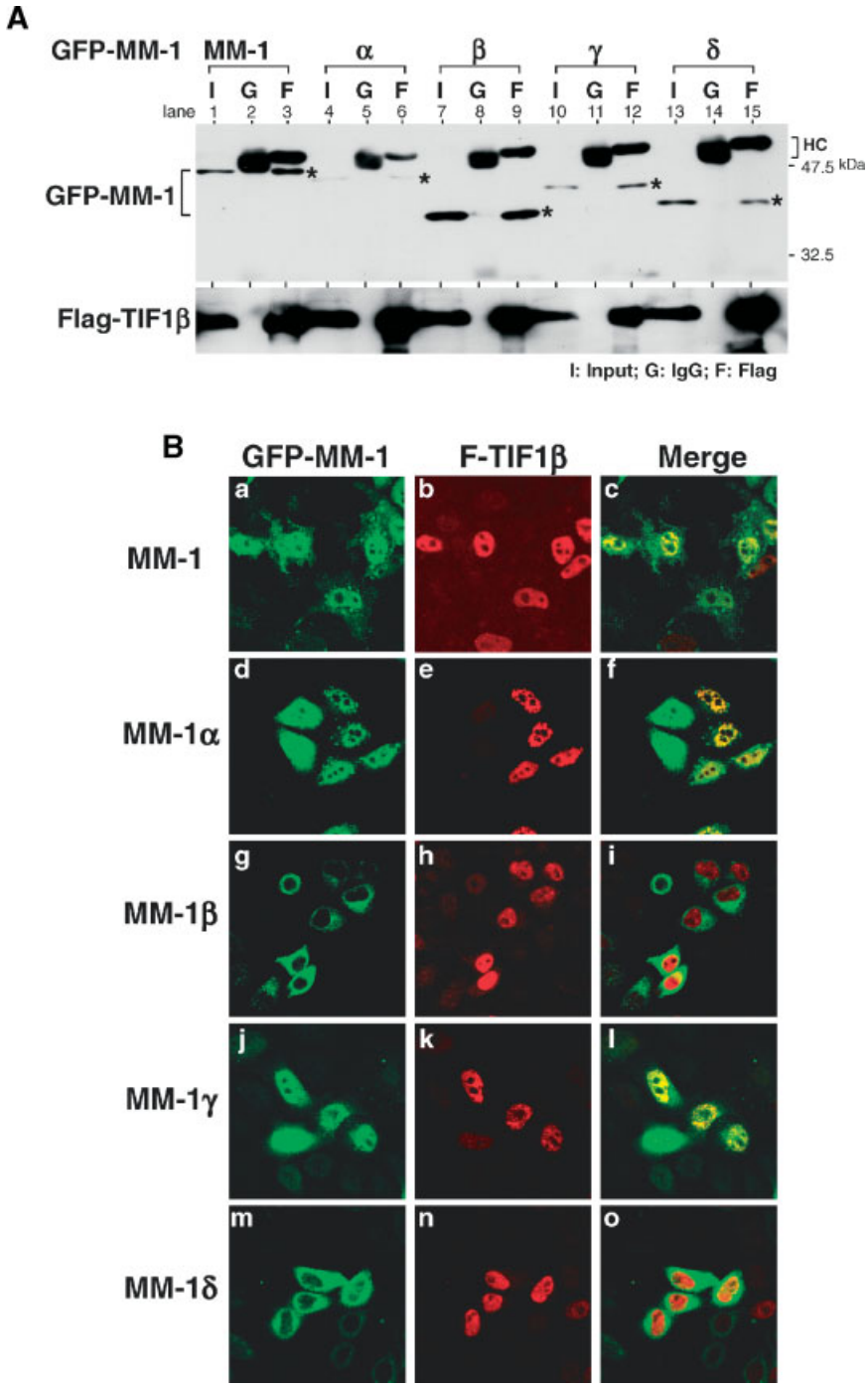


**Fig. 5.** Association and colocalization of MM-1 isoforms with TIF1β. **A**: Expression vectors for GFP- MM-1α, -MM-1β, -MM-1γ, or -MM-1δ along with Flag-TIF1β were introduced into human 293T cells by the calcium phosphate precipitation technique [Graham and Vander, 1973]. Two days after transfection, cell extracts were prepared, and the proteins in the extracts were first immunoprecipitated with an anti-FLAG monoclonal antibody (F) or non-specific IgG (G). The proteins in the precipitates were separated in a 12.5% polyacrylamide gel and analyzed by Western blotting with an anti-GFP antibody or the anti-Flag antibody (''GFP-MM-1'' and ''Flag-TIF1β'' in upper and lower parts, respectively). **B**: Expression vectors for GFP-MM-1α, -MM-1β, -MM-1γ, or -MM-1δ were cotransfected with Flag-TIF1β. The cells were reacted with an anti-Flag monoclonal antibody and visualized with a rhodamine-conjugated anti-rabbit antibody and by a self-fluorescence of GFP as described in the legend to Figure 4A. The two figures were merged (Merge).

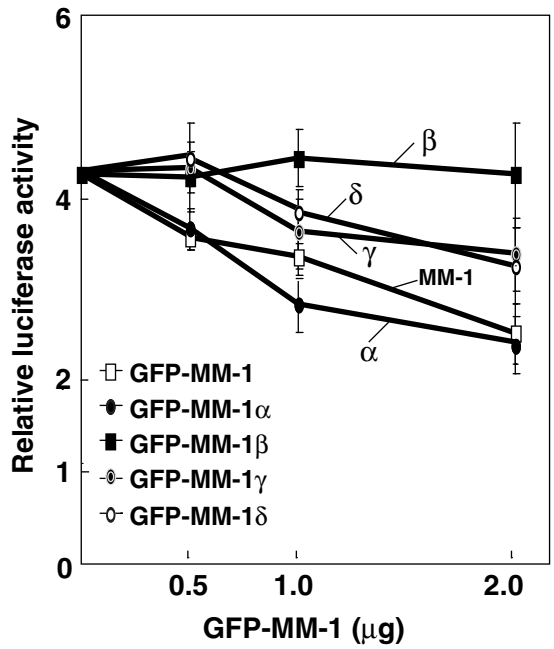


**Fig. 6.** Effect of MM-1 isoforms on transcription activity of c-Myc. HeLa cells were transfected with 0.5 μg of pCMV-β-gal, 0.5 μg of pEF-c-myc, and various amounts of pGFP-MM-1 or its isoforms together with 2 μg of p4xE-SVP-Luc by the calcium phosphate precipitation method [Graham and Vander, 1973]. Forty-eight hours after transfection, cell extracts were prepared and their luciferase activities were examined as described in Materials and Methods. Luciferase activity of cells transfected with pSVP-Luc, instead of p4xE-SVP-Luc, was set to 1. Experiments were carried out three times.

the sequences of chromosomes 14 and 12. Thirteen amino acids from chromosome 14 were found to be fused to MM-1α from chromosome 12, in which the *MM*-1 gene is located. Since mRNA of this fusion gene was found to be ubiquitously expressed in all of the human tissues examined, cDNA of MM-1 isolated from a HeLa cDNA library was concluded not to be an artifact during preparation of the cDNA library. Indeed, another group also isolated MM-1 cDNA by RT-PCR from HeLa cells [Watanabe et al., 2002]. Other tumor suppressers such as PML [de The et al., 1991; Kakizuka et al., 1991] or PLZF [Chen et al., 1993a,b, 1994] have been reported to be fused with the retinoic acid receptor, resulting in these proteins being tumorigenic. Activity of a fusion type of MM-1, therefore, might be different to that of the non-fusion type of MM-1, though expression levels of MM-1 mRNAs in normal colon and colon cancer tissues were significantly not changed. Although the reason for occurrence of and the significance of the fusion gene are not known, investigation of functional significance of this fusion gene might be important. We then identified novel MM-1 isoforms, MM-1α, MM-1β, MM-1γ, and MM-1δ, and we determined their expression profiles, cellular localizations and repression activities toward c-Myc. The results showed that all of the MM-1 isoforms possess potential binding activities to both c-Myc and TIF1β in in vivo cultured cells and are localized in fetal brain, and liver. Whereas MM1β was found to be ubiquitously expressed, except for in the small intestine, distinct expressions patterns of MM-1α, MM-1γ, and MM-1δ were observed. Regarding cellular localization, MM-1 isoforms are classified two groups: MM-1, MM-1α, and MM-1γ, isoforms, which are localized predominantly in the nucleus, and MM-1β and MM-1δ, which are localized predominantly in the cytoplasm. Since MM-1, MM-1α, and MM-1γ possess intact exon IV, exon IV is thought to determine the nuclear localization of MM-1 isoforms. It is interesting that MM-1δ is strongly expressed in the small intestine, in which MM-1β is not expressed, suggesting that compensation of function occurs between MM-1β and MM-1δ. In contrast to the findings of localization of MM-1 isoforms, all of the isoforms bound to c-Myc and TIF1β, both of which were localized in the nucleus. This discrepancy might be explained as follows. Firstly, small amounts of nucleus-localized MM-1β and MM-1δ bound to c-Myc and TIF1β. Secondly, since total cellular extracts were used in the binding reactions, the cytoplasm-localized MM-1β and MM-1δ bound to nuclear localized c-Myc and TIF1β during incubation. Since all of the MM-1 isoforms contain exon 1, the results of binding experiments in the present study are consistent with results of a previous study showing that the N-proximal region of MM-1 is necessary for MM-1 to bind to c-Myc [Mori et al., 1998].

Of the MM-1 isoforms, MM-1β did not repress transcription activity of c-Myc, a finding that is consistent with the findings of different cellular localizations of MM-1β and c-Myc or TIF1β. Although MM-1δ and MM-1γ, which are predominantly localized in the cytoplasm and nucleus, respectively, weakly repressed transcription activity of c-Myc, the degree of repression activity of MM-1δ toward c-Myc transcription was smaller than that of MM-1γ, suggesting that their different localizations

affect repression activity. Northern blot and Western blot analyses showed that MM-1α is a predominant form of the MM-1 isoforms expressed in cells as described previously [Mori et al., 1998 and data not shown]. Since all of the isoforms of MM-1 contain exon I, which encodes the c-Myc-binding domain, MM-1β, possibly MM-1δ, and MM-1γ, are thought to act as dominant-negative forms of MM-1α or MM-1 in repression activity of c-Myc. It would be interesting to clarify functional differences among MM-1 isoforms during developmental stages and in different tissues.

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