Expression of the TAF4b gene is induced by MYC through a non-canonical, but not canonical, E-box which contributes to its specific response to MYC

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Abstract. Transcription factor binding sites are short DNA sequences that interact with transcription factors and the proper control of gene expression appears to require the mechanisms including the regulation through the genome context around the transcription factor binding sites. The MYC proteins are central regulators of cell growth. Many genes have been reported to be regulated by MYC through E-box sites. However, the characters of E-box that Myc selects to function are not clear and identification of additional genes controlled by MYC will provide information to completely understand the functions of MYC. Here we report that MYC directly induces TAF4b expression. We mapped the transcription start site and characterized functional promoter elements for MYC response in the TAF4b promoter. There are several E-box sequences near the transcription start site, including canonical (CACGTG) and non-canonical (CGCGTG) ones. We found that c-MYC induces TAF4b expression through one of the non-canonical E-box sites, which is in a highly conserved region of TAF4b promoters in mammals, suggesting the importance of the genome context around the target E-box. When the non-canonical E-box in the TAF4b promoter was mutated to a canonical one, MYC functioned on both E-boxes, while another E-box-binding transcription factor, USF, did so on only the canonical E-box. These results suggest that in addition to the context where the target E-box exists, a sequence within an E-box is involved in the mechanisms by which specific E-box sites are selected by Myc.

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Introduction

Transcription factor binding sites are short DNA sequences that interact with transcription factors. Although there are a great number of the candidate binding sites in the genome, the transcription factors function only on limited number of sites. Furthermore, while some transcription factors share their target sequences, the genes that respond to them and their biological functions are different. Thus, the proper control of gene expression requires the mechanisms, by which transcription factors specifically select their target sites. Recent studies suggest that the specificity of protein-DNA interaction depends, at least in part, on the genome context around the target sites. For example, the veracity of a potential target site correlates with the GC content and CpG island (1), preferential spacing between transcription factor binding sites and transcription start sites (2), co-operation of cis-regulatory molecules (3) and clustering of degenerate transcription factor binding sites near the target sites (4). However, it is still unclear how the target specificity for each transcription factor is achieved.

The *myc* family of proto-oncogenes consists of three main genes: *c-myc*, *N-myc* and *L-myc* (5-9). The three genes have different expression patterns but appear to have basically the same biological activity. The accumulating data indicate that the *myc* family genes are central regulators of cell growth (6,7,10-14). The MYC protein encoded by *myc* is a member of the basic helix-loop-helix leucine zipper transcription factors (6,14). MYC functions as an E-box-binding transcription factor and activates transcription of a number of genes (15-24). Although there are a number of E-box sites in the genome, Myc selects only some E-boxes to function on.

Besides proteins in the *myc* proto-oncogene family, there are other classes of E-box-binding transcription factors, including USF (25) and TFE3 (26). Although these factors also transactivate gene expression through an E-box, their biological functions and the genes that respond to them are different from Myc. However, it is unclear which factors are really responsible for the E-box-binding transcription factors to select the specific target genes.

Here we report that MYC directly induces the expression of TAF4b. The sequence responsible for this induction is a non-canonical E-box and the surrounding sequence of this element is highly conserved in mammals, confirming the importance of the context where E-box exists. Although this site responded specifically to MYC, it also responded to another E-box binding transcription factor, USF, when the non-canonical E-box was mutated to the canonical one. These results suggest that the sequence within E-box have a role for the selection of the target genes by E-box transcription factors.

Materials and methods

Cell culture. Human glioblastoma cell line T98G cells and its derivative T98Gmycer-2 cells, which express c-MycER chimeric protein (23), were cultured in Eagle's medium supplemented with non-essential amino acids and 10% fetal calf serum (FCS). The African green monkey kidney fibroblast line Cos-7 were cultured in Dulbecco's modified Eagle's medium supplemented with 10% FCS. Human promyelocytic leukemia HL60 cells were cultured in RPMI-1640 medium supplemented with 20% FCS. The rat colon cancer cell line RCN-9 (Riken Cell Bank, Saitama, Japan) and the human erythroleukemia cell line HEL (Health Science Research Resources Bank, Osaka, Japan) were cultured in RPMI-1640 medium supplemented with 10% FCS.

Polymerase chain reaction (PCR). PCR amplifications were performed in 50 μl EX Taq buffer (Takara, Shiga, Japan) containing 10 pmoles of each primer, 1.2 U EX Taq DNA polymerase and 200 μM dNTP.

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 (23,24). The first PCR was performed using primers, AA287145-1st (5'-TTCTTATGCCTCTTCCTTTCTCCTG-3', a sequence in the 5' terminal region of EST clone AA287145) and the AP1 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 sec and annealing and extension at 68°C for 3 min. One μl of the first RACE-PCR product diluted 1,000 times was used as the template for nest RACE-PCR. Nest PCR was performed using the AA287145-nest primer (5'-TTGGCAAATGAGGCAGGTAGTAACA-3', which corresponds to a sequence in the upstream region of AA287145-1st of EST clone AA287145) 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 sec and annealing and extension at 68°C for 3 min. The amplified DNA fragment was cloned into a pGEM-T vector (Promega, Madison, WI) (pT/fragment TAF4b) and sequenced. The results of sequencing showed that AA287145 is part of the 3'-UTR of the TAF4b gene.

To obtain information about the 5'-terminal of TAF4b mRNA, 5'-RACE amplification was performed as described above, using the primer TAF4bRACE-1 (5'-GGTCTCGGCT CTTGTTACAGTTTGCTG-3'), a sequence in the 5'-terminal

region of TAF4b cDNA reported previously (27) and the AP1 primer provided by the supplier for the first PCR; and the TAF4bRACE-2 primer (5'-GGATTGTCGTGGTGTTGGG GGCTTTCA-3', which corresponds to a sequence in the upstream region of TAF4bRACE-1) and the AP2 primer provided by the supplier for the nest PCR. The amplified DNA fragment was cloned into a pGEM-T vector (Promega) and sequenced.

Reverse transcriptase-polymerase chain reaction (RT-PCR). Synthesis of single-strand cDNA of HEL cells was performed on total RNA (1 μg) using a Superscript first-strand synthesis system (Invitrogen, Carlsbad, CA). One μl (total 20 μl) of the resultant single-strand cDNA was used as the template for PCR. The RT-PCR primers for the amplification were TAF4b coding U (5'-CGGAATTCGAAGCTGCGAGAGGT CGGGCGGGTGTCG-3', a sequence in the 5' untranslated region plus an *Eco*RI site) and TAF4b coding L (5'-CGGAA TTCGGCAGTAAATAGCAAGGATGTGGATGGA-3', a sequence in the 3' untranslated region plus an *Eco*RI site). The temperature profile was 35 cycles of denaturing at 98°C for 15 sec, annealing at 65°C for 1 min and extension at 72°C for 2.5 min. The amplified DNA fragment was cloned into a pGEM-T vector (Promega) to produce pT/TAF4b and sequenced. The sequence was confirmed by direct sequencing using the amplified fragment.

Reporter plasmids with a TAF4b genomic DNA fragment. A genomic DNA fragment of the human TAF4b gene, which extends from the promoter region to part of exon 1, was amplified by PCR with 5'-TTTTACCATAACCTCACTTGC TGGAAGGGG-3' and 5'-TGTTGGGGGCTTTCACGGCG ACTATCTG-3'. The 2.2-kb amplified fragment was cleaved with *Sac*I and *Nco*I and inserted into the 4.8-kb fragment of pGL3 containing firefly luciferase (Promega) cut with *Sac*I and *Nco*I to produce pTAF4b(W)luci. The 0.6 kp fragment of TAF4b(W)luci cut with *Sma*I and *Nco*I was inserted into the 4.8-kb fragment of pGL3 cut with *Sma*I and *Nco*I to produce pTAF4b(dSma)luci. The 0.6 kp fragment of TAF4b(W)luci cut with *Xho*I and *Nco*I was inserted into the 4.8-kb fragment of pGL3 cut with *Xho*I and *Nco*I to produce pTAF4b(dXho) luci. pTAF4b(dSma)luci was cleaved with *Xho*I and *Nco*I, blunted with Klenow enzyme and self-ligated to produce pTAF4b(Sma-Xho)luci. pTAF4b(dSma)luci was also cleaved with *Pvu*II and *Nco*I, blunted with Klenow enzyme and self-ligated to produce pTAF4b(Sma-Pvu)luci.

Further deletion mutants, pTAF4b(117Pvu)luci, pTAF4b (114Pvu)luci, pTAF4b(102Pvu)luci, pTAF4b(73Pvu)luci, pTAF4b(43Pvu)luci, pTAF4b(Sma118)luci, pTAF4b (Sma87)luci, pTAF4b(Sma74)luci, pTAF4b(Sma68)luci, pTAF4b(Sma59)luci and pTAF4b(Sma45)luci were produced by PCR with appropriate primers using pTAF4b(Sma-Pvu) luci as a template and the subsequent introduction of the resultant fragments into a pGL3 vector.

Mutation was introduced at E-box sites of pTAF4b(W)luci using a Gene Editor™ *in vitro* site-directed mutagenesis system (Promega) to produce pTAF4b(mE1/2)luci, in which two E-boxes (CACGTG elements) were mutated to CACCTG. Mutation was also introduced at non-canonical E (NCE)-box sites (CGCGTG) of pTAF4b(Sma-Pvu)luci to produce

pTAF4b(Sma-Pvu mNCE1/2)luci, pTAF4b(Sma-Pvu mNCE1)luci, and pTAF4b(Sma-Pvu mNCE2)luci, in which one or two NCE-boxes (CGCGTG elements) were mutated to CGCCTG. The non-canonical E (NCE)-box site 2 (NCE-box 2) in pTAF4b(Sma-Pvu)luci was mutated to CACGTG to produce pTAF4b(Sma-Pvu CanoE2)luci. The DNA sequences in the produced plasmids were confirmed by sequencing.

Sequence of the rat and monkey TAF4b promoters. A 1.2 kb fragment of the rat TAF4b promoter was isolated as follows: First, the mouse DNA sequence homologous to the human TAF4b gene was selected (GenBank Accession no. AK012135). The two regions in the mouse DNA sequence whose nucleotide sequences exactly match the human TAF4b genomic sequences were selected, and oligonucleotides rat TAFII-F (5'-GCGCACGTGTGAGCGCCGCTGAGG-3') and rat TAFIIPF-1 (5'-CTGACAGGAGCCTTAGTCAC-3') were synthesized. With these oligonucleotides as primers, the rat TAF4b genomic DNA was amplified using rat colon cancer cell RCN-9 genomic DNA as a template. The amplified DNA fragment was sequenced and the upstream region of this sequence was further cloned by promoter find kit (Clontech) with *Dra*I and *Ssp*I libraries as templates using primers rat TAFIIPF3 (5'-GCCGGGGCGGGCGGCAGGAGACTCG-3') and rat TAFIIPF4 (5'-CGGGCACTCCCTCAGCGGCGCTCAC-3'), whose sequences were in the rat TAF4b DNA sequence identified above and AP1 and AP2. The amplified rat TAF4b genomic fragments were sequenced.

The monkey TAF4b promoter was amplified with TAF-primer 1 (5'-GACACAAGGAGAGGAACACGGATGC-3') and TAF-primer 2 (5'-GCCTGGGCTGCCCCCGGAGCGACAC-3') matching the human TAF4b gene using the genomic DNA of Cos-7 cells as a template and sequenced.

Other plasmids. pRL-CMV containing the *Renilla reniformis* luciferase gene under control of the CMV promoter was purchased from Promega. CMV-c-Myc, pCMV-USF and pCMV-TFE-3 were gracious gifts from Dr Martin Eilers (28).

RNA preparation. Total RNA was isolated from cells by the acid guanidium thiocyanate-phenol/chloroform extraction method using a DEPC-treated RNA preparation solution set (Nakalai, Kyoto, Japan).

Differential display using DNA chip (cDNA micro array). cDNA micro array was performed as described previously (23). Total RNA was isolated from untreated T98Gmycer-2 cells and from cells treated by 4-hydroxytamoxifen (OHT) for 20 h. Poly(A+) RNA was recovered and subjected to differential display using a DNA chip (Incyte Genomics, Palo Alto, CA). About 9,000 kinds of cDNAs including expressed sequence tag (EST) clones were plated on the chip (UniGEM Human V Ver. 2).

Northern blot analysis and preparation of probe DNAs. RNA was electrophoresed in an agarose gel containing formalde-hyde, transferred to Hybond-N (Amersham Biosciences) and probed with ^{32}P-labeled cDNA. Probes were labeled with [α-^{32}P]-dCTP using a Multiprime labeling kit (Amersham Biosciences). The results were quantified using a BAS2000

image analyzer (Fuji Photo Film, Tokyo, Japan). The DNA probe for c-*myc* was described previously (18). The DNA probe for TAF4b was a 0.6 kb *Sac*I fragment from pT/fragmentTAF4b.

Transient expression assay. Transient expression reporter assay in T98Gmycer-2 cells was performed as described previously (23). The effects of MYC and other transcription factors on the TAF4b promoter were investigated by transfection of the plasmids encoding the transcription factors. T98G cells were grown in medium supplemented with 10% FCS. Cells (3x10⁴) were plated into a dish (12-well plate; well diameter 22 mm) and cultured for 20-24 h. Transfections were carried out using FuGENE 6 reagent (Roche Diagnostics, Indianapolis, IN) with 1 μg of the reporter plasmids, 20 ng of pRL-CMV as an internal transfection marker and various amounts of plasmids expressing the transcription factors. The total amount of DNA in each transfection was kept constant by adding the empty expression vector. One day later, cells were collected and the luciferase activities were determined as described previously (18).

Chromatin immunoprecipitation. Chromatin immuno-precipitation assay was performed basically as described previously (23). Immunoprecipitated DNA fragments were detected by PCR. The PCR primers were 5'-GACACAAGG AGAGGAACACGGATGC-3' (TAF4b-primer 1) and 5'-GCC TGGGCTGCCCCCGGAGCGACAC-3' (TAF4b-primer 2), which amplify a 521-bp fragment containing the transcription start site of the human TAF4b gene (TAF4b primers) and 5'-TTACAGGTAAGCCCTCCAATGACC-3' and 5'-GCAA AGCTACCATTTAGGAACCC-3', which amplify the genomic sequence of a region containing an E-box in chromosome 22 (control primers). This E-box is located in a chromosomal region without any detectable genes (20).

Antibodies. Antibody-1 against c-MYC was described previously (18). Anti-c-MYC antibody-2 (N262) (Santa Cruz Biotechnology, Santa Cruz, CA) was purchased.

The sequence information. The sequence data in this study have been submitted to the DDBJ databases under accession No. AB234096 for the DNA sequence of full-length human TAF4b, accession No. AB234097 for the DNA sequence of the human TAF4b promoter, accession No. AB234098 for the DNA sequence of the rat TAF4b promoter and accession No. AB234099 for the DNA sequence of the monkey TAF4b promoter (Cos-7 cells).

Results

c-MYC induces the expression of TAF4b. To conditionally induce c-MYC activity, the estrogen-inducible MYC system (23,29) 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 analogous molecule 4-hydroxytamoxifen (OHT) binds to the chimeric protein, it becomes free to function as c-MYC. The human glioblastoma cell line T98G was used as the

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-1573                                 GAGCTCAGGAATTGGAGACCAGCCTGGACAATATAACGAGATCCCGTTTTTGC
-1520 TAGGCATAGTGCCGCTCCCCTGTGGTTCCAGCTACTCGGGAATCTGAGGTAGGAGGGTAGGATGATCACTTGAGCCCAGG
-1440 AGGTGGAGGCTGCAATGAGCCAATGGTGCCACTGCACTCCAGCCTGGGTGACAGAGTTAGATCCTGTTTCCAAAACAAAA
-1360 CAAAACAAAACAAAACAAAACAAAACACCACAACATTCAGATCATAGCATCTGCCTACTTGTAAAATCGCCTTTCAGTGC
-1280 AGTGTTGGTTCCTACAAACATACAATATAATGACTTTAGCTTGAAATTAAGGCATCTTCTCTGACTGCATTACAAACAGG
-1200 CAAGTTTTTTCATATCGATTCACTCAACACCTTTTATTTATTCCAGATAATATCTAGGATCACGCTTAATATCTAAGTGCT
-1120 AGAATCTAATTATATAGATATCAAAAGGCATTAAGTTTTTCAGAAGACAGACATACAATTTTAATGTTTAGAGGTGATAG
-1040 AGACACCTATAGCAGCACCAAAGAAGAAACGATTTTCCATTTAAGATTTTAGGCAGTTTAACTCTAAAATATGAAAATAG
 -960 GCACATCTCACAGACTATAAAACTATACGCCATTTTAATTTTTACAATGTCAATTCTAACTGCGAATTTTAGCAACGCTT
 -880 CAGTGTGAAGACAGCAGAAAAACACTGAAAATGCGTGATTGAATTCTTATTGAGTCACTCTTCTCGGAAATCAGTCCT
 -800 CCCCATTTTCTCCTTAAAAAATAGAATCCTGGAGATAGTCTAGTGCTGGCTTTTCGAGGAACACACTACAGGGGACACAA
 -720 GGAGAGGAACACGGATGCCGCAGTTTTCCAAGTGCAGCTTGCCCCTCCCACGTGATAGGTTTCTGGCATCGCCCCTCCTT
 -640 GCTGGCGATTCTGGGGAAGATTTTAACGCTCCCTGGAGCCGTCCCCAGTGACGTCACGGGCGCGGCTCCGCTCCTTGGCC
 -560 AATCACGCGGCCGGATCCCGGGGAAACCCGGGCGGTGGCGGCGGCCTGGGCGCGTGCGCGAGGTCTCCGCGTGGCTATA
     TATA                     SmaI              NCE-box 1            NCE-box 2    TATA
 -480 TAAACATGGCTGGCGTGGCCGCGCGGCGGGGGCGGTGGCGGCCGTCCGGCCAATCGCGCGTAGGGGGCACTCGGGGTTCGTAGTTT
     TATA
 -400 TGAAATTTCTGGCGGGGGAGCAGCTGCGCAGTTAGGCTCGAGGTGTGAGCGACGACCTTCCGGGAGCCGCAAGTCCAGGC
           PvuII         XhoI
 -320 TCCCCCGCAGCGGGACCCGAGCCTGAGGCGCAGGGCTGAGGCAGCCCACGTGTGAGCGCCGCTGAGGAAGCTGCGAGAGG
                                           E-box 2
 -240 TCGGGCGGGTGTCGCTCCGGGGGCAGCCCAGGCTCGCGCGGACGAGAGGAAGGTCCGGGACGCGCGTGTCCTGCCGTGCA
 -160 GCGGGCGCCCGTCACTGACTTCGCTGCTGCGGCCCCGGCGTCTCCCCGACGCGATGCTGTGGAACCCGAACCGCACCGGA
  -80 GTCGGCTGCCGCGCGCCAAGCCTCCCCTCACCTCTGCTCCCGGAACCGCAGCGCCAAAGCTGCCGCTGAGCCCCTGGGGG
   +1 ATGCCCGCCGGCCTCACCGAACCCGGCCGGCGCCGGCTCCCCCGGCTGCTGTGAGCGCCTCGGGGACCGTGACCATGGCCCC
      M  P  A  G  L  T  E  P  A  G  A  A  P  P  A  A  V  S  A  S  G  T  V  T  M  A  P

  +81 GGCCGGGGGCGCTGCCGGTGCGGGTGGAGAGCACTCCGGTGGCCCTGGGCGCCGTGACTAAGGCTCCTGTCAGCGTCTGCG
      A  G  A  L  P  V  R  V  E  S  T  P  V  A  L  G  A  V  T  K  A  P  V  S  V  C

 +161 TGGAGCCCACGGCGTCCCAGCCCCTGCGGTCCCCCGTGGGG
      V  E  P  T  A  S  Q  P  L  R  S  P  V  G
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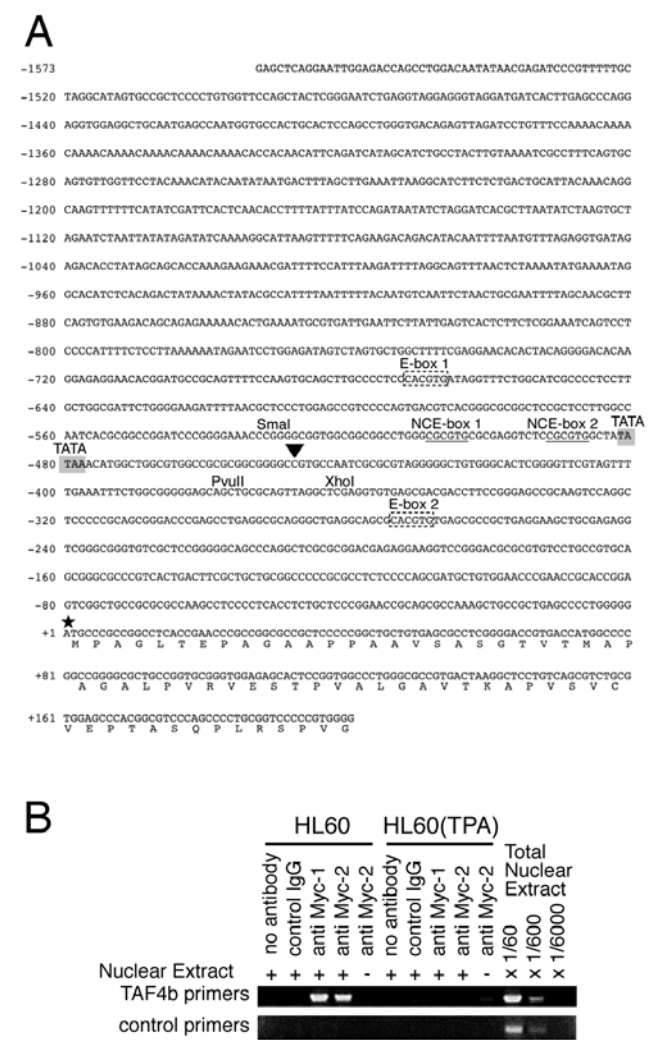
B

Figure 1. The genomic DNA sequence of the human TAF4b promoter and chromatin immunoprecipitation experiments. (A) The genomic DNA sequence of the human TAF4b promoter and part of exon 1. The amino acid sequence of the N-terminal end, which had not been reported, is also shown under the DNA sequence with one letter notation. The transcription and translation start sites are indicated by an arrowhead and an asterisk, respectively. E-box sites, E-box 1 and E-box 2 (surrounded by dotted lines), non-canonical E (NCE)-box sites, NCE-box 1 and NCE-box 2 (underlined), TATA box sequence (shaded), a *Sma*I site (CCCGGG), a *Pvu*II site (CAGCTG), and a *Xho*I site (CTCGAG) are also shown. (B) Chromatin immunoprecipitation using anti c-MYC antibody. HL60 cells in the proliferating phase (TPA) and those treated with 10 nM TPA for 24 h (+TPA) were fixed with 1% formaldehyde, nuclear extracts were collected, and chromatins were immunoprecipitated using antibodies against c-MYC (anti-c-MYC antibody-1 reacting with the N-terminal half of c-MYC and anti-c-MYC antibody-2 reacting with the C-terminal half of c-MYC), control antibody, or no antibody (no antibody). In the presence of TPA, *c-myc* expression was greatly reduced (Fig. 2A). Mock immunoprecipitation, in which no nuclear extract was added, was also performed (nuclear extract -). Following DNA purification, samples were subjected to PCR with primers designed to amplify a DNA fragment of the TAF4b promoter region (TAF4b primers) or primers to specifically amplify a DNA fragment containing a chromosome 22 E-box (control primers) (20).

parental cell into which ectopic c-MYC activity was introduced. A T98G cell line expressing c-MycER protein (T98Gmycer-2 cells) was established previously (23). Total RNAs from T98Gmycer-2 cells in an exponentially growing phase in the presence or absence of OHT for 20 h were subjected to cDNA micro array analysis. Specific signals for

MYC target genes, *ornithine decarboxylase* (17), *nucleolin* (19) and *mina53* (23) were increased by 2.6-, 1.6- and 1.9-fold, respectively, with c-MycER activation. These results suggest that MYC target genes could be detected in this experimental system.

We found that the signal for EST clone AA287145 was stimulated by 2.3-fold with c-MYC activation. cDNA encoding the 5' upstream part of EST clone AA287145 was isolated using the 5'-RACE protocol from a library of human erythroid leukemia (HEL) cells, and it was found that AA287145 was part of the 3' non-coding region of the TAF4b gene (previously called TAF$_{II}$105) (27). Because the translation and transcription start sites of the human TAF4b gene had not been reported, we isolated cDNA encoding the 5'-end of the transcript by 5'-RACE experiments. The transcription start site was mapped on the TAF4b genomic sequence, which is indicated by an arrowhead in Fig. 1A. Using primers consistent with the 5' and 3' sequences of TAF4b mRNA, a 3.2-kb length cDNA was amplified from total RNA of HEL cells by RT-PCR protocol and sequenced. Although the entire nucleotide sequence was almost the same as the previously reported one (27), we found an additional 66-amino acid sequence extending from the N-terminal amino acid glycine of TAF4b reported previously (Fig. 1A), and several amino acids were different (the sequence was deposited in GenBank as accession no. AB234096). The cDNA clone encodes a protein of 862 amino acids with a predicted molecular weight of 91090.52 Da and PI of 9.59.

Expression of TAF4b mRNA. Human promyelocytic leukemia HL60 cells are terminally differentiated by 12-O-tetra-decanoylphorbol-13-acetate (TPA), in which the *c-myc* expression level decreases (30,31), after a transient stimulation (32). This experimental system was used to investigate whether MYC-target genes are affected during the shut-off of MYC (33,34). As shown in Fig. 2A, the level of *c-myc* mRNA had doubled at 1 h after addition of TPA, then started to decline at 3 h, and reached one-fifth at 7 h. The level of TAF4b mRNA had increased to one and a half-fold at 3 h after addition of TPA. At 6 h it started to decline, and reached one-tenth at 9 h. These results show that the expression of TAF4b closely followed that of *c-myc*.

Next, the effect of c-MYC activation on TAF4b mRNA was investigated (Fig. 2B) using the MycER system. The level of TAF4b mRNA had increased to 2- and 3-fold at 3 and 7 h, respectively, in OHT-treated T98Gmycer-2 cells. The OHT treatment of T98G parent cells did not stimulate the TAF4b mRNA level. As shown in Fig. 2C, induction of TAF4b mRNA by OHT in T98Gmycer-2 cells was maintained even in the presence of the protein synthesis inhibitor cycloheximide. Treatment with cycloheximide had little effect on the TAF4b mRNA level in T98G parent cells (data not shown). These results indicate that the TAF4b gene is a direct target of MYC.

c-MYC binds to the TAF4b promoter region in vivo. To examine c-MYC protein binding to the endogenous TAF4b gene *in vivo* during proliferation of HL60 cells, chromatin immunoprecipitation was performed as described previously (35,36). After immunoprecipitation, enrichment of the
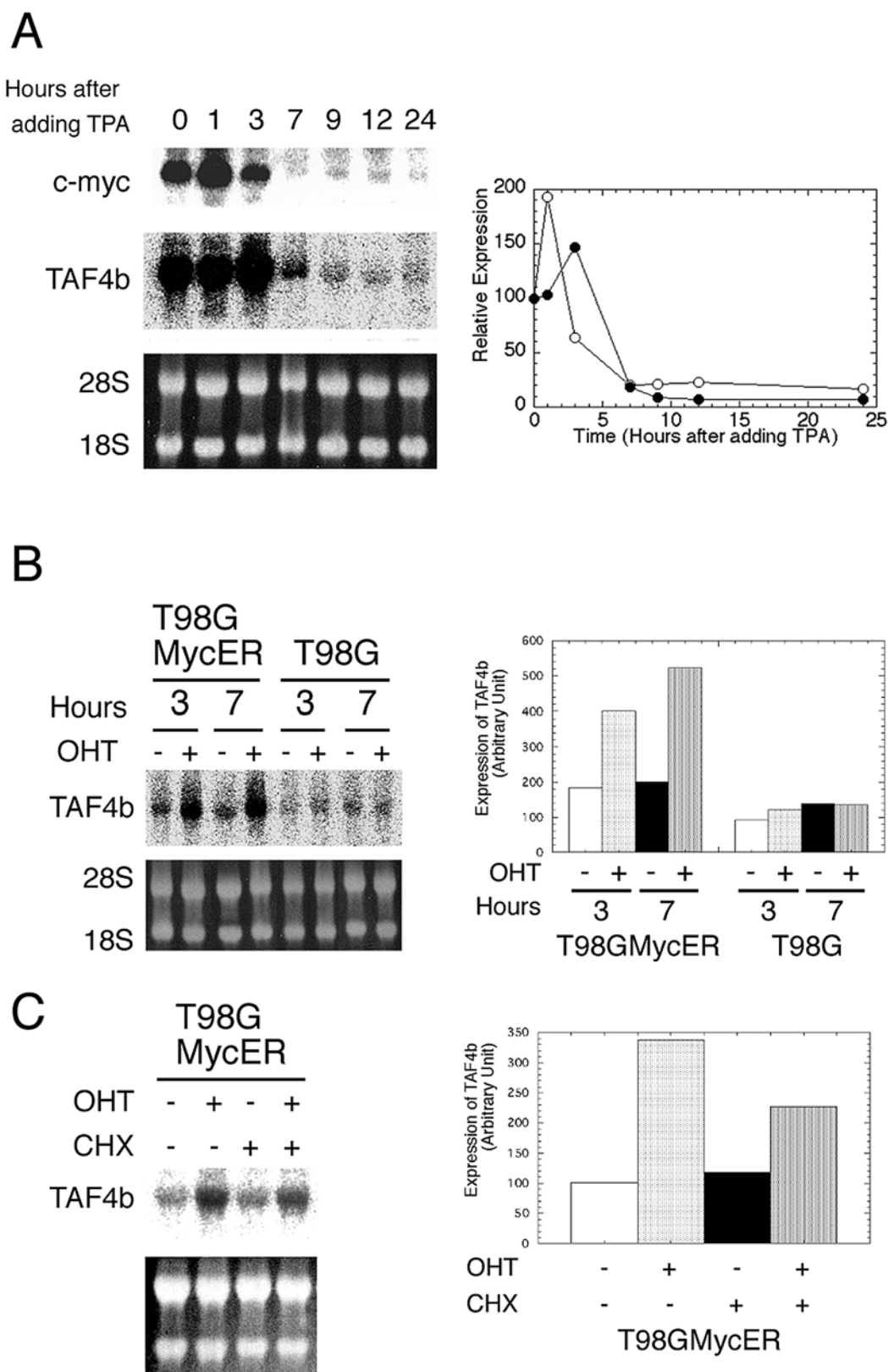
Figure 2. Expression of TAF4b mRNA. (A) The changes of TAF4b and c-*myc* mRNA in human promyelocytic leukemia HL60 cells after TPA treatment. RNA was isolated at the indicated time points after exposure to 10 nM TPA and analyzed by Northern blotting to detect TAF4b and c-*myc* mRNAs (left). 28S and 18S ribosomal RNAs are also shown. The results were quantified and plotted (right). (B) Increase of TAF4b mRNA levels in T98G cells by activation of MycER protein. After T98Gmycer-2 cells (T98GMycER) and parental cells (T98G) were cultured in the medium supplemented with 0.25% serum for 40 h, cells were cultured in the presence (+) or absence (-) of 200 nM OHT. RNA was isolated at the indicated time points and analyzed by Northern blotting to detect TAF4b mRNA (left). 28S and 18S ribosomal RNAs are also shown. The results were quantified and expressed as a bar graph (right). (C) The increase of TAF4b mRNA levels due to activation of MycER protein was maintained in the presence of the protein synthesis inhibitor. After T98Gmycer-2 cells were cultured in the medium supplemented with 0.25% serum for 40 h, cells were treated with 200 nM OHT for 8 h. RNA was isolated and analyzed by Northern blotting to detect TAF4b mRNA (left). When indicated as + in the row of CHX, 20 µg/ml cycloheximide was added 20 min before the addition of OHT. 28S and 18S ribosomal RNAs are also shown. The results were quantified and expressed as a bar graph (right).
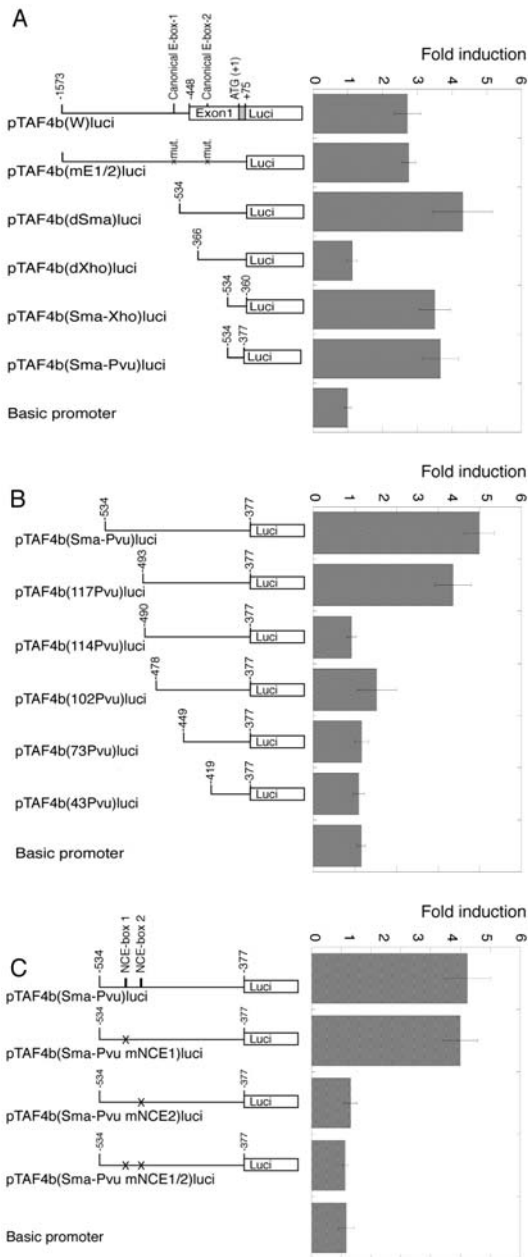
Figure 3. Transient expression from the TAF4b promoter. (A) The reporter plasmid [pTAF4b(W)luci] was constructed to detect the TAF4b promoter activity. In pTAF4b(mE1/2)luci, two E-boxes (CACGTG elements) of pTAF4b(W)luci were mutated to CACCTG (see Fig. 1A). Deletion mutants of pTAF4b(W)luci, pTAF4b(dSma)luci, pTAF4b(dXho)luci, pTAF4b(Sma-Xho)luci, and pTAF4b(Sma-Pvu)luci were also produced. T98Gmycer-2 cells were transfected with the reporter plasmids. One day later, OHT was added at the final concentration of 200 nM to activate the MycER chimeric protein, and cells were further cultured for 17 h. Cell extracts were analyzed for activities of firefly luciferase. The promoter activities were determined as the ratio to activities without MycER activation. To normalize the effect of OHT not through MycER activation, the values from T98Gmycer-2 cells were divided by those from T98G parent cells, which were treated by exactly the same procedures as T98Gmycer-2 cells described above. The basic promoter was a pGL3 plasmid without any insert. Values are the means of four separate experiments. The bars indicate the standard errors. (B) pTAF4b(Sma-Pvu)luci were deleted from the 5'-end to produce pTAF4b(117Pvu)luci, pTAF4b(114Pvu)luci, pTAF4b(102Pvu)luci, pTAF4b(73Pvu)luci, and pTAF4b(43Pvu)luci. The promoter activation by MYC was measured as described in 'A'. (C) Non-canonical E-box sites, NCE-box 1 and NCE-box 2 (see Fig. 1A) of pTAF4b(Sma-Pvu)luci were mutated to produce pTAF4b(Sma-Pvu mNCE1)luci, pTAF4b(Sma-Pvu mNCE2)luci, and pTAF4b(Sma-Pvu mNCE1/2)luci, in which one or both non-canonical E-boxes (CGCGTG elements) were mutated to CGCCTG. The promoter activation by MYC was measured as described in 'A'.

endogenous TAF4b gene fragment in each sample was monitored by PCR using primers that specifically amplify DNA around the transcription initiation site in the TAF4b gene. As shown in Fig. 1B, two different anti-c-MYC antibodies immunoprecipitated the TAF4b DNA fragment from HL60 cells in the proliferating phase, while the same antibodies did not immunoprecipitate detectable levels of DNA fragments from cells treated with TPA, in which c-*myc* expression became very low. Enrichment of TAF4b genomic DNA fragments is dependent on c-MYC binding to the TAF4b gene because a nonspecific antibody did not immunoprecipitate TAF4b DNA fragments. Additionally, binding of c-MYC detected in the TAF4b gene is specific, since antibodies against c-MYC did not enrich the genomic DNA fragment containing an E-box that is located in a chromosomal region without any detectable gene in chromosome 22. These results demonstrate that the TAF4b gene around the transcription initiation site is bound by c-MYC specifically at the proliferating phase of HL60 cells.

Identification of c-MYC-responsive element in the TAF4b promoter. To determine the DNA element(s) through which c-MYC controls the expression of TAF4b, the promoter region of the human TAF4b gene was isolated. In Fig. 1A, 'A' in the first ATG was designated as +1 (indicated by an asterisk). The transcription initiation site was at -460 bp, which is indicated by an arrowhead in Fig. 1A. The TATA box sequence was found ~30 bp upstream of the transcription initiation site.

To investigate the activity of the TAF4b promoter, the genomic DNA fragment including a 1.6-kp region from the upstream region of exon 1 to part of exon 1 was ligated to firefly *luciferase* cDNA to construct a reporter plasmid, pTAF4b(W)luci (Fig. 3A). Transient expression assays in T98Gmycer-2 cells indicated that the DNA fragment has promoter activity. After c-MycER activation by OHT, the luciferase activity was increased up to 2.5-fold (Fig. 3A). This stimulation is comparable to that observed for an increase in the TAF4b mRNA level by OHT in T98Gmycer-2 cells (Fig. 2B).

There are two canonical E-box sites (CACGTG elements) near the transcription initiation site. We designated the first and second CACGTG elements as E-box 1 and E-box 2, respectively. Since it had been reported that CACGTG elements function as MYC-responsive elements in many MYC-responsive genes, we examined whether the E-box sites were involved in activation of the promoter activity by c-MYC. When E-boxes 1 and 2 were mutated, however, luciferase activity was still increased by MYC as in the wild-type reporter plasmid (Fig. 3A, lane for pTAF4b(mE1/2)luci).

Next, the region responsible for MYC stimulation was investigated by using deletion mutants (Fig. 3A). While deletion of the region between -1575 and -535 [pTAF4b (dSma)luci] did not decrease stimulation of the promoter by MYC, deletion of the DNA fragment between -1575 and -367 [pTAF4b(dXho)luci] abolished MYC stimulation, suggesting the importance of the region between -534 and -367. Then we constructed two additional reporters, which contained the sequences between -534 and -360 [pTAF4b(Sma-Xho)luci] and -534 and -377 [pTAF4b(Sma-Pvu)luci], respectively. The expression of these two reporters was well stimulated by MYC.
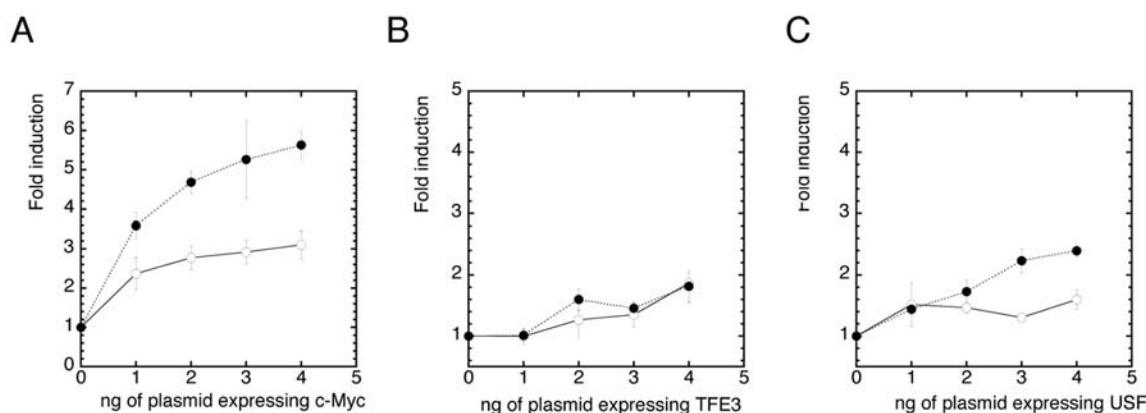
Figure 4. Comparison of the wild-type TAF4b promoter to the one with the canonical E-box. (A-C) The effects of c-MYC, TFE3, and USF expression on the TAF4b promoter activity, pTAF4b(Sma-Pvu)luci (○) and pTAF4b(Sma-Pvu CACGTG)luci (●), in which NCE-box 2 (CGCGTG element) was mutated to the canonical one (CACGTG), were examined. T98G cells were transfected with the reporter plasmids and pCMV-c-Myc (A), pCMV-TFE3 (B), or pCMV-USF (C), together with pRL-CMV (for standardization of the transfection efficiency). Luciferase activities were expressed as the ratio to expression without the effector plasmids. The amounts of the effector plasmids are shown on the X-axis. Values are the means of four separate experiments. The bars indicate the standard errors.

To find the responsible element for MYC-activation, the DNA fragment in the reporter [pTAF4b(Sma-Pvu)luci] was deleted sequentially from the 5'-end of the TAF4b promoter (Fig.3B). While the deletion of the region between -534 and -492 [pTAF4b(117Pvu)luci] marginally reduced the elevation by MYC, deletion of -534 to -490 [pTAF4b(114Pvu)luci] abolished the elevation by MYC. The expression by the reporters with further deletions [pTAF4b(102Pvu)luci, pTAF4b(73Pvu)luci and pTAF4b(43Pvu)luci] was not elevated by MYC. These results suggest the importance of the region between -534 and -490, especially between -492 and -490.

The region between -492 and -490 is in a non-canonical E-box element CGCGTG (-492 to -487 in Fig. 1A). It has been reported that c-MYC could bind this sequence (6). Then we tested whether CGCGTG elements were involved in activation by MYC. There are two CGCGTG elements very near the TATA box sequence (Fig. 1A). We designated the first and second CGCGTG elements as non-canonical E (NCE)-box 1 (-509 to -504) and NCE-box 2 (-492 to -487), respectively. We constructed the reporter plasmids pTAF4b(Sma-Pvu, mNCE1)luci, pTAF4b(Sma-Pvu, mNCE2)luci and pTAF4b (Sma-Pvu, mNCE1/2)luci, whose one or both CGCGTGs were mutated to CGCCTG (Fig. 3C). While mutation of NCE-box 1 had little effect on the stimulation of promoter activity by c-MYC, mutation of NCE-box 2 or of both NCE-box 1 and NCE-box 2 completely abolished the MYC-stimulation. These results indicate that c-MYC transactivates the TAF4b promoter through NCE-box 2.

The response of CGCGTG and CACGTG elements to E-box binding transcription factors. In many genes, a sequence of MYC responsive E-box is CACGTG, rather than CGCGTG. To test whether the sequence CGCGTG has any biological significance, NCE-box 2 (CGCGTG) was mutated to the canonical E-box (CACGTG). The promoter with CACGTG element was stimulated by c-MYC and the elevation was higher than that of the wild-type promoter (Fig. 4A).

Besides proteins in the *myc* proto-oncogene family, there are other E-box-binding transcription factors, including USF

(25) and TFE3 (26). Then we examined how these E-box-binding transcription factors responded to CACGTG and CGCGTG elements. As shown in Fig. 4B and C, the wild-type TAF4b promoter was hardly stimulated by TFE3 and USF. These results suggest that the TAF4b promoter specifically responds to MYC. However, when the CGCGTG element was mutated to the canonical CACGTG, the expression from the TAF4b promoter was stimulated by USF. TFE3 still hardly stimulated the expression from the reporter.

The MYC-responsive E-box element and the surrounding sequence in the TAF4b promoter is conserved in mammals. Since the sequence between -493 and -461 appears to be critically important for MYC activation, it was curious to test whether this sequence also exists in other species. We isolated a 1.2 kb fragment of the rat TAF4b genomic DNA containing the promoter region, and sequenced it. Fig. 5A shows the difference in the nucleotide sequences between the human and rat TAF4b promoter regions. The nucleotide diversity was calculated using the average number of nucleotide differences in sliding and overlapping windows of 100 bp, placed at 10 bp intervals. In this analysis, an insertion/deletion (indel) was treated as a single mutation. The minimum values of nucleotide diversity, namely the strongest homology, were observed around NCE-box 2 (location 792). Low values of nucleotide diversity were also observed around location 1000. This may be attributed to the fact that, in those locations, there are several large indels, which caused the nucleotide diversity to be underestimated. When the human TAF4b promoter sequence was compared to that of mouse, whose DNA sequence was recently deposited in the GenBank (accession no. AC133172.4), similar results were obtained (data not shown).

We isolated the TAF4b promoter DNA fragment of monkey from African green monkey kidney fibroblast Cos-7 cells and sequenced it. The nucleotide sequences around NCE-box 2 in human, monkey, rat and mouse are compared in Fig. 5B. The 29 bp sequence containing NCE-box 2 and the TATA box element are completely conserved among the four species studied.
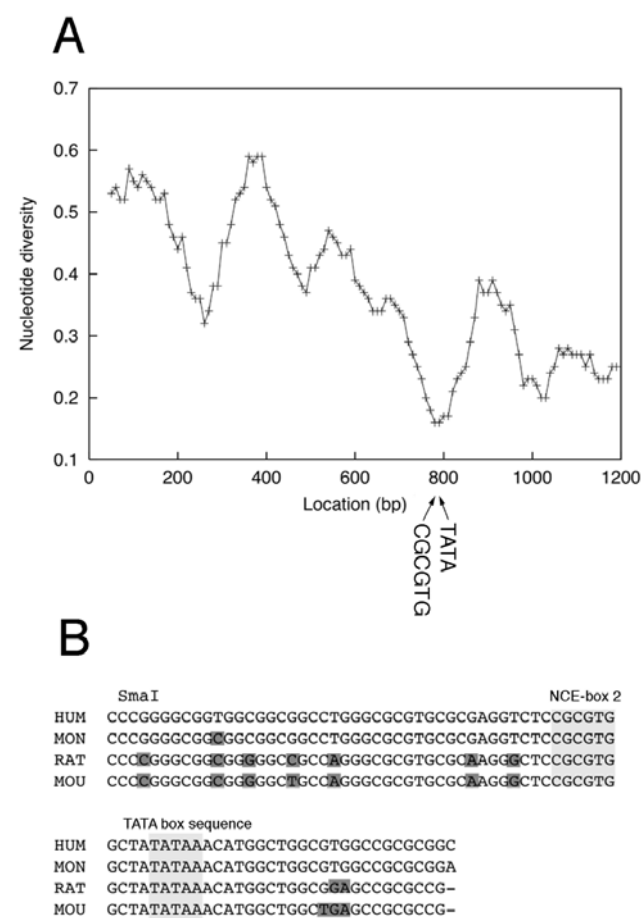
A

B

Figure 5. TAF4b promoters of other species. (A) Comparison of the human and rat TAF4b promoter sequences. In this analysis, highly homologous regions give a low score (see text). The position of NCE-box 2 (CGCGTG) and the TATA box sequence (TATA) are indicated (see Fig. 1A). (B) Comparison of the sequences around the CGCGTG element in the human (HUM), monkey (MON), rat (RAT), and mouse (MOU) TAF4b promoters. NCE-box 2 and TATA box sequences are lightly shaded. The nucleotides that are different from the human TAF4b promoter are darkly shaded. The *Sma*I site in the human promoter is also shown.

Discussion

c-MYC induces TAF4b expression through an E-box site, which is in a highly conserved region of TAF4b promoters in mammals. We found evidence that MYC directly induces the expression of TAF4b. After treatment with TPA, the expression of TAF4b mRNA closely followed that of c-*myc* in HL60 cells (Fig. 2A). Stimulation of the expression of TAF4b mRNA was observed when c-MYC was activated in the c-MycER chimeric protein (Fig. 2B). This induction was maintained even in the presence of a protein synthesis inhibitor (Fig. 2C), indicating that c-MYC directly controls the expression of TAF4b. c-MYC protein bound the TAF4b promoter *in vivo* in proliferating HL60 cells (Fig. 1B). The TAF4b promoter activity was elevated by c-MYC (Fig. 3). This elevation was mediated through an E-box (NCE-box 2; a CGCGTG element) (Fig. 3).

All E-boxes are not always MYC-responsive elements. The MYC-responsive E-box in the TAF4b promoter exists in the most conserved region between human and rat (Fig. 5A) where GC content is high (Fig. 1A). The 29 bp-nucleotide

sequence covering the MYC-responsive element and TATA box is completely conserved in human, monkey, rat, and mouse (Fig. 5B), suggesting that the distance from the E-box site to the transcription start site is conserved between species. The high conservation of the DNA sequence suggests that the interacting proteins on the DNA region are conserved. There are several E-boxes besides the MYC-responsive E-box near the promoter region. Thus, the characteristics proposed to be used for specific protein-DNA interaction, including high GC content (1), preferential spacing between transcription factor binding sites and transcription start sites (2), co-operativity of cis-regulatory molecules (3), and clustering of transcription factor binding sites near the target sites (4), are observed around this E-box site in the TAF4b promoter and may be involved in the mechanisms by which NCE-box 2 specifically functions as a MYC-target site.

The MYC responsive E-box in the TAF4b promoter is a non-canonical (CGCGTG) element. When the CGCGTG element was mutated to the canonical CACGTG, c-MYC still up-regulated the promoter activity (Fig. 4A). While USF, another E-box-binding transcription factor, hardly stimulated the wild-type TAF4b promoter, it up-regulated the activity of the promoter with the CACGTG element (Fig. 4C). These results suggest that in addition to the context where the target E-box exists, a sequence within an E-box is involved in the mechanisms by which the E-box site is selected specifically by MYC.

When the CGCGTG element was mutated to the canonical CACGTG, c-MYC more strongly up-regulated the promoter activity (Fig. 4A). It was shown that c-MYC binds to CACGTG E-box element with higher affinity than other E-boxes (37). The decreased sensitivity of the non-canonical E-box with lower affinity to MYC may contribute to the proper gene activation by MYC. This idea is consistent with a recent study, which indicates that the affinity of binding sites is a primary determinant of the level of physiological stimulus necessary for substantial gene activation, contributing to fine-tuning the responses (38). TFE3, another E-box-binding transcription factor, activated the TAF4b promoter with neither CACGTG element nor CGCGTG element (Fig. 4B). Together, these results indicate that the sequences within E-box differently affect the activities of E-box-binding transcription factors and function as a factor to determine the spectrum of genes stimulated by the transcription factors.

TAF4b is a Myc-target gene. In eukaryotes, protein-encoding genes are transcribed by RNA polymerase II (RNA Pol II). The molecular machinery that guides RNA Pol II to initiate transcription of a specific gene is composed of multiple classes of regulatory proteins (39). TFIID is a core component of the RNA Pol II regulatory machinery, which is conserved from *Saccharomyces cervisiae* to humans and is a large multi-protein complex composed of the TATA box-binding protein (TBP) and several TBP-associated factors (TAFs). Although TFIID was initially thought to be ubiquitous in expression and function, identification of tissue-specific TAFs suggested that a specialized TFIID complex could play a direct role in regulating tissue-specific programs of gene expression. The first cell type-specific TFIID subunit identified was TAF4b

(previously called TAF$_{II}$105) (27), which is a homologous protein of a ubiquitously expressed factor, TAF4 (previously called TAF$_{II}$130). The comparison between the three dimensional structure of the TFIID complex with TAF4b and that with original TAF4 suggest that distinct localized structural changes in TFIID may cause a difference in their activity (40). Indeed, it was reported that TAF4b and TAF4 proteins share a common non-redundant function in regulating gene expression control (41,42). These findings show that the expression control of TAF4b is a physiologically important issue. However, there have been no studies on the mechanisms of the TAF4b expression control.

It is well established that MYC is a central regulator of cell growth. There are studies describing functions and expression of TAF4b, which suggest the functional and expressional connection between TAF4b and MYC. In adult mouse testes, TAF4b was required for germ cell proliferation (43), and the expression of TAF4b and c-*myc* was detected in premeiotic spermatogonia (43-45). When primary B lymphocytes were activated by bacterial lipopolysaccharide (LPS), expression of TAF4b (46) and c-*myc* (47) was increased. TAF4b can induce a number of genes involved in the control of cell growth (41). It was also reported that TAF4b elevates the expression of VEGF (42), while vasculogenesis and angiogenesis were induced by MYC, which can indirectly induce the expression of VEGF (48). Therefore, TAF4b may be involved in the induction of VEGF expression by MYC (49). Together, the above studies suggest that the induction of TAF4b induced by MYC may contribute to cell growth control and tumorigenesis.

Acknowledgements

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