Repression of the c-*fms* gene in fibroblast cells by c-Myc-MM-1-TIF1β complex

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Received 8 June 2004; revised 25 June 2004; accepted 6 July 2004

Available online 26 July 2004

Edited by Lukas Huber

Abstract MM-1 has been reported to repress the E-box-dependent transcription activity of c-Myc by recruiting histone deacetylase 1 complex via TIF1β/KAP1. In this study, to identify target genes for c-Myc-MM-1-TIF1β, we established rat-1 cells harboring the dominant-negative form of TIF1β to abrogate the pathway from TIF1β to MM-1-c-Myc. This cell line, in which transcription activity of c-Myc was activated, was found to be tumorigenic. By DNA-microarray analysis of this cell line, expression and promoter activity of the c-*fms* oncogene were found to be upregulated. Of the two promoters, pE1 and pE2, in the c-*fms* gene, pE1 promoter activity was found to be activated in an E-box-dependent manner.

Keywords: c-Myc; MM-1; Transformation; c-*fms*; Transrepression

1. Introduction

The proto-oncogene product c-Myc 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 [1–3]. 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 [4,5]. As for the MM-1-dependent transrepression pathway of c-Myc, we have shown that MM-1 recruited histone deacetylase (HDAC) complex to c-Myc via TIF1β, a corepressor [6]. MM-1 has been reported to bind to p73, a member of the p53 family of proteins, to modulate its function [7], and to be a component of the PFD/Gim complex, a chaperon that carries unfolded proteins to a shaperonin [8,9]. In this study, to identify genes that are regulated by the c-Myc-MM-1-TIF1β complex, we established a fibroblast cell line harboring a dominant-negative form of TIF1β to abrogate this complex. The results showed that the c-*fms* oncogene is a target gene for c-Myc-MM-1-TIF1β to be suppressed in fibroblast cell lines.

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Abbreviations: HDAC1, histone deacetylase 1; HA, hemagglutinin antigen; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; M-CSF-1, macrophage colony stimulating factor; 4-OHT, 4-hydroxyl tamoxifen

2. Materials and methods

2.1. Establishment of cell lines

Rat Myc-ER cells cultured in Dulbecco's modified Eagle's medium with 10% calf serum in the presence of 2.5 μg/ml of puromycin were transfected with pEFTIF1βΔN-HA, which expresses the C-terminal region of TIF1β (TIF1βΔN), possessing the region spanning amino acid numbers 470–835 [6], or with pEF-HA together with pSV2-bsr, an expression vector for blasticidin S, and the cells were cultured in the medium in the presence of 8 μg/ml of blasticidin S for 14 days. The cells that were resistant to the drug were then selected and expression of the C-terminal region of TIF1β was examined by Western blotting with an anti-hemagglutinin antigen (HA) antibody.

2.2. Luciferase activity

Reporter genes for the human c-*fms* gene promoters, pE1-Luc and pE2-Luc [10], were kindly provided by E. Sapi. Myc-ER cell lines harboring TIF1βΔN subconfluent in a 6-cm dish were transfected with 1 μg of p4xE-SVP-Luc [4], pE1-Luc or pE2-Luc together with 1 μg of pCMV-β-gal by the calcium phosphate precipitation method. Forty eight hours after transfection, cell extracts were prepared and their luciferase activities were examined after standardization of the transfection efficiencies by β-galactosidase assays as described previously [6].

2.3. RT-PCR and plasmid construction

Nucleotide sequences of the oligonucleotide used for PCR primers were as follows:

M-CSF-1-up, 5′-GCGATGTGTGAGCAATGGCAGT-3′;
M-CSF-1-down, 5′-AGACCGTTTTGCGTAAGACCTG-3′;
GAPDH-up, 5′-CTCATAGACAAGATGGTGAAGGTCG-3′;
GAPDH-down, 5′-GGTCCAGGGTTTCTTACTCCTTGG-3′;
pGLprimer1, 5′-TGTATCTTATGGTACTGTAACTG-3′;
pGLprimer2, 5′-CTTTATGTTTTTGGCGTCTTCCA-3′;
box1-sense, 5′-TTGGCTATGGACACCCTCCCCTC-3′;
box1-antisense, 5′-GGGTGTCCATAGCCAAAGCCAAA-3′;
box2-sense, 5′-GTGACAGGCTAGAGCCCAGATTC-3′;
box2-antisense, 5′-GGCTCTAGCCTGTCACAGAGCAC-3′;
box3-up, 5′-CCAGATTCAGTCATGATCAGGCC-3′;
box3-down, 5′-TCATGACTGAATCTGGGCTCTAG-3′.

RT-PCRs, to amplify cDNAs of rat c-*fms* or rat *GAPDH*, were carried out using total RNA from Myc-ER cell lines with M-CSF-1-up and M-CSF-1-down or with GAPDH-up and GAPDH-down as templates and primers, respectively. Reporter plasmids of luciferase-deletion mutants of the c-*fms* promoter were constructed using

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doi:10.1016/j.febslet.2004.07.034

pE1-Luc and primers other than those for RT-PCR as templates and primers, respectively.

2.4. Colony forming and tumor forming assays

Myc-ER cell lines were cultured in a medium containing agarose for 14 days as described previously [11]. A total of 1×10^7 Myc-ER cells were injected subcutaneously into 6-week-old mice. Eight weeks after injection, the numbers of tumors that had been developed in the mice were counted.

2.5. Chromatin immunoprecipitation assays

Nucleotide sequences of the oligonucleotide used for PCR primers were as follows:

ODC E-box up, 5′-GGCACGTGTGCGCGCCTCGCC-3′;
ODC E-box down, 5′-AAATTGGTGTCATCTGCTCTGTAG-3′;
ODC intron up, 5′-GTTGTTACTCTCTTCCCTTCTCCG-3′;
ODC intron down, 5′-ATAATTTGGGGCTAACTTCACCAA-3′;
c-fms E-box up, 5′-AGGCTCTGGTGCTGAGGCGT-3′;
c-fms E-box down, 5′-CTGAGATGCTGCAGCCGTGA-3′;
c-fms intron up, 5′-AGTGACCTGAGTCCTACTACTAT-3′;
c-fms intron down, 5′-CTGAGTCACATCACAGGACT-3′.

Chromatin immunoprecipitation (Chip) assays were carried out using HeLa nuclear extract and the above primers according to the supplier's protocol (Chip Assay Kit, Upstate).

3. Results and discussion

3.1. Activation of c-myc promoter in cells harboring a dominant-negative form of TIF1β

MycER cells are Rat-1 cells harboring a chimeric protein of c-Myc fused with the estrogen receptor (MycER), and MycER complexed with Hsp90 is localized in the cytoplasm in the absence of 4-hydroxyl tamoxifen (4-OHT), thereby inactivating transcription activity of Myc-ER. After addition of 4-OHT to the medium, Myc-ER is translocated into the nucleus to be activated [12]. MycER cells have therefore been used for an Myc-specific expression system. In order to identify target genes for the c-Myc-MM-1-TIF1β complex, MycER cells were transfected with expression vectors for the HA-tagged C-terminal region of TIF1β (TIF1βΔN-HA), which is a dominant-negative form of TIF1β [6], or a vector alone together with blasticidin S. Blasticidin S-resistant cells were then selected and expression of TIF1βΔN-HA in each cell line was examined by Western blotting using an anti-HA-antibody (Fig. 1A). While protein concentrations determined by amounts of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were almost the same in the cell lines, TIF1βΔN-HA was expressed in MycER cells transfected with TIF1βΔN-HA (MycER-TIF1ΔN cells) but not in parental MycER and MycER cells transfected with a vector alone (MycER-vec cells). It was confirmed that amounts of TIF1βΔN-HA in MycER-TIF1ΔN cells did not change in the presence or absence of 4-OHT as described previously [12]. The amounts of endogenously expressed TIF1β and c-Myc also did not change in each cell line (data not shown). To examine the transcription activity of c-Myc, these cell lines cultured in the presence or absence of 4-OHT were transfected with reporter genes containing the 4 x E-box linked to the luciferase gene and their luciferase activities were examined 48 h after transfection (Fig. 1B). In the absence of 4-OHT, the luciferase activity level of MycER-TIF1ΔN cells was found to be higher than that of MycER or MycER-vec cells as in the case of transient transfection experiments [6], indicating that transcription activity of endogenous-Myc was stimulated in MycER-TIF1ΔN cells, in which repressed transcription activity of c-Myc by MM-1-TIF1β complex was abrogated by

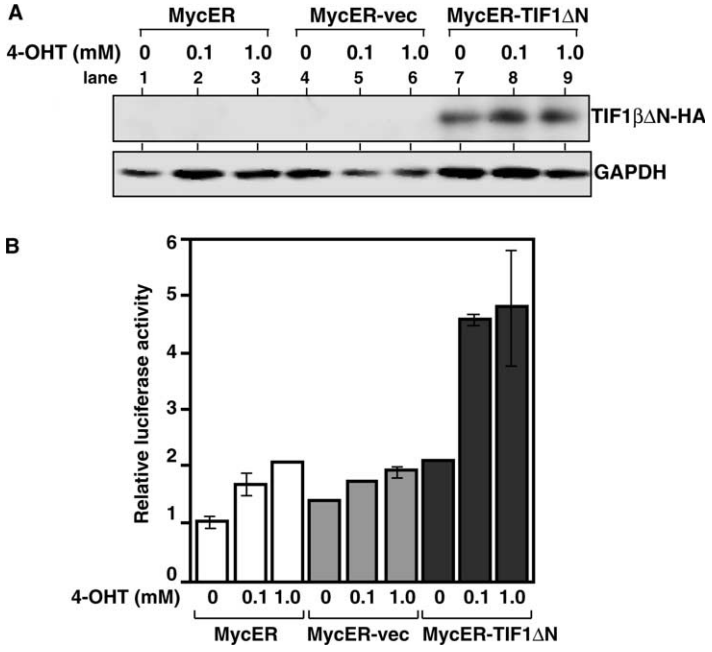

Fig. 1. Establishment of a cell line harboring a dominant-negative form of TIF1β. (A) Myc ER cell lines harboring the C-terminal region of TIF1β (MycER-TIF1ΔN cells) or vector alone (MycER-vec cells) were established as described in Section 2. Expression of the C-terminal region of TIF1β and GAPDH in these cells cultured in the presence of 4-OHT was analyzed by Western blotting using an anti-HA antibody (12CA5, Roche) and an anti-GAPDH antibody (Chemicon). (B) MycER, MycER-vec and MycER-TIF1ΔN cells were transfected with p4xE-box-SVP-Luc and pSV-β-gal. Forty eight hours after transfection, cell extracts were prepared and their luciferase activities were examined as described in Section 2. More than five experiments were carried out.

TIF1ΔN. Since exogenously added Myc-ER was activated by 4-OHT, the results also showed that transcription activities of c-Myc in all of the MycER cell lines were stimulated by 4-OHT and that transcription activity of c-Myc in MycER-TIF1ΔN cells was a sum of activities of endogenous c-Myc and exogenous MycER.

To characterize MycER-TIF1ΔN cells in terms of oncogenesis, MycER-TIF1ΔN and MycER-vec cells were cultured in a medium containing agarose in the absence of 4-OHT. The results showed that MycER-TIF1ΔN grew and formed colonies, while MycER-vec cells died (Fig. 2A). Furthermore, when MycER-TIF1ΔN and MycER-vec cells were injected subcutaneously into mice, only MycER-TIF1 ΔN cells were found to induce tumors (Fig. 2B). MycER-TIF1 ΔN cells cultured in the presence of 4-OHT were also found to give colonies in soft agar medium and to induce tumors more than did MycER-TIF1ΔN cells cultured in the absence of 4-OHT (data not shown), which was parallel with transcription activity of c-Myc as described in Fig. 1B. Although efficiencies of MycER-TIF1ΔN cells to form colonies and tumors were found not to be high compared to those of cells transformed with c-*myc* and activated H-*ras* genes, these results suggest that MycER-TIF1ΔN cells are tumorigenic and that stimulated transcription activity of c-Myc leads to upregulation of some genes that are related to cell transformation.

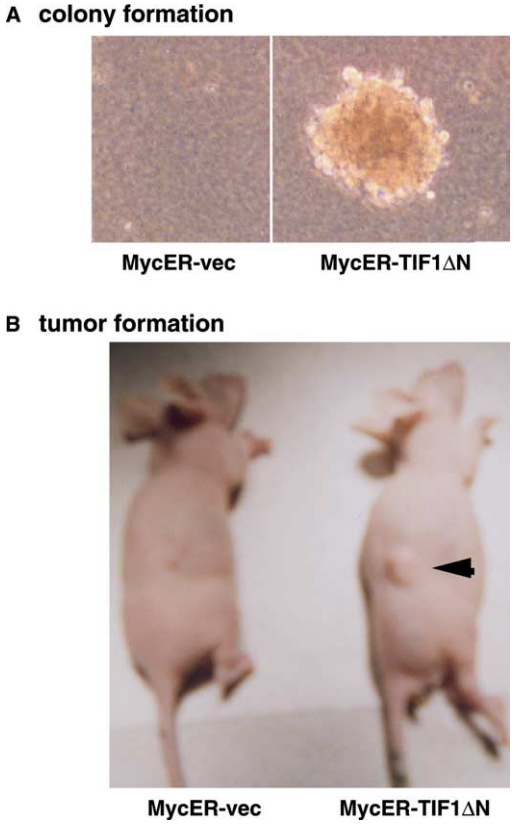

Fig. 2. Tumorigenicity of MycER-TIF1ΔN cells. (A) MycER-vec and MycER-TIF1ΔN cells were cultured in a medium containing agarose, and colonies formed were examined at 14 days after seeding. Photographs of cells are shown. (B) 10^7 MycER-vec and MycER-TIF1ΔN cells were injected subcutaneously into 6-week-old mice. Photographs of tumors that had developed in the mice at eight weeks after injection are shown. Two experiments were carried out both in (A) and (B).

3.2. *Identification of the c-fms oncogene as a target gene for the c-Myc-MM-1-TIF1β pathway*

To identify genes whose expressions are upregulated in MycER-TIF1ΔN cells, DNA-microarray analysis of genes between MycER-TIF1ΔN and MycER-vec cells was carried out using a Rat microarray glass filter, in which 1081 genes were spotted (Rat 1.0 Microarray, Clontech). Five genes were found to be expressed at levels more than 2.5-times higher in MycER-TIF1ΔN cells than in MycER-vec cells, and expressions of these genes were further examined by RT-PCR using total RNAs from both cell lines. The results showed that of the five genes, expression of the c-*fms* gene, which encodes macrophage colony stimulating factor 1 (M-CSF-1) receptor, was reproducibly stimulated in MycER-TIF1ΔN cells (Fig. 3A). It

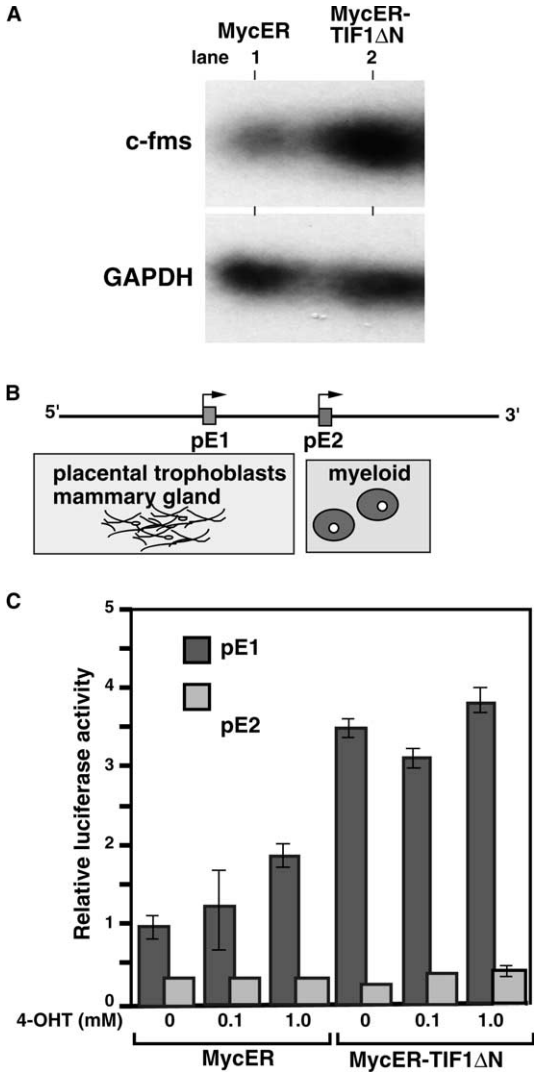

Fig. 3. Activation of c-Myc transcription activity in MycER-TIF1 ΔN cells. (A) Total RNAs were extracted from MycER and MycER-TIF1ΔN cells, and expression of c-*fms* and *GAPDH* genes was analyzed by RT-PCR as a described in Section 2. After 10 cycles of PCR, DNAs extracted from the mixtures were analyzed by Southern blotting using ^{32}P-labeled cDNAs of c-*fms* and *GAPDH* genes as probes. (B) Schematic drawing of the promoter region of the human c-*fms* gene. (C) MycER and MycER-TIF1ΔN cells were transfected with pE1 or E2-Luc and pSV-β-gal. Forty eight hours after transfection, cell extracts were prepared and their luciferase activities were examined as described in Section 2. Five experiments were carried out.

is known that the c-*fms* gene is expressed strongly in macrophage and myeloid cells and weakly in fibroblast cells such as Rat-1 cells and that overexpression of the c-*fms* gene transforms macrophage and myeloid cells [13–15]. The human and mouse c-*fms* genes contain two promoters, pE1 and pE2, and these are necessary for the c-*fms* gene to be expressed specifically in fibroblastic cells, including placental trophoblasts and mammary gland, and myeloid cells, respectively [10] (Fig. 3B).

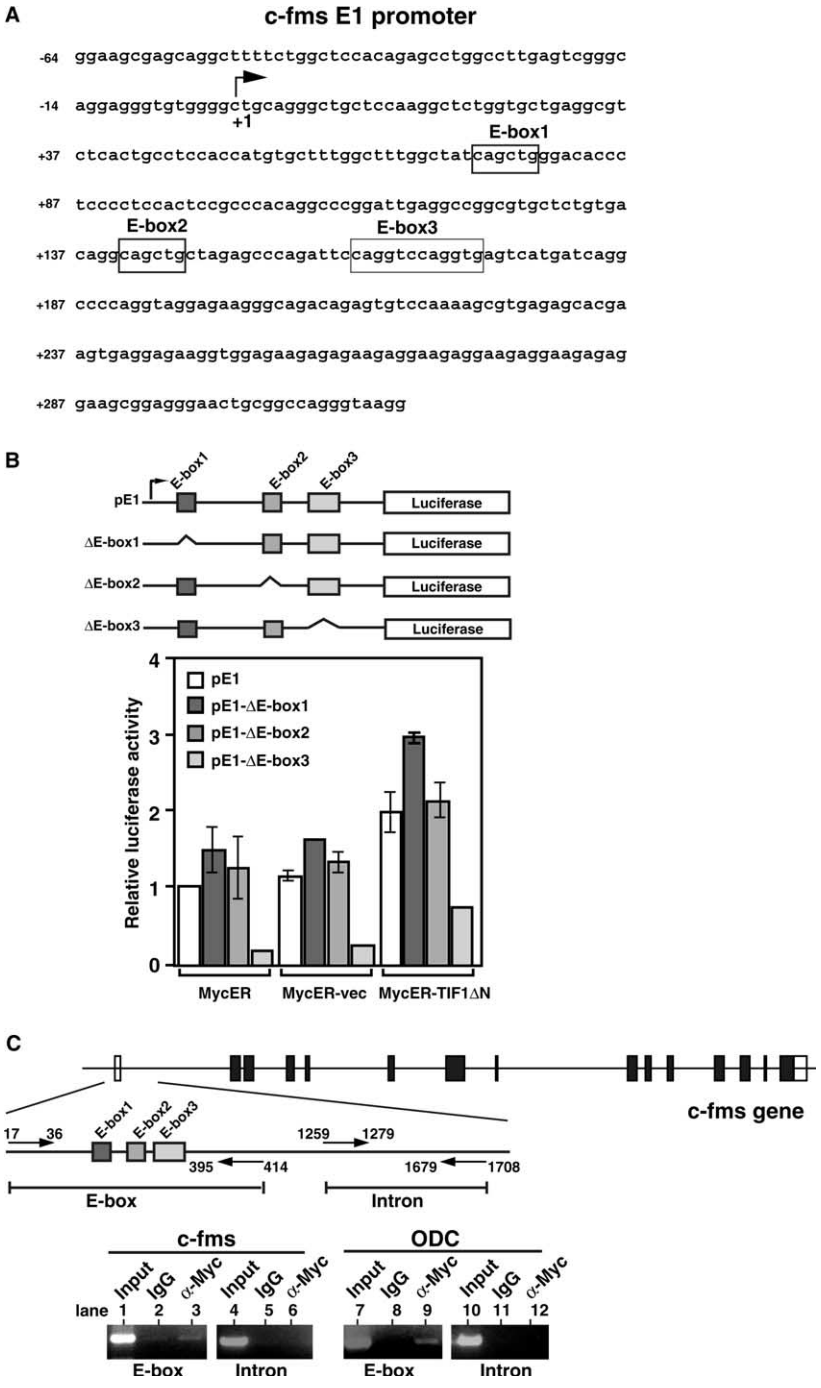

Fig. 4. Identification of the E-box sequence for expression of the c-*fms* gene in MycER cells. (A) Nucleotide sequence of the promoter region of the human c-*fms* gene. Squares indicate E-box sequences. (B) A schematic drawing of deletion mutants of the pE1 promoter is shown in the upper part of the figure. MycER, MycER-vec and MycER-TIF1ΔN cells were transfected with deletion mutants of pE1-Luc and pSV-β-gal. Forty eight hours after transfection, cell extracts were prepared and their luciferase activities were measured as described in Section 2. Five experiments were carried out. (C) Chip assays were carried out using HeLa cell extracts and primers specific to an exon and intron of human c-*fms* and *ODC* genes. The human c-*fms* gene was schematically described and the regions amplified by PCR with specific primers in the c-*fms* gene, E-box and Intron, were also described. Nucleotide numbers indicated at both ends of primers correspond to those in A. The regions to be amplified by PCR in the *ODC* gene were described previously [18]. The nuclear extracts containing chromatin were prepared from HeLa cells and immunoprecipitated with an anti-c-Myc antibody (C33, Santa Cruz) or non-specific IgG according to the supplier's protocol (Chip Assay Kit, Upstate). PCR was carried out using DNAs extracted from the mixtures and specific primers as described in Section 2. The products were then run on agarose gels containing ethidium bromide and visualized under UV-light.

To examine which promoters are used in MycER and MycER-TIF1ΔN cells, reporter genes containing pE1 or pE2 linked to the luciferase gene were transfected into MycER and MycER-TIF1ΔN cells and their luciferase activities were examined (Fig. 3C). The results clearly showed weak luciferase activities of pE2 in both cell lines compared to those of pE1 even in the presence of 4-OHT, which activates transcription activity of MycER. Promoter activities of pE1 in MycER cells were found to be stimulated by 4-OHT in a dose-dependent manner, and those in MycER-TIF1ΔN cells in the absence of 4-OHT, on the other hand, were found to be higher than those in MycER cells in the presence of 1 mM 4-OHT (Fig. 3C). These results suggest that transcription activity of the c-*fms* gene in MycER cells depends on pE1 and that abrogation of the c-Myc-MM-1-TIF1β pathway stimulates pE1 activity.

A search for recognition sequences for transcription factors in the E1 promoter region of the human c-*fms* gene was made, and three E-box sequences, which are candidates for the recognition sequence of c-Myc, were identified (Fig. 4A). These were termed E-box 1, E-box 2 and E-box 3, and the E-box 3 contains two E-box sequences. To identify the E-box that contributes to expression of the c-*fms* gene in MycER cells, deletion mutants of reporter genes lacking each E-box sequence, pE1ΔE-box 1, pE1ΔE-box 2 and pE1ΔE-box 3, were constructed and their promoter activities were examined after transfection of these reporter genes into MycER cell lines (Fig. 4B). The results clearly showed that while promoter activities of pE1ΔE-box 1 and pE1ΔE-box 2 did not change, or rather were stimulated, compared to those of the wild-type E1 promoter, promoter activity of pE1ΔE-box 3 lacking the E-box 3 was found to decrease in all of the cell lines, indicating that the E-box 3 is responsible for the E1 promoter in MycER cells. Furthermore, to determine whether c-Myc binds to the region containing the E-box in the c-*fms* gene, chromatin immunoprecipitation assays were carried out using HeLa cell extracts. Since reporter gene assays described above used the human c-*fms* gene and the genomic structure of the rat c-*fms* gene has not been characterized, we used HeLa cells for chromatin immunoprecipitation assays. Expression of the c-*fms* gene was not detected in HeLa cells and primers that amplify the regions corresponding to the E-box or intron of the human c-*fms* or *ornitin decarboxylase* (ODC) gene as a positive control (Fig. 4C). The results showed that an anti-c-Myc antibody but not non-specific IgG precipitated regions containing E-box sequences of c-*fms* and ODC genes, while the anti-c-Myc antibody did not precipitate intron sequences of c-*fms* and ODC genes. Furthermore, gel mobility shift assays showed that purified c-Myc and Max bound to the E-box 3 (data not sown). These results indicate that c-Myc binds to E-box sequences of both genes.

In this study,we identified the c-*fms* oncogene that is regulated by the c-Myc-MM-1-TIF1β complex through the E-box sequence in fibroblasts. Abrogation of this pathway in RatER cells, a fibroblast cell line, resulted in the cells becoming tumorigenic with strong transcription activity of c-Myc. Since the c-*fms* gene is usually expressed in macrophage or myeloid cells and overexpression of the c-*fms* gene transforms these cells [13–15], these findings suggest that the MM-1-TIF1β complex plays a role in suppression of oncogenes that are not expressed or only weakly expressed in cells other than myeloid cells and also that contain an E-box sequence, a recognition sequence for c-Myc. The findings in this study also suggest that even when c-Myc binds to the E-box sequence in these oncogenes, the MM-1-TIF1β complex suppresses c-Myc transcription activity, thereby preventing cells from becoming tumorigenic.

Although transrepression pathways through basic-helix-loop-helix-zip domains in the C-terminal region of c-Myc have been well characterized, those from the N-terminal region containing myc box I and myc box II domains have not been well investigated. From this point of view, MM-1 is thought to be the most characterized protein that binds to the N-terminal transrepression region of c-Myc. In addition to MM-1, two proteins, Pag [16] and Krim1 [17], that have characteristics similar to those of MM-1, have been reported. Although target genes for Pag and Krim1 have not been identified, investigation of these proteins, including MM-1, should shed light on the repression pathway from the N-terminal region of c-Myc.

Acknowledgements: We thank E. Sapi for providing pE1 and pE2. We thank Yoko Misawa and Kiyomi Takaya for their technical assistance. This work was supported by grants-in-aid from the Ministry of Education, Science, Sport, Culture and Technology of Japan.

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