サイトカイン依存性白血病細胞株の分化誘導の解析と顆粒球系分化特異的転写因子の同定

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研究発表

口頭発表

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The establishment of eosinophilic differentiation system with IL-3 receptor α transfectant of human leukemia cell line, MB-02, and the analysis of eosinophilic lineage specific differentiation factors.

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Abstract

The roles of cytokines on cell survival, proliferation, and differentiation commitment (especially myeloid) was analyzed with a human leukemia cell line, MB-02. GM-CSF supported long term cell proliferation of MB-02 cells. In contrast, IL-3 did not support proliferation but induced either myeloid differentiation or apoptosis. The differentiated cells were mostly eosinophils since their cytoplasmic granules were well stained with antibodies against both major basic protein of eosinophil (MBP) and eosinophilic peroxidase (EPOX), although very small fraction showed basophilic characteristics as detected with electron micography. On the contrary, GM-CSF could not induce the differentiation under various conditions tested.However, a lot of MB-02 cells showed the sign of apoptosis, because they usually contains a small fraction of IL-3 receptor α-positive cells. To clarify this point, MB-02 cells were transfected with cDNA for IL-3 receptor α to circumvent cell loss or apoptosis of MB-02 cells cultured with IL-3. A stable transfectant, MB-02-IL-3 receptor α, proliferated in response to IL-3 as well as to GM-CSF and was resistance to apoptosis. In low concentration of IL-3, in which the transfectant cells could not proliferate but survived, the cells could differentiate into eosinophils. These results, combined with previous report, clearly indicate that MB-02 cells have the potential for tri-lineage differentiation, i.e., erythroid, megakaryocytic and eosiniophilic and that the signaling system mediated by IL-3 determines the balance between differentiation and proliferation.

Using this eosinophilic differentiation system, the identification of eosinophilic differentiation specific transcription factors were tried. According to the DNA sequence of 5' promoter of MBP, 250 bp of 5' flanking region was prepared and was used to detect DNA binding proteins. Until now, we could not identify new band showing the appearance of new DNA binding proteins upon eosinophilic differentiation, which could be the candidates of eosinophilic specific transcription factor. So, we are concentrating our effort to detect qualitative differences. According to the literature, we are especially interested with C/EBP proteins that bind with CAAT box. Simultaneously, we are trying to reanalyze the promoter region of MBP gene expression by expanding the DNA probe to 2 kb of 5' flanking region. For this purpose, various size of deletion fragment prepared from DNA fragment, which was described above, were ligated with luciferase gene, and was transiently transfected for the analysis of luciferase activity. These projects were still on going and we are trying to get answers.

Materials and Methods

Cells and reagents MB-02 cells were cultured in 10 % human serum in RPMI1640 (Gibco Laboratories, Grand island, NY) as described previously (11). Recombinant human cytokines, GM-CSF, IL-3, erythropoietin (EPO) , stem cell factor (SCF) were the generous gifts of Kirin Brewery Co. (Tokyo, Japan). Mouse monoclonal antibodies against CD34 (HPCA1 and 2), Glycoprotein IIb/IIIa (GP IIb/IIIa) (TP80), major basic protein of eosinophil (MBP), eosinophil peroxidases (EPOX), human IL-3 receptor a, human α globin were purchased from Becton Dickinson (Mountain View, CA, USA), Nichirei Co. (Tokyo, Japan), Santa Cruz Biotechnologies Inc. (Santa Cruz, CA, USA), Cosmo Bio Co. Ltd. (Tokyo, Japan), respectively.

In some experiments, Dynabeads ^{TM} M-450 (coated with sheep anti mouse IgG) (Dynal, Oslo, Norway) ware used to separate antigen positive and negative cells according to manufacture's manual. Phorbol ester, TPA, and chymopapain were purchased from Sigma (St. Louis, Mo, USA). G-418, serum reduced medium OPTI^{R}1, and Lipofectin^{R} reagent were derived from Gibco BRL Co. (Gaithersburg, MD, USA). Polybrene was from Aldrich Japan Inc (Tokyo, Japan).

Differentiation lineage marker : Liquid benzidine staining was done as described before (5,7). Cytospin slides was made using Shandon Cyto-centrifuge (Shandon Inc., Pittsburgh, PA). In some experiment, anti-human a globin monoclonal antibody was used for the detection of hemoglobin with Dako's LSAB immuno-staining kit (Carpinteria, CA, USA). Cytoplasmic granules as the marker of myeloid differentiation was observed after May-Giemsa staining. GP IIb/IIIa staining was done using anti-human GP IIb/IIIa monoclonal antibody (TP80) as described before (6). CD34 was detected with the mixture of monoclonal antibodies, HPCA1 and 2. Differentiation into eosinophils were confirmed by staining with anti-MBP and anti-EPOX antibodies after cold acetone fixation for 10 minutes.

Transfection: IL-3 receptor α cDNA (13) was derived from Drs. K. Arai and T.Yokota (Tokyo Univ., Tokyo, Japan). Expression vector PCXN2 (PcAGGS + MC_1-neo polyA) was a gift of Dr. J. Miyazaki (Tokyo Univ., Tokyo, Japan) (14). XhoI fragment of IL-3 receptor α cDNA was cloned into the same cloning site of the vector. After ampicillin selection, the orientation of the insert of each colony was determined by Bgl II and ApI digestion. Transfection was performed with LIPOFECTIN^{R} Reagent from Gibco BRL. MB-02 cells (2 x10^6/ml) were suspended in serum reduced medium OPTI MEM^{R}1. Two μg of constructed expression vector in 100 μl of OPTI MEM^{R}1 and 12.5 μl of lipofection reagent in 100 μl of OPTI MEM^{R}1 were mixed and incubated for 15 minutes at room temperature. A mixture was added to cell suspension, and incubated for 24 hour at 37℃. DNA containing medium was replaced to ordinary serum containing medium and after another 24 hours, G418 (final concentration 600 μg/ml) was added. 7 days after, medium was changed and incubated thereafter with the gradual decrease of G418 concentration. GM-CSF was present during the whole selection period. When significant cell growth was observed, a limiting dilution was done to get stable transformant clones.

DNA ladder formation DNA ladder formation as the marker of apoptic cell death was detected according to Sellins et al.(15) with minor modification. Briefly, cells (1 x 10^6) were collected by centrifugation and was lysed with 5 ml of a lysis buffer containing 5 mM Tris, pH8.0, 10 mM EDTA, 0.5 % Triton X-100, and were centrifuged at 15000g for 20 min. One hundred ul of Proteinase K (10mg/ml) and 1 % sodium dodecyl sulfate (SDS) were added to the supernatant. After 12 to 18 hours incubation at 37℃, the lower molecular weight DNA was extracted with phenol/chloroform, precipitated with ethanol and dissolved in TE buffer. After treatment with RNase A, DNA samples were electrophoresed in 3 % agarose gel and was visualized with ethidium-bromide staining.

Immunomagnetic bead selection: The procedure of positive selection of CD 34 positive cells

was performed as described before (16). Briefly, 200 μl of MB-02 cell suspension was mixed with 30 ul each of mouse monoclonal antibodies (HPCA1 and 2) and was incubated for 30 minutes at 4℃ After washing, immunomagnetic beads coated with anti mouse IgG antibody (Dynabeads M-450) were added to 1 ml of the cell suspension at the cell-to-beads ratio of 1:5 (number) and then incubated for 30 minutes at 4℃. The cells forming rosettes with beads were collected with a magnetic particle concentrator (MPC-1, Dynal). The collected cells were resuspended in 1 ml of phosphate-buffered saline (PBS) containing 100 U/ml of chymopapain (Sigma) and were incubated for 15 minutes at 37℃. Cell and beads were finally separated with chymopapain treatment. After these procedure, CD34 positive fraction and CD34 negative fraction were separated and used for further analysis. In some cases, anti-IL-3 receptor α antibody (Santa Cruz Biotechnologies Inc.) was used instead of anti CD34 antibody for separating IL-3 receptor α positive and negative cells using the same method.

Gel shift assay In order to detect the DNA binding protein, gel shift assay was performed according to Watanabe et al. (17). Nuclear extract was prepared with the method of Dignam et al.(18). Briefly, 5μg of nuclear extract was incubated with radiolabeled DNA fragment of 250 bp of 5' promoter region of MBP gene reported by Li et al. (19).(shown in Fig 7.) After 1 hour incubation at 37℃, the mixture was electrophoresed with 30 mA of constant current. Gel was dried and autoradiograph was taken.

Long polymerase chain reaction and luciferase assay To obtain genomic fragment of MBP promoter region (2.0 kb), Genome Walker^{TM} Kits (Clonotech, USA) was used according to the manufacturer's recommendation. Long PCR was performed with an LA PCR Kit Version 2 (Takara, Otsu, Japan). Genomic DNA digested with Eco RV was ligated with adaptors; then was subjected to PCR with adaptor primer (forward) and promoter side primer (reverse) as shown in Fig. 8. PCR condition was 30 cycles of denaturation for 40 s at 94℃, annealing for 30 s at 55℃, and extension for 5 min at 72℃ in a Thermal Cycler 9600. The sequence of long-PCR products were directly analyzed by the dye terminator cycle sequencing method. Unidirectional deletions of the 2 kb MBP/luciferase construct were prepared using Exonuclease III as previously described (20). Each deletion fragment was confirmed its nucleotide sequences with direct sequencing.The promoterless luciferase plasmid pXP2 was used for all promoter studies. Murine fibroblast cell line, NIH3T3 cells ($2x10^5$ each), were cultured in 6 cm culture dishes for transient transfection assay. Each expression construct was transfected with reporter plasmid by calcium phosphate method (21), cells were dissolved 48 hours after transfection, and luciferase activity was measured using PicaGene luciferase assay system (Toyo Ink Mfg. Co. Ltd, Tokyo, Japan) and LUMAT measuring instruments (Berthold, Postfach, Germany).

Results and Present Status

1. ***Myeloid differentiation of original MB-02 cells.*** When cultured with GM-CSF, MB-02 cells proliferated well in blast-like morphology (Figure 1A). With 10 ng/ml of IL-3, however, MB-02 did not grow and showed different morphology: nuclear maturation and the appearance of cytoplasmic granules. Furthermore, smaller population showed a feature of typical apoptic cell death (Figure 1B). The cytoplasmic granules were well stained with antibodies against MBP (Figure 1C) and EPOX (Figure 1D). The eosinophillic granules were also seen in IL-3 + SCF treated cells but very few in IL-3 + GM treated cells (data not shown).

The immunohistochemical detection of IL-3 receptor α of original MB-02 revealed that only 0.5 - 1 % of original MB-02 cells were weakly positive (Figure 4). Immunomagnetic bead selection of IL-3 receptor positive cells among IL-3 treated MB-02 cells showed that most of these myeloid differentiated MB-02 cells cultured with IL-3 were IL-3 receptor α-positive (Figure 1E), while IL-3 receptor α-negative cells were either blast like or apoptic cells (Figure 1F).

Electron-microscopic examination also showed that typical blast like cell (Figure 2A) was converted to either cytoplasmic granule-positive cells (Figure 2B) or apoptic cells (Figure 2C). The high magnification of cytoplasmic granules suggested that most of granules were those of eosinophils (Figure 2D), while few of them were basophil-like by their morphology (Figure 2E).

2. The relationship between CD34 expression and myeloid differentiation. Original MB-02 cells express CD 34 antigen which is one of the character of normal hematopoietic stem cells (17). When cultured with GM-CSF, the percentage of CD34 positive cells remained high and the percentage of positive cells for each lineage marker was low (Table 1).Using immunomagnetic bead selection with anti-CD34 antibody, we confirmed that CD 34 positive MB-02 cells cultured with GM-CSF were literally negative for each lineage marker (data not shown). When incubated with IL-3, the percentage of CD34-positive cells decreased, and the percentage of myeloid cells rose up to 25-30 %. Hemoglobin positive cells also increased to 17 %. Without cytokine addition, cell showed rapid apoptosis and any sign of cell differentiation was not observed (data not shown).

3. Transfection of IL-3 receptor a and IL-3 receptor a expression. For more complete understanding of this IL-3 induced differentiation, it is ideal to avoid cell loss due to apoptosis. We tried transfection of IL-3 receptor α to MB-02 cells to confer IL-3 dependency. Figure 3 shows the constructs of the expression vectors of IL-3 receptor α cDNA. We could get several stable transfectants. After checking IL-3 receptor-α expression of each transfectant with immuno-histochemical method, we finally pick up a representative clone, MB-02 sc 3R-7 for further analysis , because the expression of transfected gene was highest in this clone.

IL-3 receptor α expression as well as May-Giemsa staining of original MB-02 and IL-3 transfectant, MB-02 sc 3R-7 was shown in Figure 4. Only MB-02 sc 3R-7 showed high (85-90 %) and strong receptor expression. As mentioned above, only a few of MB-02 (less than 1 %) showed weak positive staining of IL-3 receptor α. May-Giemsa staining of two cell lines showed that they were morphologically indistinguishable.

4. Cell proliferation and differentiation pattern of IL-3 receptor a transfectant.

Next, we analyzed cell growth of MB-2 and IL-3 receptor-α transfectant, MB-02 sc 3R-7 (Figure 5A). Cytokine depletion induced rapid decrease of cell number and the appearance of apoptic cells (as shown in Figure 2C) of both MB-2 and IL-3 receptor-α transfectant. IL-3 receptor-α transfectant showed active cell growth in response to IL-3 (more than 10 ng/ml), and can be passaged with IL-3 without changing their blastic morphology.

One of the characteristics of apoptosis is the fragmentation of DNA, which can be detected with gel electrophoresis. DNA ladder formation as shown in Figure 5B confirmed apoptosis induction of

both MB-02 and IL-3 receptor-α transfectant when incubated with EPO or no addition. IL-3 receptor α transfectants could not proliferate well with 1 or 2 ng/ml of IL-3 but could be maintained with their viability and showed myeloid-like differentiation as observed in original MB-02 cells cultured with 10 ng/ml of IL-3. Less than 1 ng/ml of IL-3 induced apoptosis of IL-3 receptor α transfectant. More than 10 ng/ml of IL-3 that can greatly stimulated the proliferation of MB-02 IL-3 receptor α transfectant inhibited myeloid differentiation as observed in cultures with low concentration of IL-3 (Figure 6).

The percentage of CD34 (+) cells of MB-02 IL-3 receptor α transfectant decreased in accordance with the inhibition of cell growth when incubated with IL-3 concentration. In accordance with the cessation of cell growth, the percentage of benzidine positive cells also increased up to 30 % (data not shown) suggesting that proliferation and differentiation are opposing process in this cell line.

5. The analysis of eosinophil differentiation commitment factor: With the 250 bp fragment of 5' promoter of MBP gene (Fig 7.), DNA binding protein was analyzed using nuclear extract (5ug)of both low dose of IL-3 treated MB-02 3R and control cells cultured with GM-CSF. We could observe 5 or 6 band under our experimental conditions (data not shown). However, we could not detect the appearance of new bands in low dose IL-3 stimulated samples.

6. Promoter activity of 2 kb of 5, flanking sequence of MBP gene: To confirm the relative importance of MBP promoter, 2 kb of 5' flanking sequence of MBP gene was cloned according to the materials and methods. Various deletions (as shown in Fig. 8) were ligated to luciferase gene, and was introduced into NIH3T3 cells with calcium phosphate method. After 48 hours, luciferase activity was assayed. Figure 8 shows our preliminary data of relative promoter intensity. It shows the importance of 250 bp of 5'flanking region of MBP gene as the promoter activity. However, repeated analysis is necessary. The experiments are now on going.

Discussion and Future Aspects

MB-02 cells were reported to be dependent on GM-CSF in proliferation and to have at least two-lineage differentiation potential (11). Our observation showed that IL-3 could not support cell growth of MB-02 cells but induced apoptosis in certain population and myeloid differentiation in other population (Figure 1 and 2). Cytoplasmic granules that appeared upon treatment with IL-3 well resembled to those of eosinophils (Figure 1B, 2B and 2D). The immunohistochemical staining of myeloid-like MB-02 cells with antibody against MBP, which is the major component of eosinophil cytoplasm (23) as well as with anti eosinophlic peroxidase antibody (24) confirmed its eosinophilic character (Figure 1C and D).

Morgan et al. (11) observed that primary cells from the patient could be induced to eosinophils and basophils for a limited period of time in response to IL-3 but gradually lost that capacity . Our results, combined with previous report (11), indicate that MB-02 possesses tri-lineage differentiation potentials [erythroid, myeloid (eosinophilic) and megakaryocytic], although IL-3 induced myeloid differentiation is accompanied with apoptosis.

MB-02 cells cultured in GM-CSF expressed high percentage of CD34 antigen, one of markers for normal stem cells (22). The differentiation marker analysis in combination with magnetic beads selection (Table 1 and data not shown) showed the inverse relationship between the percentage of CD34 positive cells and that of lineage marker, suggesting that MB-02 can mimic, at least partially, differentiation process of normal hematopoietic stem cells. GM-CSF inhibits the differentiation induction by either IL-3, EPO or even TPA (data not shown). The similar hierarchy of cytokines in their roles of differentiation induction was previously reported in GM-CSF-dependent human leukemia cell line, UT-7 (25).

Eosinophil-like differentiated cells among IL-3 treated cells are mostly IL-3 receptor positive (Figure 1E), whereas most of MB-02 cells were IL-3 receptor α-negative with the immuno-histochemical detection method. Under our experimental conditions, significant myeloid differentiation was not observed in cytokine-depleted or low concentration of GM-CSF treated MB-02 cells (data not shown). At present, the inductive role of IL-3 was evident in eosinophilic differentiation of MB-02 cells. However, the cessation of cell growth and apoptosis was often observed simultaneously or sequentially as in the case of IL-3 treated MB-02 cells. Thus, the loss (or limited exhibition) of multipotentiality of MB-02 cells might be connected with the loss of IL-3 dependency due to loss of IL-3 receptor α.

Stable transfectant with IL-3 receptor α cDNA showed IL-3 dependency when cultured more than 10 ng/ml of IL-3. Myeloid (eosinophilic) differentiation was also observed in IL-3 receptor α transfectant, only when incubated with low concentration (1-5 ng/ml) of IL-3 but not high concentration of IL-3. In transfectant, erythroid differentiation was also enhanced when cell growth was inhibited, but further analysis was necessary for the mechanism of differentiation lineage selection (eosinophilic or erythroid).

The fact that cessation of cell growth enhanced myeloid differentiation of IL-3 R α transfectant treated with IL-3 is consistent with our previous finding in erythroleukemia cells treated with chemical inducers (5,7) and those of Fairbairn et al. utilizing bcl-2 transfection (26) Previous transfection experiments using, for example, c-fms, CSF-1 receptor, or G-CSF shows various results concerning enforced receptor expression and differentiation. Bourette et al.(27), and Kato et al. (28) reported no differentiation occurred in transfected cells, if cell growth was maintained with CSF-1. Similarly, in NFS 60, AML-193 and OCI/AML 1a cells, the major effect of the cytokine appears to be stimulators of growth (29,30,31). With WEHI-3B D+, 32DC13 and HL60 cells, the predominant effect of G-CSF may be to induce maturation (32,33,34). Rohrschneider et al. (35) reported that both proliferation and differentiation occurred simultaneously.

However, previous transfection experiments sometimes lacked precise analysis of cytokine

concentration in the differentiation induction as well as the data of single cell level. Recent reports have shown that cytokine receptor has distinct domain involved in the induction of proliferation and maturation (36,37). The different attitude observed between MB-02 and its IL-3 receptor transfectant toward sensitivity to apoptosis, differentiation and proliferation, may be primarily determined by the net effect of IL-3 concentration and the receptor number on each cell surface and that IL-3 induced signal was essential in myeloid differentiation observed in this cell line. In other words, weak signal induced with IL-3 prevents apoptosis and induces differentiation, and strong signals triggered by high dose of IL-3 can erase the differentiation signal triggered by the same cytokines and induces cell proliferation strongly. Taken together, our present data shows that MB-02-3R subclone is useful for the analysis of eosinophilic differentiation induced with IL-3.

Using this system, we started the analysis to detect possible candidates for eosinophilic lineage commitment factor using gel shift assay. Under our experimental condition, we could observe several proteins to bind with 250 bp of 5' flanking region of MBP gene. However, our repeated analysis could not detect any new band upon stimulation of low dose IL-3 treatment (eosinophilic differentiation condition). This may be the limitation of the gel shift assay for the detection of subtle differences, or our subline, MB-02-3R may possess several transcription factor even in uninduced state. We are now concentrating our effort to detect significant quantitative changes of each band between control and eosinophilic differentiation stimulation. We are especially interested in the report that C/EBP can induce eosinophilic differentiation (38), and started preliminary experiment to detect C/EBP protein using monoclonal antibody against C/EBP in our system

Knowing that the analysis of eosinophilic lineage commitment factor using gel shift assay is not easy, we modified the strategy of our analysis. We tried to analyze the precise evaluation of each promoter area. This analysis is now on going. We could only show our preliminary data in Fig 8. The data confirmed the importance of 250 bp of 5' flanking region of MBP gene. However, our present analysis could not pick up the major factor of eosinophilic differentiation (in our case, MBP gene expression). The simple way of explanation was the coordinated (quantitative) expression of several factors that were already present in uninduced cells induce eosinophilic differentiation commitment.

We could not get the concrete conclusion of either cis (promoter sequence) or trans (DNA binding transcription factor) elements. The new approaches we are now considering are (1) to detect the precise qualitative change of shifted bands in gel shift assay (with the special emphasis of C/EBP protein), (2) the introduction of DNAse I protection assay to elucidate the protein-DNA interaction clearly (39), (3) the analysis of the involvement of known transcription factors, such as GATA1, GATA2, GATA3, NF-E2 and PU-1.

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Figure legends

Figure 1. Morphology of MB-02 cells. May-Giemsa staining of MB-02 cells treated with 5 ng/ml of GM-CSF (A), 10 ng/ml of IL-3 (B) for 5 days are presented. C and D shows immuno-histochemical staining of MB-02 cells cultured with IL-3 using Dako's LSAB staining kit. C represents a staining with anti-MBP antibody and D represents a staining with anti-EPOX antibody, respectively. Immunomagnetic bead selection of IL-3 treated MB-02 cells with anti IL-3 receptor α antibody as the primary antibody is shown in E and F. MB-02 cells were cultured with IL-3 for 5 days. Cells were collected and treated with anti-IL-3 receptor a antibody followed by Dynabeads M450 as described in the Materials and Method. E shows IL-3 receptor α receptor positive fraction and F shows negative fraction. Magnification x400.

Figure 2. Electron micrographs of MB-02 cells treated with various cytokines. **A;** A cell with blastic appearance. The cell has no distinguished features. It is aguranular and has high nuclear to cytoplasmic ratio. **B;** A matured myelocyte with many granules, rough endoplasmic reticulum and many other organelles in the cytoplasm. Nucleus is invaginated and heterochromatin in the nucleus is observed. **C;** An apoptic cell which shows condensed and fragmented nucleus with intact plasma membrane. **D;** Higher magnification of the granules in **B**. **E;** Higher magnification profile of granules appeared in basophilic granulocytes. The occurrence of this basophil-like cells is quite rare in the present study. Magnification **A-C** x 12,000. **D** and **E;** x20,000.

Figure 3 The constructs of IL-3 receptor α expression vectors

Figure 4. IL-3 receptor α expression, May-Giemsa staining and bcl-2 gene expression of MB-02 cells and IL-3 receptor α transfectant, sc 3R-7. Upper part ; IL-3receptor α expression as detected by LSAB staining methods of MB-02 (A) and MB-02 sc 3R-7 (B). Magnification was 200x. Lower part; May-Giemsa staining of respective cells.

Figure 5. A: Cell growth curve of MB-02 cells and its transfectants. Original MB-02 and MB-02 sc 3R-7 (2.0×10^5/ml) were cultured with GM-CSF (5 ng/ml), IL-3 (30 ng/ml) or without any cytokine addition, respectively. Viable cell number was counted sequentially. Experiments were repeated three times with the similar data. **B:** DNA ladder formation. MB-02 cells and MB-02 sc 3R-7 were cultured with GM-CSF (5 ng/ml), IL-3 (30 ng/ml), EPO (5U/ml) or without any cytokine addition. Cells were collected on day 2, and processed as described in the Materials and Method. The low molecular weight DNA was visualized after gel separation. MW means molecular weight size marker, and the number denotes base pair.

Figure 6. Differentiation characteristic of MB-02-IL-3receptor α transfectant. MB-02 sc 3R-7 was cultured with various doses of IL-3. Cell number and the percentage of myeloid differentiated cells as detected with May-Giemsa staining was counted on day 5. APO denotes the defect of the data due to severe apoptosis. Experiments were repeated three times with the similar results.

Figure 7. Nucleotide sequence of MBP promoter region Transcription start sites are indicated by asterisks. Potential binding sites for different transcription factors are indicated.

Figure 8. Functional activity of the MBP/pXP2 luciferase constructs in NIH3T3 cells Luciferase activities have been normalized as compared to the mock transfected sample. the data was expressed as the percent activity relative to the mean activity of the longest 2 kB MBP promoter construct. The

mean ± S.E. for three replicate experiments is shown.

	CD 34	Myeloid	Hemoglobin	GP IIb/IIIa
GM-CSF	78 ± 5.1	0.6 ± 0.7	3.0 ± 2.2	6.3 ± 3.5
IL-3	18 ± 5.5	29 ± 6.2	17 ± 5.5	10 ± 3.2

Table 1. Differentiation potential of MB-02 cells cultured with GM-CSF or IL-3.

Two x 10^5/ml of MB-02 cells were cultured with 5 ng/ml of GM-CSF or 10 ng/ml of IL-3 for 5 days. On day 5, cell concentrations were 1 x10^6/ml and 1.5 x 10^5/ml, respectively. Each differentiation marker was analyzed on day 5. Myeloid cells were identified with May-Giemsa staining as shown in Figure 1B. Hemoglobin - positive cells were identified with liquid benzidine staining. GP IIb/IIIa positive cells were counted using immunohistochemical staining as described in the Materials and Methods. The number was the mean percent± SD of three different samples.

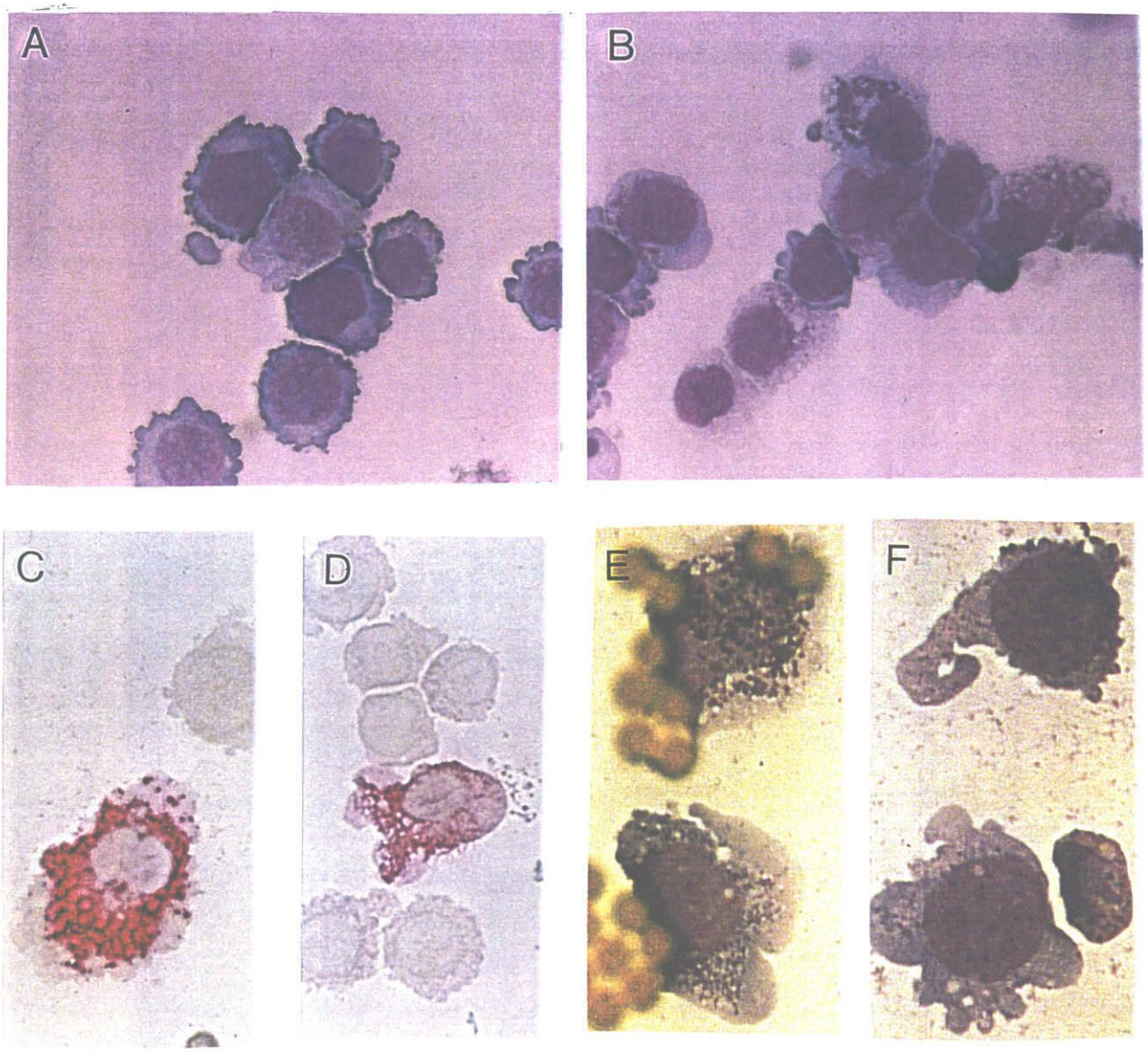

Figure 1.

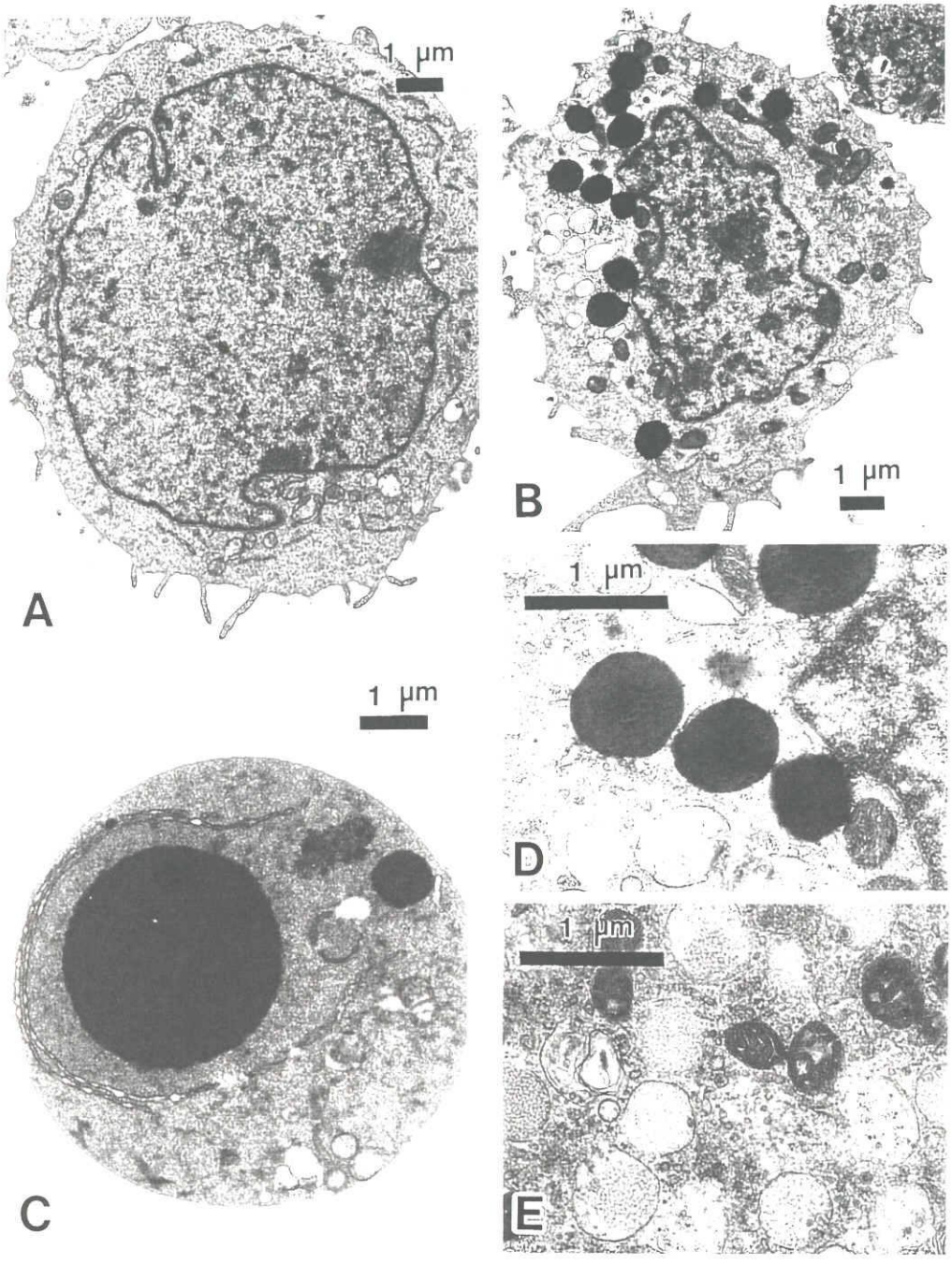

Figure 2

IL-3 Receptor-α

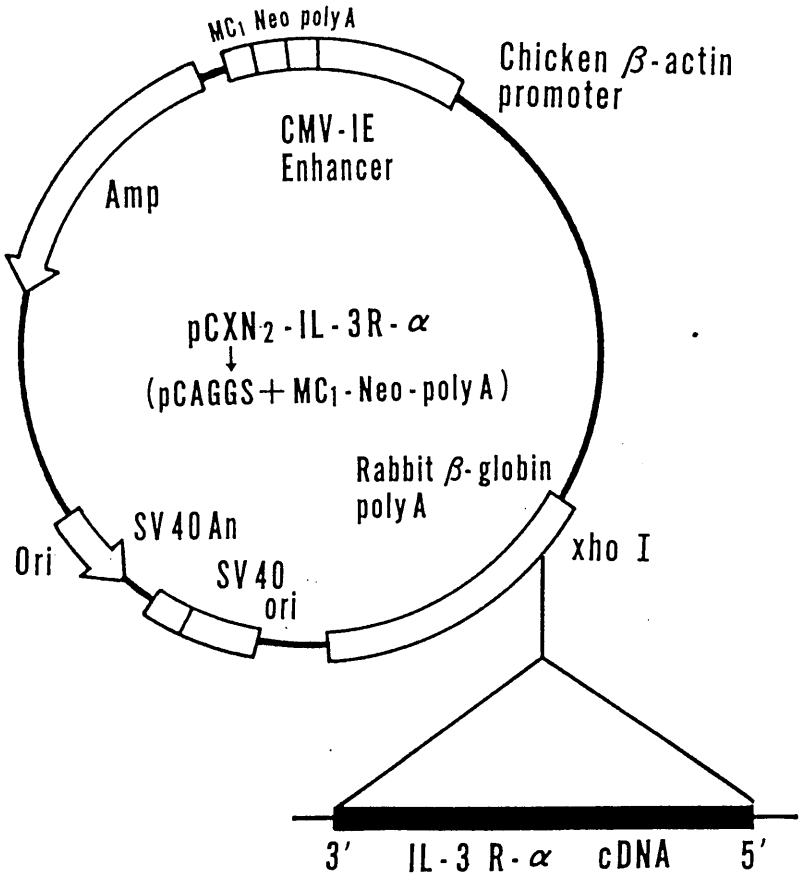

Figure 3

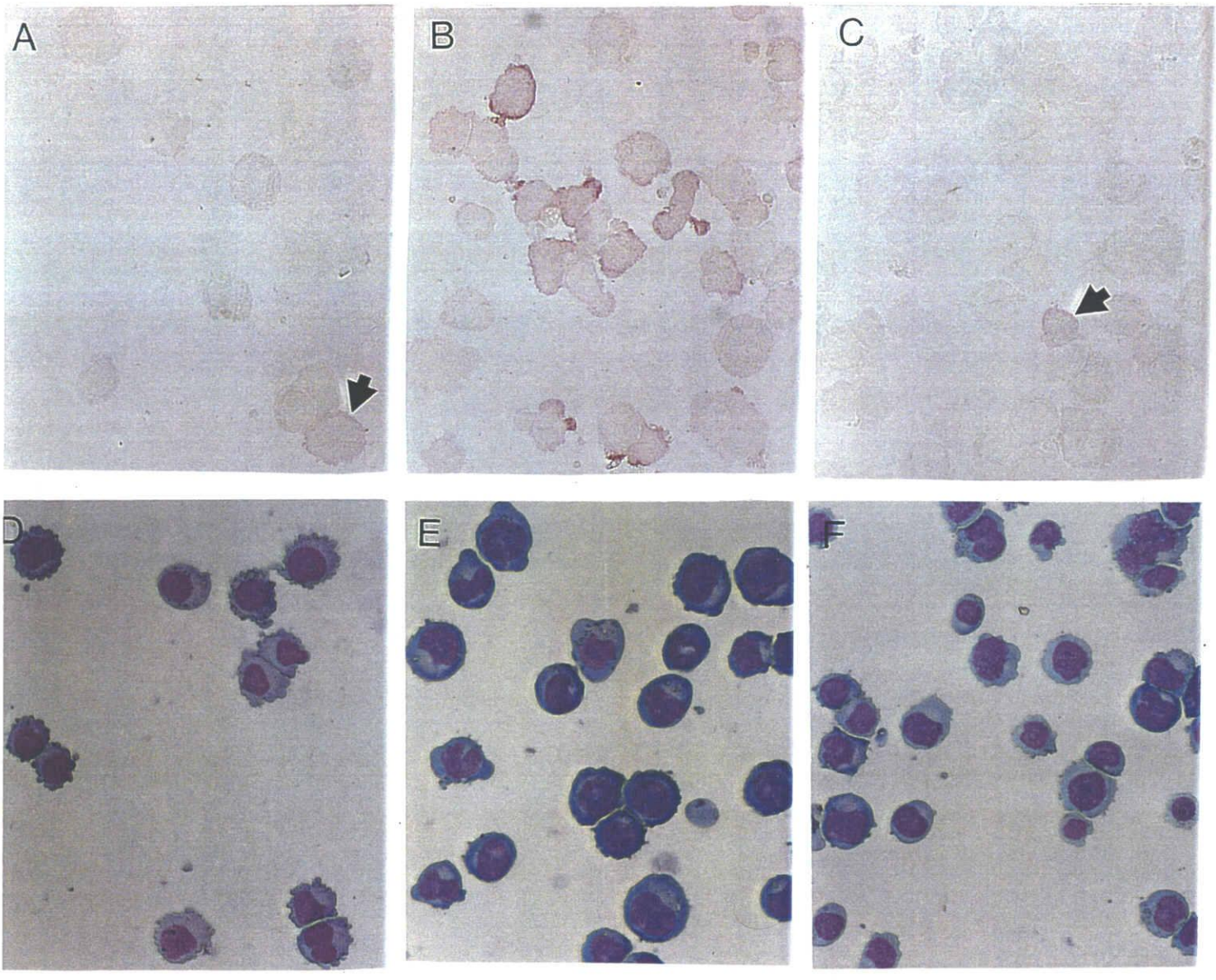

Figure 4

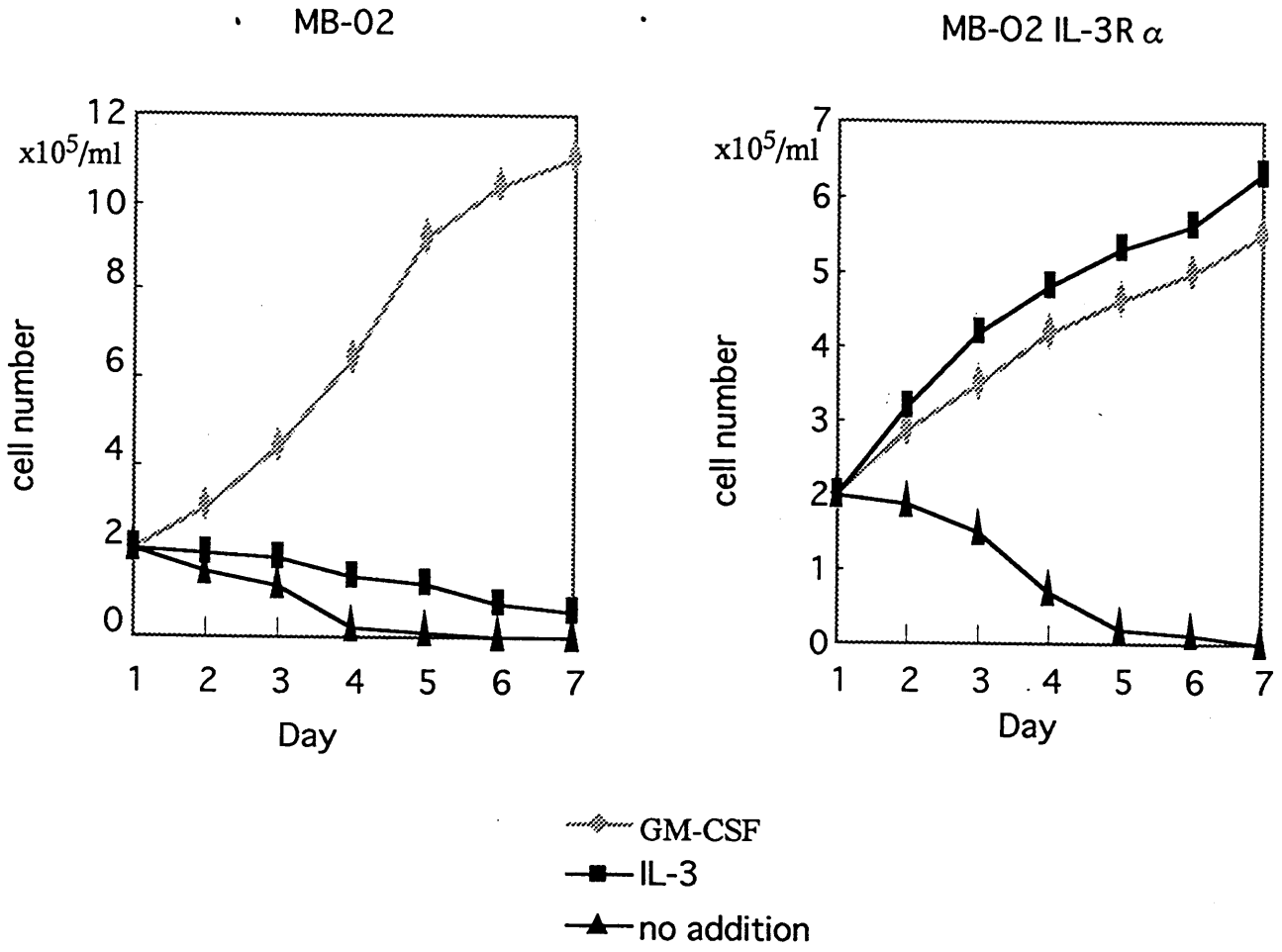
MB-02
MB-02 IL-3R α
x10^5/ml
cell number
Day
GM-CSF
IL-3
no addition

Figure 5A

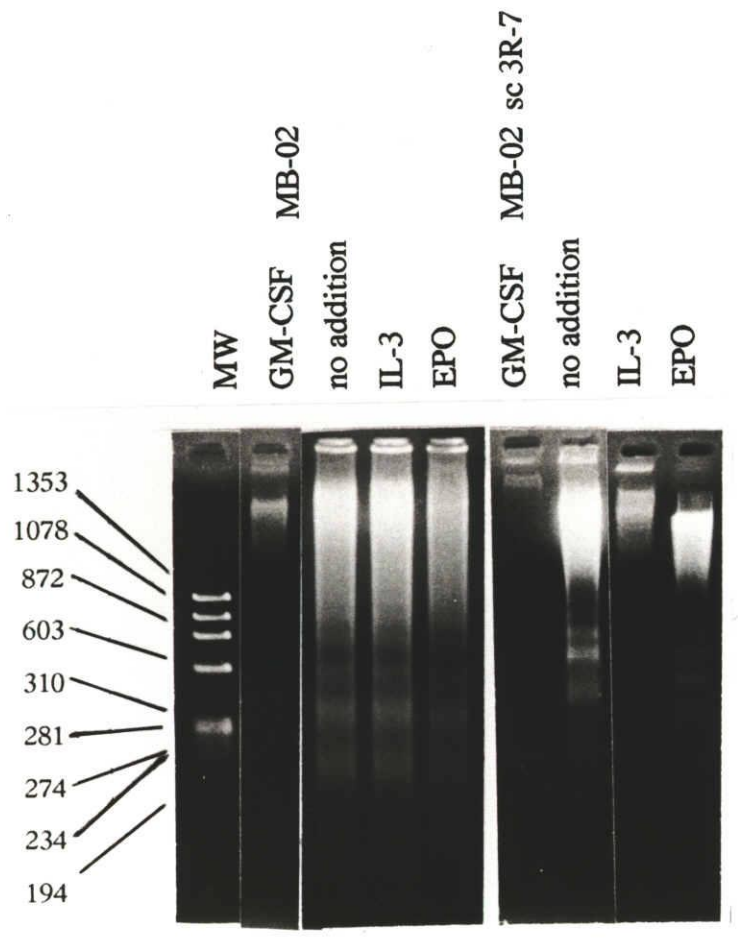
MW
GM-CSF
MB-02
no addition
IL-3
EPO
GM-CSF
MB-02
sc 3R-7
no addition
IL-3
EPO
1353
1078
872
603
310
281
274
234
194

Figure 5B

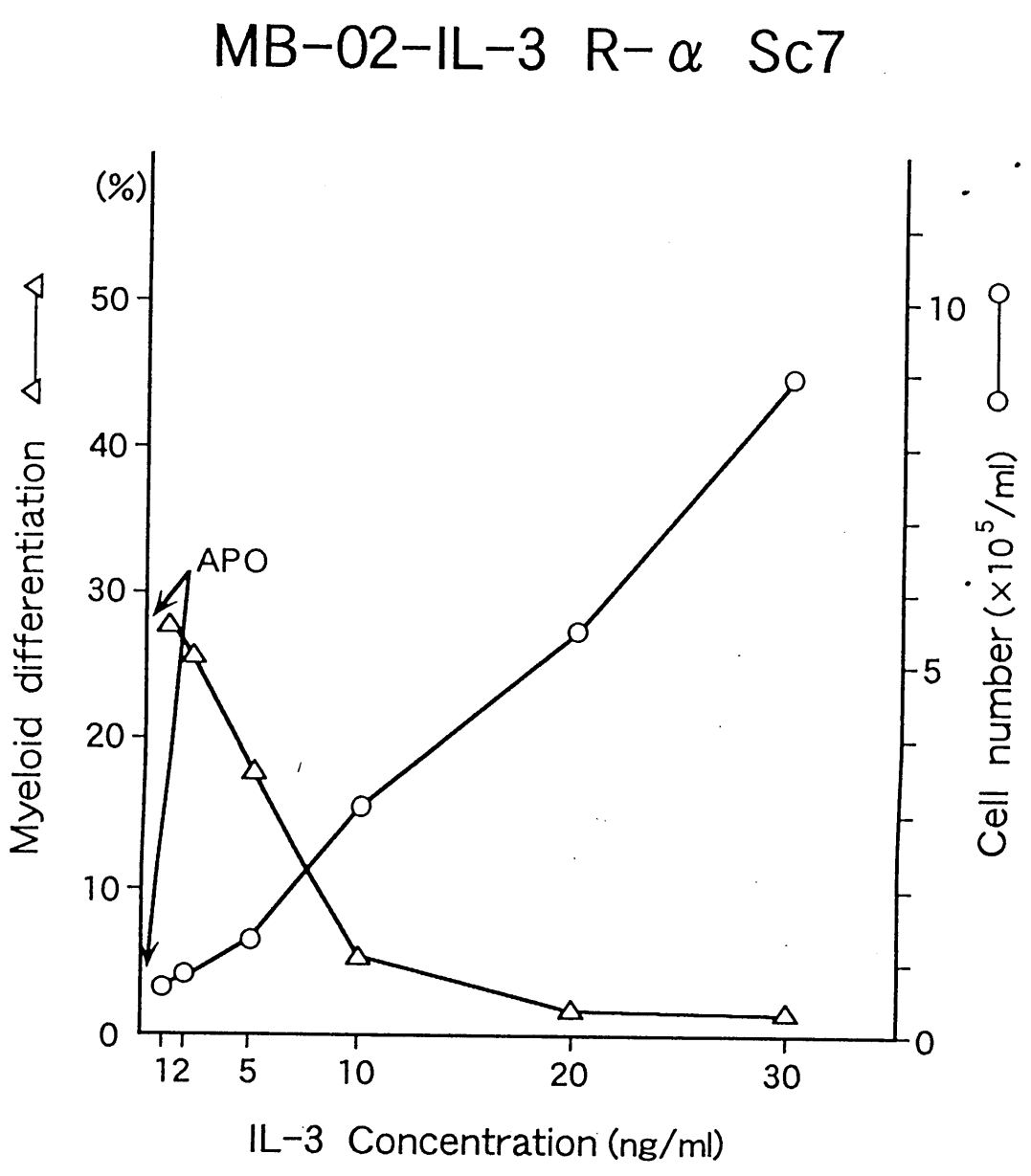
MB-02-IL-3 R-α Sc7
(%)
50
40
30
20
10
0
APO
Myeloid differentiation
10
5
0
Cell number (×10⁵/ml)
12
5
10
20
30
IL-3 Concentration (ng/ml)

Figure 6

Figure 7. Transcriptional start sites for the 1.6 kb cDNA transcript of MBP and probable promoter sites

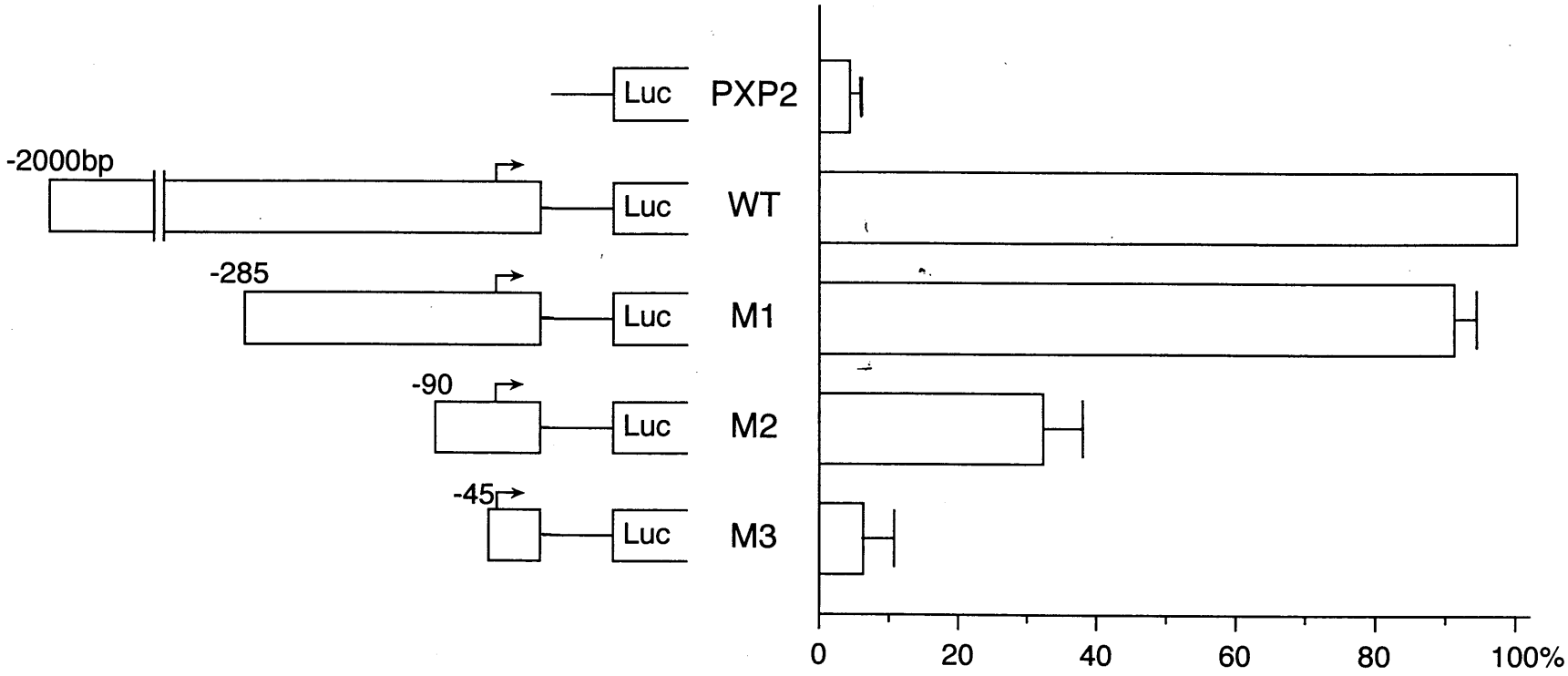
Luc
PXP2
-2000bp
Luc
WT
-285
Luc
M1
-90
Luc
M2
-45
Luc
M3
0
20
40
60
80
100%

Figure 8