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Self-Regulation of Mouse p45/NF-E2 during Murine Erythroleukemia Cell Differentiation

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Tung-Liang Lee, Shau-Ching Wen, Wei-Yuan Hsiao, Che-Kun James Shen, and Yu-Chiau Shyu (2009) Self-regulation of mouse p45/NF-E2 during murine erythroleukemia cell differentiation. *Zoological Studies* **48**(3): 362-369. The complicated process of murine erythroleukemia (MEL) cell differentiation is precisely controlled by a group of transcription factors. One of those transcription factors, p45/NF-E2, is important for globin gene expression. We analyzed the structure of the mouse *p45* gene which contains a putative nuclear factor erythroid-derived 2 (NF-E2) which binds to the Maf recognition element (MARE) located upstream of the erythroid-specific *p45* promoter. Chromatin immunoprecipitation (ChIP) assays showed that p45/NF-E2 bound to this MARE-like region during the period of dimethyl sulfoxide (DMSO)-induced MEL differentiation. Moreover, the ChIP analysis also showed that p45/NF-E2 binding to the GATA-1 binding region of the erythroid-specific *p45* promoter was similar to the binding to the MARE-like region. Analysis of the *p45* expression profile corresponded to the *p45* promoter binding capacity of p45/NF-E2 during MEL cell differentiation. This evidence suggests that the MARE-like binding site might function as an enhancer that interacts with the GATA-1 binding motif within the *p45* promoter to mediate the upregulation of *p45* mRNA in erythroid differentiation. Furthermore, we also found that the MARE binding repressor, Bach1, did not bind to the *p45* promoter, thus excluding any involvement of Bach1 in *p45* gene regulation before MEL differentiation. Together these results suggest that p45/NF-E2 self-regulation is a positive enhancer regulatory mechanism, which differs from the MARE-dependent regulatory mechanism that contributes to the rapid upregulation of p45/NF-E2 required for erythroid differentiation. http://zoolstud.sinica.edu.tw/Journals/48.3/362.pdf

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The hematopoietic activator nuclear factor erythroid-derived 2 (NF-E2) is a functional heterodimer, composed of an erythroid-specific p45 subunit, and ubiquitously expresses small Maf family subunits, which play critical roles in erythroid differentiation and megakaryocyte maturation (Andrews et al. 1993, Andrews 1998). NF-E2 is a positive regulatory transcription factor that recognizes the specific Maf recognition element (MARE) sequence, GCTGA(G/C)TCAGCA, present in the β globin gene and several genes essential to the heme biosynthesis pathway (Mignotte et al. 1989b, Ney et al. 1990, Cox et al. 1991, Tugores et al. 1994). The importance of NF-E2 is evident in the *p45*-deficient mouse erythroleukemia cell line (CB3) that shows deficient erythroid differentiation. *p45*-null CB3 cells fail to express large amounts of globin messenger (m)RNA, although this can be rescued by reintroducing the expression of p45/NF-E2 (Lu et al. 1994, Kotkow and Orkin 1995). The abnormal expression of p45/NF-E2 has been shown to have different effects on cell proliferation and differentiation (Labbaye et al. 1995, Sayer et al. 2000, Li et al. 2001). Overexpression of NF-E2 promotes erythropoietin (EPO)-independent erythroid maturation and also reprograms precursor cells differentiating into erythroid and megakaryocytic lineages (Sayer

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et al. 2000). Downregulation of *p45* expression diminishes erythroid colony formation and promotes tumorigenesis by accelerating the cell proliferation rate (Labbaye et al. 1995, Li et al. 2001). In terminal erythroid differentiation of erythroid cell lines including MEL and K562, NF-E2 is activated, and levels rise following treatment with specific chemical inducers such as dimethyl sulfoxide (DMSO), hexamethylene bisacetamide (HMBA), and hemin (Ross and Sautner 1976, Fibach et al. 1977, Nudel et al. 1977). After erythroid differentiation, NF-E2 binds to MARE on the enhancer or promoter region, and then turns on the expressions of erythroid-specific genes required for hemoglobin synthesis (Mignotte et al. 1989a, Andrews 1994, Tugores et al. 1994, Surinya et al. 1997, Chenais 1998). Thus, precise regulation of NF-E2 expression is critical for erythroid differentiation.

The *p45* gene has a GATA-1-dependent alternative promoter (promoter 1b) that regulates the transcription of alternative isoforms. One of the *p45* transcripts regulated by promoter 1b has been suggested to be more erythroid-specific (Moroni et al. 2000, Toki et al. 2000). The GATA-1 binding motif is known to be a critical *cis*-element for erythroid-specific *p45* promoter activity. Although *p45* expression obviously increases after erythroid differentiation, the transcriptional regulation of *p45* expression during erythroid differentiation has not yet been investigated and remains unclear (Nagai et al. 1998, Chenais 1998, Brand et al. 2004).

We investigated the mechanisms underlying the regulatory mechanism of *p45* gene expression during early to late erythroid differentiation of MEL cells. MEL cells are widely used as a model of *in vitro* erythroid differentiation and provide a useful system to study molecular regulatory mechanisms of terminal erythroid differentiation (Harrison 1976). In this study, we used chromatin immunoprecipitation (ChIP) to analyze the upstream region of the *p45* gene. The first intron region of the *p45* gene shows a putative MARE-like sequence, which potentially binds small Maf-associated transcription factors including NF-E2, Bach1-Maf, Nrf1, and Nrf2 (Nagai et al. 1998, Igarashi et al. 1998, Igarashi 2000). The transcriptional activity of MARE-dependent genes is regulated by recruiting these repressors or activators (Brand et al. 2004, Sun et al. 2004): for example, the enhancer activity of the α globin, HS-40, and β globin, LCR, are regulated by the binding exchange between NF-E2 and Bach1-MafK that mediates the expression of α and β globins (Igarashi et al. 1998, Sawado et al. 2001, Brand et al. 2004, Tahara et al. 2004).

We first demonstrated the binding of endogenous p45/NF-E2 to the MARE-like motif located in the first intron region of the *p45* gene after MEL differentiation. In addition to the binding of the MARE-like region, p45/NF-E2 also bound to the GATA-1-binding region after DMSO-induced MEL differentiation, and these bindings were more enriched at a later stage of erythroid differentiation. In contrast to the regulatory mechanism of the β globin, LCR, the long distance between the MARE-like motif and promoter 1b implies that this p45/NF-E2-bound MARE-like region has an enhancer function for promoter 1b. This interesting observation suggests that this self-regulating mechanism might lead to the speedy revival of *p45* mRNA levels to overcome the dramatic drop in transcription of the *p45* gene at the beginning of DMSO-induced erythroid differentiation and further enrich the expression of *p45* for erythroid differentiation.

MATERIALS AND METHODS

Cell culture

The murine adult erythroleukemia cell line (MEL; Nudel et al. 1977) was cultured in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Carlsbad, CA, USA) containing 10% fetal bovine serum (FBS) (Hyclone, Logan, UT, USA), 50 U/ml of penicillin, and 50 μg/ml of streptomycin (Invitrogen). MEL cell differentiation was induced by DMSO treatment: MEL cells were cultured in the presence of 2% DMSO at a cell density of 5 x 10^5 confluent cells. Cells were cultured at 37°C in a 5% CO_2 atmosphere and harvested at various times after induction.

Antibodies. The anti-p45/NF-E2 rabbit antibody, C-19, was purchased from Santa Cruz (Santa Cruz, CA, USA) and the anti-Bach1 antibody (polyclonal rabbit antiserum A1-6) was kindly provided by Dr. K. Igarashi (Tohoku Univ., Sendai, Japan) (Sun et al. 2004).

Chromatin immunoprecipitation (ChIP)

A ChIP analysis was conducted as described in Daftari et al. (1999). MEL cells were cultured to confluent growth (5 x 10^6 cells/ml) in DMEM without or with DMSO induction for 24, 48, and

72 h. Cells were harvested and adjusted to 10^7 cells/ml then fixed in 10 ml of DMEM containing 1% formaldehyde for 10 min at room temperature. After sonication to break the DNA into small pieces, protein-DNA complexes were immunoprecipitated with the anti-p45 and anti-Bach1 antibodies. The precipitated chromatin DNAs were purified and amplified by a polymerase chain reaction (PCR) consisting of 95°C for 7 min, followed by 34 cycles at 94°C for 30 s, 55°C for 40 s, and 72°C for 40 s, using the following mouse-specific primers: β-actin (sense: 5'-ATTGTGATGG ACTCCGGAGAC-3' and antisense: 5'-GCCAATA GTGATGACCTGGC-3'), p45/NF-E2 (sense: 5'-CT GGAGGAGCAGGGGAGTGAA-3' and antisense: 5'-CCTCC TGCTACTAGCAGCCAAG-3'), promoter 1b (sense: 5'-GATAAAGGGTAAAGGTCCAGTGT C-3' and antisense: 5'-TGTGTTGTGGGAAGTGTT AGTCAG-3'), and HS2 (sense: 5'-CACTTCTTCAT ATTCTCTCTCTAG-3' and antisense: 5'-CTTATTT TCTTTTCACCTTCCCTG-3'). The β-actin intensity was used as the reference for the amounts of DNA in the PCR. Different target/β-actin ratios were further normalized against input samples. The relative intensities of the positive signals are shown as histograms of the relative increase over the pre-immune samples, where each bar represents the mean ± SEM of data derived from 2 or 3 PCR analyses from at least 2 different ChIP samples.

Northern blot analysis

Northern blot hybridization followed the procedures of Liu et al. (2003). Total RNAs were extracted with commercial Trizol reagent (Invitrogen) from MEL cells. Aliquots (5 μg) of each RNA sample were separated on a 1% agarose, 1 x MOPS (20 mM MOPS, 1 mM EDTA, and 5 mM sodium acetate), and 6% formaldehyde gel, transferred to a nylon membrane, prehybridized at 37°C for 6 h, and then hybridized with radioactive DNA probes at 37°C overnight. The probes were labeled with ^{32}P-dCTP by a random primer labeling system (GE Healthcare Life Science, NJ, USA). The radioactive signal was visualized by autoradiography, and GAPDH was used as a control.

Real-time PCR and data analysis

For the gene expression analysis, total RNA was isolated from cells using the Trizol reagent and reverse-transcribed using SuperScriptII according to the manufacturer's protocol (Invitrogen). Mouse p45 and GAPDH mRNA levels were determined by real-time PCR using appropriate primers from Mission Biotech (Taiwian, ROC): mouse p45 (sense: 5'-CCCCGTGTCCTCCTCAGCAGAA-3' and antisense: 5'-GGTGGGTGCTTGAGGCTCA A-3') and mouse GAPDH (sense: 5'-GCTACACT GAGGACCAGGTTGTC-3 and antisense: 5'-GAA GGTGGAAGAGTGGGAGTTG-3). PCR assays were performed using an Applied Biosystems 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) with the following profile: 1 cycle at 50°C for 2 min, 1 cycle at 95°C for 10 min, and 40 cycles each at 95°C for 15 s and 60°C for 1 min. The threshold cycle (Ct) was calculated by the instrument's software (7500 System SDS software vers. 1.3.1). GAPDH served as an internal control, and the relative amount of *p45* mRNA was normalized to GAPDH. Data are presented as histograms where each bar represents the mean ± SEM of data derived from 3 real-time PCR analyses.

RESULTS

Binding of p45/NF-E2 to the MARE-like region of the *p45* gene *in vivo*

The mouse p45 genomic sequence was previously identified (Moroni et al. 2000); the 1st promoter/1st exon a (promoter 1a) and 2nd promoter/1st exon b (promoter 1b) regions function as alternative promoters that regulate the splicing of the *p45* gene to form 2 different transcripts (Fig. 1). The 1b transcript was abundant in erythroid cells including MEL cells (lower transcript of Fig. 1) (Moroni et al. 2000). Here, we focused on the transcriptional regulation of this erythroid-specific *p45* transcript. The 2nd alternative promoter was demonstrated to contain a GATA-1-binding motif, and GATA-1 binding activates the activity of promoter 1b of *p45* (Moroni et al. 2000). Further analysis of the first intron region of the *p45* gene revealed a putative MARE-like sequence (nucleotides 580-587, TGCTGACT) (Fig. 1), which can potentially bind different transcriptional activators and repressors. Moreover, this MARE-like sequence was about 3 kilobase pairs (kb) away from the downstream promoter 1b, which suggests that it might function as an enhancer and be regulated by MARE binding factors.

Most MARE-dependent genes are regulated by recruiting activator or repressor complexes. In contrast to the upregulation of NF-E2 after

erythroid differentiation, the presence of the MARE-like sequence implies that regulation of *p45* expression might also be coordinated by replacing activators and repressors on MARE. A more-interesting possibility is self-regulation due to the binding of NF-E2 itself. To test whether the p45/NF-E2 complex can bind to this MARE-like region, ChIP assays were carried out using a p45/NF-E2 antibody to analyze the *p45* gene in MEL cells treated with DMSO. Surprisingly, p45/NF-E2 bound to this MARE-like region after DMSO induction, but this event was not observed in non-induced MEL cells (Fig. 2A).

p45/NF-E2 binding to the GATA-1 region of promoter 1b

The promoter 1b contains a tandem palindromic GATA-binding site, which bound to GATA-1. In this study, we assumed that the p45/NF-E2-bound MARE-like region might act as an enhancer, and the present evidence suggests that enhancers can functionally interact with promoters independent of orientation and distance. We applied a p45 ChIP assay to the GATA-1-binding region to examine whether p45/NF-E2 can interact with promoter 1b through physical interaction between the enhancer and promoter. The results showed an elevated binding capacity of p45/NF-E2 to the GATA-1-binding region within the promoter 1b region, even though there was no p45/NF-E2 binding sequence in this region (Fig. 2B). These results suggest that this MARE-like motif could be an enhancer element which interacts with GATA-1 to co-regulate *p45* gene expression.

The transcriptional repressor, Bach1, did not bind to the MARE-like region of the *p45* gene

Switch activation or silencing of MARE-dependent genes invariably proceeds through binding of antagonistic transcription factors. In order to determine whether *p45* expression is also modulated by this mechanism, we estimated the binding of the p45/NF-E2 antagonistic repressor, Bach1, to the same MARE-like site. The Bach1 ChIP result showed no binding to the same region of the *p45* gene in either non-induced or induced MEL cells (Fig. 2C). These results indicate that p45/NF-E2 binding to this MARE-like region during

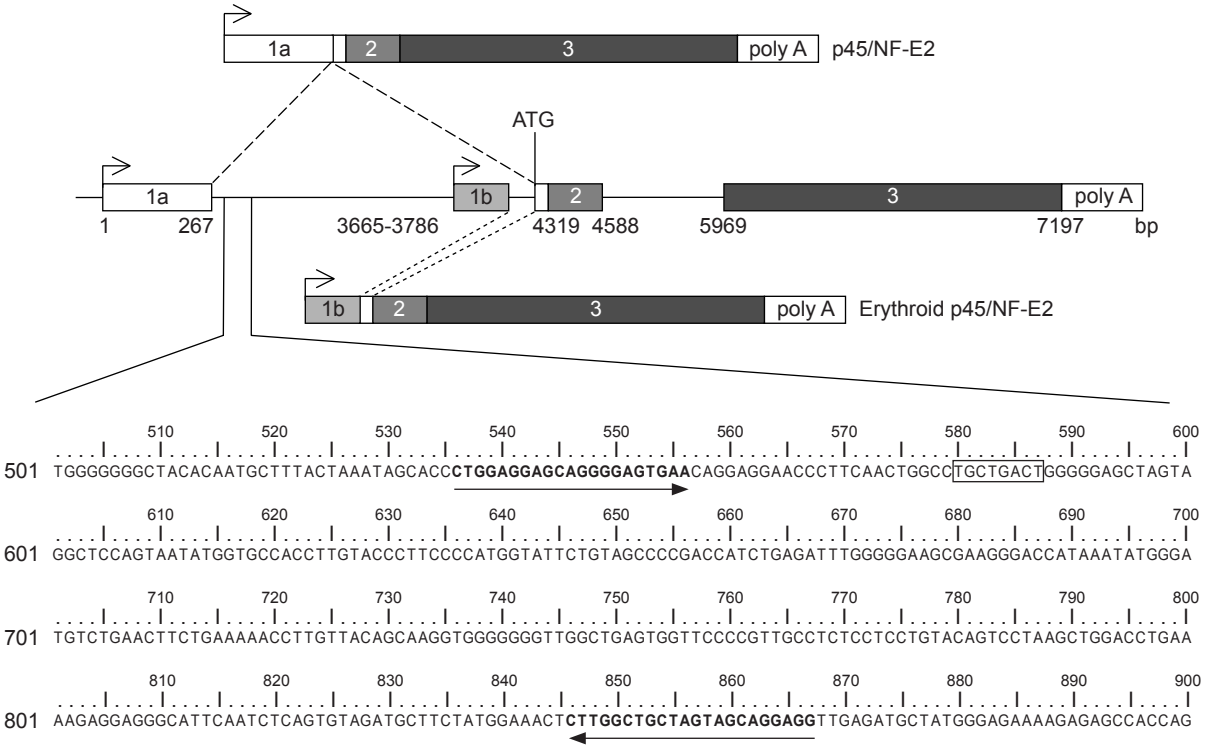

Fig. 1. Schematic of the *p45* genomic sequence. The 2 alternative splicing isoforms of p45/NF-E2 (A, top and B, bottom) transcribed from the *p45* gene (middle). Arrows indicate the transcription start site; the putative MARE-like sequence is boxed; and the primer locations for the chromatin immunoprecipitation (ChIP) assay are indicated by underlined arrows.

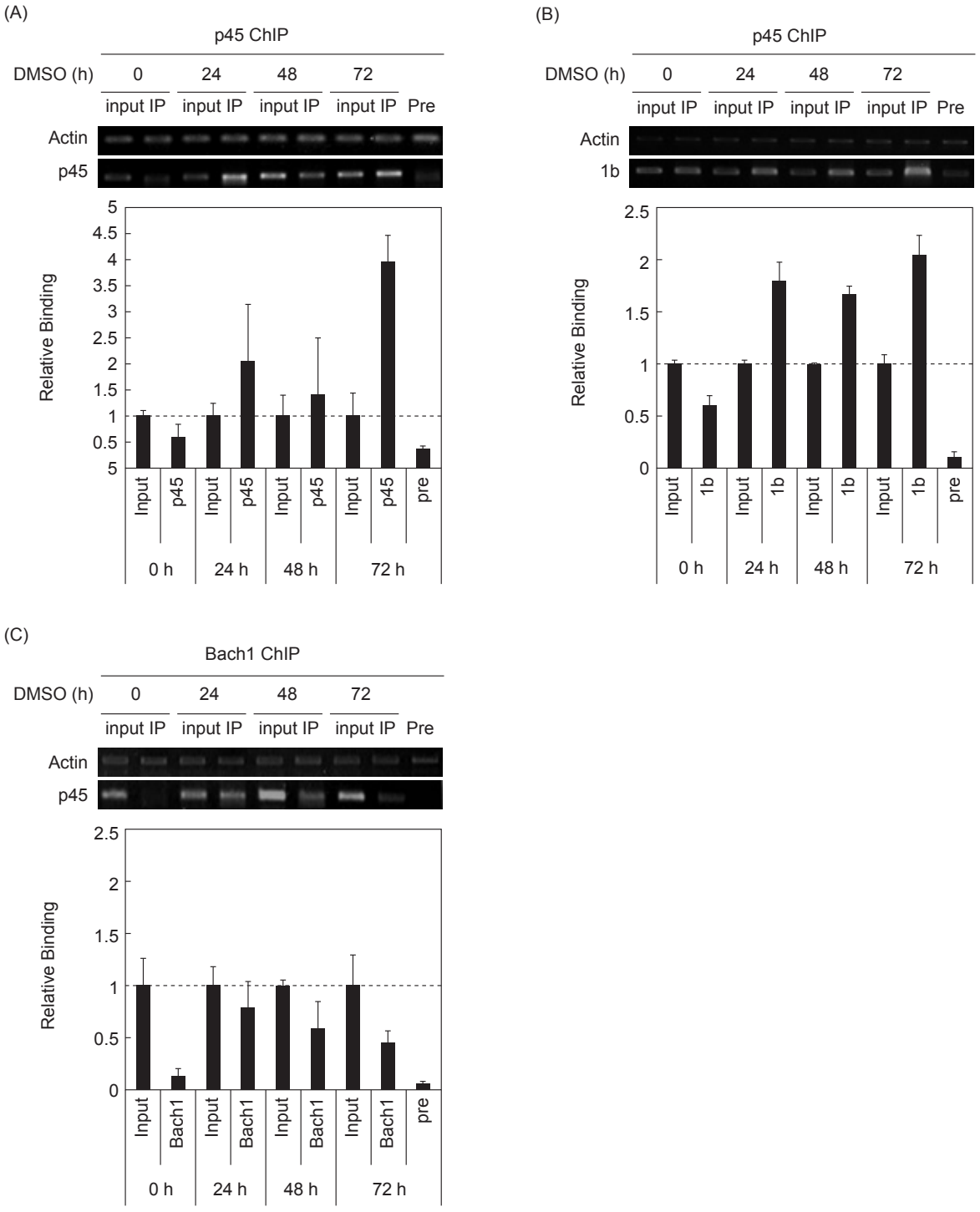

Fig. 2. Chromatin immunoprecipitation (ChIP) assay of the association of p45/NF-E2 with the mouse *p45* gene *in vivo* in DMSO-induced MEL cells. DNA fragments from MEL cells before and 24-72 h after DMSO induction were precipitated using an anti-p45 antibody (A, B) or an anti-Bach1 antibody (C) then analyzed by PCR using primers specific for the *p45* gene. The MARE region of the *p45* gene (A, C) and GATA-1-binding region of the *p45* promoter 1b (B) were targeted for PCR amplification. The PCR products were analyzed by 1.5% agarose gel electrophoresis. The DNA amounts used for PCR were quantified by the intensities of the β-actin signals. The PCR signals were first normalized to those from the β-actin region. Different target/β-actin ratios were then further normalized against the target/β-actin ratios of the input samples, and used to plot the histograms. The relative intensities of PCR signals from the p45 ChIP assay (A, B) and Bach1 ChIP assay (C) were compared to the pre-immune samples. Each histogram shows the mean ± SEM of 2 or 3 PCR analyses of chromatin DNA precipitated from 3 individual differentiation experiments. Negative controls of the ChIP samples were prepared with pre-immune serum.

erythroid differentiation is Bach1-independent, and show that the general competition mechanism between activator and repressor binding to MARE is not a factor in this self-regulation of *p45* gene expression during differentiation. This p45/NF-E2 self-regulation mechanism might be similar to the binding of GATA-1 to its promoter during erythroid differentiation (Tsai et al. 1991, Kobayashi and Yamamoto 2007).

Upregulation of mouse *p45* gene expression after MEL differentiation

Although previous studies illustrated an increase in *p45* expression after erythroid differentiation, no evidence was presented of the particular mechanism of this regulation. To further confirm that the binding of p45/NF-E2 to its MARE-like motif also plays a positive regulatory role, we carefully observed *p45* expression during DMSO-induced MEL cell differentiation. We estimated the *p45* transcript level during DMSO-induced MEL differentiation by Northern blot and real-time PCR analyses. Both assays showed that *p45* was upregulated after DMSO induction for 24 h (Figs. 3A, B), which agrees with previous studies showing increased *p45* mRNA after erythroid differentiation for 72 and 96 h (Chenais 1998, Brand et al. 2004). Moreover, we assessed the expression during the first 24 h of DMSO induction, which revealed that *p45* mRNA expression quickly recovered after DMSO treatment for 24 h (Fig. 3C). This result suggests that the p45/NF-E2 self-upregulation mechanism might also function in quickly increasing the amount of p45/NF-E2, so that it is sufficient for erythroid differentiation.

DISCUSSION

The aim of this study was to establish whether the MARE-like sequence located within the first intron of the *p45* gene can bind to p45/NF-E2 and upregulate *p45* expression, which is

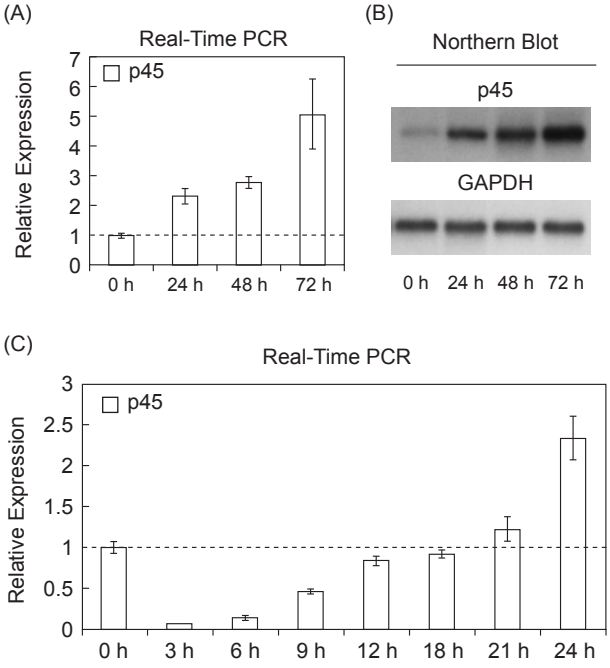

Fig. 3. Comparison of *p45* expression levels in response to MEL differentiation. Gene expression level of *p45* in MEL cells before and after DMSO induction were analyzed by (A) real-time PCR and (B) Northern blotting, respectively. (C) *p45* mRNA expression levels during the first 24 h of DMSO induction were also analyzed by real-time PCR. As the control in the Northern blot and real-time PCR, expression levels of GAPDH were also analyzed.

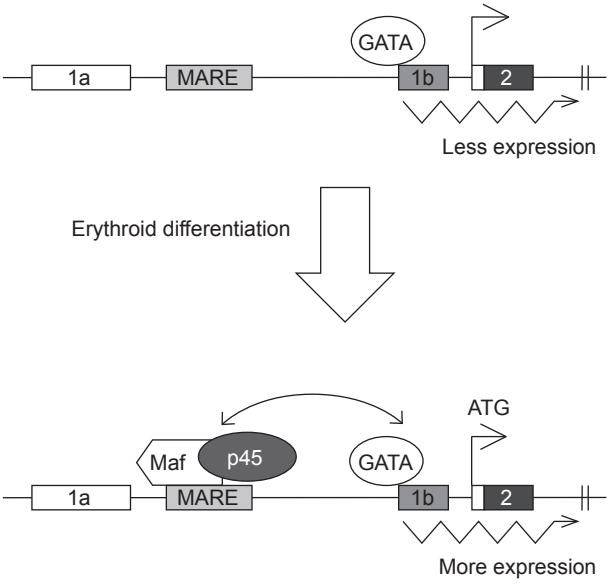

Fig. 4. Hypothetical model of p45/NF-E2 self-upregulation during MEL cell differentiation. *p45* expression is regulated by the GATA-dependent promoter 1b at lower expression levels before MEL cell differentiation. Upon induction by DMSO, p45/NF-E2 is urgently needed to turn on hemoglobin synthesis-related genes, and thus p45/NF-E2 binding to its MARE-like motif functions as an enhancer and further accelerates GATA-dependent promoter 1b mediated erythroid-specific *p45* mRNA expression during erythroid differentiation.

similar to other specific targets of NF-E2 during MEL cell differentiation. We demonstrated that p45/NF-E2 was efficiently bound to this MARE-like region specifically during MEL differentiation. In contrast to p45/NF-E2 binding to 5'HS2 of the β globin LCR and the β major promoter of the β globin gene (Sorrentino et al. 1990, Sawado et al. 2001), we suggest that p45/NF-E2 binding to the GATA-1 region of *p45* promoter 1b also through physical and functional interactions between the enhancer and promoter.

These observations suggest that p45/NF-E2 functions as an activator that potentially regulates itself through binding to the MARE-like motif to quickly upregulate *p45* mRNA levels after erythroid differentiation. This p45/NF-E2 self-regulatory mechanism is similar to the binding of GATA-1 to its promoter during erythroid differentiation (Tsai et al. 1991, Kobayashi and Yamamoto 2007). In contrast to the continually low level of GATA-1-activated *p45* expression, the self-regulation of p45/NF-E2 may be important for the accelerated expression of erythroid-specific *p45* transcripts and may be required for activation of numerous p45/NF-E2-regulated genes which initiate erythroid differentiation. This interpretation of all these results suggests a simple model of *p45* regulation through 3 steps during erythroid differentiation (Fig. 4). First, the GATA-1-dependent *p45* transcript is constitutively expressed at a low level before differentiation. During the early differentiation stage, p45/NF-E2 binding to the *p45* MARE-like motif cooperates with GATA-1 to accelerate the expression of *p45* mRNA to recover the rapid decrease in mRNA at the beginning of DMSO treatment. Finally, more NF-E2 binds to the *p45* promoter to achieve a sufficient amount of NF-E2 during erythroid differentiation. These findings also give further insights into burst upregulation of essential genes for hemoglobin synthesis during erythroid differentiation.

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