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NF-M (chicken C/EBPβ) induces eosinophilic differentiation and apoptosis in a hematopoietic progenitor cell line

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CAAT/enhancer binding proteins (C/EBPs) are transcriptional activators implicated in the differentiation processes of various cell lineages. We have shown earlier that NF-M, the chicken homolog of C/EBPβ, is specifically expressed in myelomonocytic and eosinophilic cells of the hematopoietic system. To investigate the role of NF-M in hematopoietic cell lineage commitment, we constructed a conditional form of the protein by fusing it to the hormone binding domain of the human estrogen receptor. This construct was stably expressed in a multipotent progenitor cell line transformed by the Myb–Ets oncoprotein. We report here that both NF-M-dependent promoter constructs and resident genes could be activated by addition of β-estradiol to the NF-M–estrogen receptor expressing progenitors. At the same time, we observed a down-regulation of progenitor-specific surface markers and the up-regulation of differentiation markers restricted to the eosinophil and myeloid lineages. In addition to the onset of differentiation, cell death was induced with typical apoptotic features. Our results suggest that NF-M plays an important role in commitment along the eosinophil lineage and in the induction of apoptosis.

Keywords: cell transformation/nuclear oncogenes/stem cells/transcription factors

Introduction

Transcription factors of the C/EBPβ (CAAT/enhancer binding protein) family are involved in differentiation and growth arrest of several cell types (Umek *et al.*, 1991; Freytag and Geddes, 1992). Common structural features of these proteins are highly conserved basic DNA binding and leucine zipper dimerization domains at the carboxyl-terminus and divergent amino-termini containing regulatory and transactivation domains (Landschulz *et al.*, 1988, 1989; Pei and Shih, 1991; Kowenz-Leutz *et al.*, 1994). The first member of this family, C/EBPα, was originally identified in liver tissue but is now known also to be expressed during later stages of adipocyte differentiation. Ectopic expression of C/EBPα in adipoblasts inhibits cell proliferation (Umek *et al.*, 1991) and activates genes characteristic for differentiated fat cells (Christy *et al.*, 1989; Herrera *et al.* 1989; Kaestner *et al.*, 1990; Freytag *et al.*, 1994). Conversely, C/EBPα antisense RNA blocks terminal differentiation of adipoblasts, indicating that it is essential for adipocyte differentiation (Lin and Lane, 1992). Similarly to what has been found in adipocyte differentiation, cessation of cell proliferation correlates with C/EBPβ accumulation in the liver and ectopic expression of C/EBPβ arrests hepatoma cell growth (Buck *et al.*, 1994).

Another tissue in which C/EBP-type factors play a role is the hematopoietic system. As observed for adipocyte maturation, myelomonocytic differentiation is paralleled by a distinct expression pattern of C/EBP proteins which, however, is different from that in adipocytes (Cao *et al.*, 1991; Natsuka *et al.*, 1992; Scott *et al.*, 1992). Thus, C/EBPβ has been shown to be expressed and active in macrophages during the inflammatory reaction and acute phase response (Akira *et al.*, 1990; Poli *et al.*, 1990; Akira and Kishimoto, 1992; Juan *et al.*, 1993; Katz *et al.*, 1993; Ramji *et al.*, 1993; Pope *et al.*, 1994). We have previously shown that NF-M, the chicken homolog of C/EBPβ, is expressed in the myelomonocytic lineage of the chicken hematopoietic system (Sterneck *et al.*, 1992a,b; Katz *et al.*, 1993). More recently, we also found it to be expressed in Myb–Ets-transformed eosinophils (Kulessa *et al.*, 1995).

A role for NF-M during lineage commitment was suggested by the observation that NF-M expression was detected at an early stage during phorbol ester-induced differentiation of Myb–Ets-transformed multipotent precursor cells (MEPs) into eosinophils and myeloblasts (Graf *et al.*, 1992; Katz *et al.*, 1993). Furthermore, NF-M was found to collaborate with both cellular and several viral forms of the Myb oncoprotein to activate the resident Myb target gene *mim-1* even in heterologous cell types such as fibroblasts (Burk *et al.*, 1993; Ness *et al.*, 1993). These data suggested that the combinatorial action of Myb and C/EBP proteins constitutes a bipartite switch which serves as a signal for the induction of myeloid-specific genes (Ness *et al.*, 1993). In addition, the finding that NF-M–C/EBPβ is regulated by activating signal transduction pathways (Wegner *et al.*, 1992; Nakajima *et al.*, 1993; Trautwein *et al.*, 1993; Kowenz-Leutz *et al.*, 1994) suggests that regulation of the factor is tightly linked to various onco-developmental cues.

Here we describe that activation of NF-M–estrogen receptor (ER) by estrogen in the MEP cell line HD57 caused a suppression of MEP-specific antigens and the expression of eosinophilic/myelomonocytic differentiation markers. In addition, we observed massive cell death with typical features of apoptosis. Our data suggest a role for NF-M (C/EBP β) in hematopoietic differentiation as well as in programed cell death.

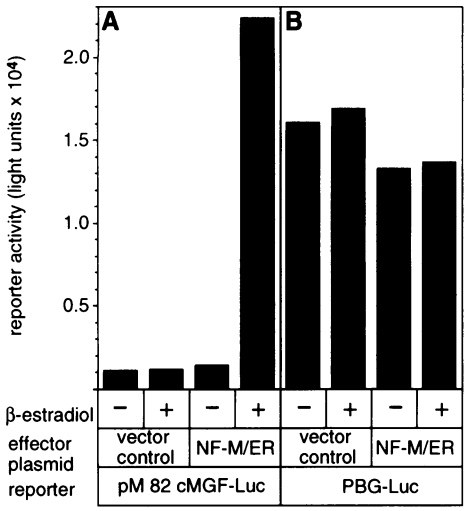

Fig. 1. Transient transfection of the NF-M–ER fusion protein. The erythroblast cell line HD3 was transiently cotransfected with the NF-M–ER expression vector and two different luciferase reporter constructs containing either a palindromic NF-M binding site derived from the cMGF promotor (pM82cMGF) (**A**) or the erythroid-specific porphobilinogen deaminase gene promotor (PBG Luc) (**B**) which is not NF-M responsive. Transfected cells were treated with β-estradiol (1 μM) or mock treated. Luciferase activity was determined 40 h after transfection and plotted as the mean of duplicates.

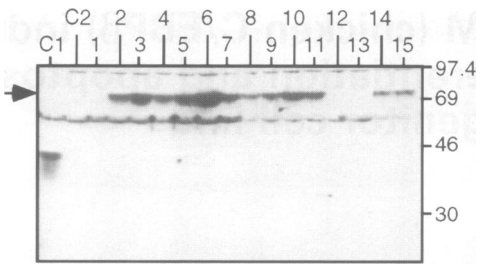

Fig. 2. NF-M–ER fusion protein expression in HD57 MEP cells. HD57 MEP cells were transfected with the NF-M–ER construct and selected in the presence of histidinol in semi-solid medium. Fifteen resistant colonies (#1–15) were isolated and expanded in liquid medium. Cell lysates were separated by gel electrophoresis, blotted and subjected to immunodetection using a polyclonal NF-M antiserum. Lysates from HD11 macrophages which express NF-M (C1) and from HD57 MEP cells which are devoid of NF-M (C2) were included as positive and negative controls, respectively. Twelve out of 15 clones express the NF-M–ER construct (electrophoretic mobility is indicated by an arrowhead on the left). Molecular weight standards are indicated on the right.

Results

Construction of a hormone-inducible form of NF-M

To investigate the function of NF-M in hematopoietic differentiation, we constructed a conditional form of the transactivator protein. For this purpose, we fused the C-terminus of NF-M to the hormone binding domain of the human estrogen receptor (Greene *et al.*, 1986). The chimeric construct is driven by the chicken β-actin promoter and carries the histidinol resistance gene as a selectable marker. The effects of NF-M–ER were tested using a luciferase reporter driven by the cMGF promoter (pM82) which contains a palindromic NF-M binding site (Sterneck *et al.*, 1992b; Katz *et al.*, 1993). Cotransfection of NF-M–ER and the reporter construct into the erythroblast cell line HD3 (Beug *et al.*, 1979) showed an ~20-fold increase of reporter expression after addition of β-estradiol as compared with the same cells in the absence of hormone or with cells transfected with the vector control (Figure 1A). In contrast, a reporter containing an NF-M-unresponsive promoter (PBG luc containing the erythroid porphobilinogen deaminase gene promoter) (Frampton *et al.*, 1990) was not activated by the NF-M–ER construct (Figure 1B). This experiment showed that the activity of the NF-M–ER fusion protein is hormone inducible and specific for NF-M target sites.

NF-M induces the expression of mim-1 in a transformed multipotent cell line

To determine whether NF-M expression can induce the differentiation of multipotent hematopoietic cells, we transfected the NF-M–ER vector into the MEP cell line HD57 and seeded the cells in semi-solid medium in the presence of histidinol. This cell line, which had been derived from chicken blastoderm cells transformed by the Gag–Myb–Ets fusion protein of the E26 virus, can be induced to differentiate into cells of the myelomonocytic and eosinophilic lineages (Graf *et al.*, 1992; Frampton *et al.*, 1995; Kulessa *et al.*, 1995). We obtained 15 stably transfected clones, 12 of which expressed the NF-M–ER fusion protein as determined by Western blot analysis (Figure 2). Three of these clones (cl.3, cl.6 and cl.14) were used for further analysis.

Both the NF-M–ER-expressing clones and the parental HD57 MEP cells were treated with β-estradiol for different time periods and examined by Northern analysis for expression of *mim-1* as a myeloid/eosinophil-specific NF-M target gene (Figure 3). In β-estradiol-treated NF-M–ER cells, *mim-1*-specific message was detectable within 1 h and still increased after 34 h of hormone treatment. In contrast, *mim-1* message was neither detected in the untreated NF-M–ER-expressing HD57 MEP cells nor in the parental cell line treated with β-estradiol (Figure 3). This result shows that NF-M induces the expression of a myeloid/eosinophilic marker in a multipotent precursor cell line.

Next we asked whether the induction of genes is a direct effect of the transactivation potential of the chimeric NF-M–ER protein. To examine this possibility, protein synthesis of cl.6 NF-M–ER cells was blocked with cycloheximide before treatment of the cells with β-estradiol. The expression of *mim-1* was then determined by Northern blotting in NF-M–ER cells as well as in the parental cell line used as a control. As can be seen in Figure 4, the treatment with cycloheximide at a concentration that inhibits >90% of protein synthesis (Kraut *et al.*, 1995) did not inhibit the induction of *mim-1* message by the hormone. As expected, no *mim-1* RNA was detectable in the parental cell line after the same treatment. These results support the notion that NF-M directly induces the expression of a myeloid/eosinophil-specific gene in MEP cells.

NF-M induces the down-regulation of MEP/thrombocyte-specific markers and the up-regulation of eosinophilic markers

To determine whether forced NF-M expression alters the phenotype of HD57 MEP cells, we examined expression of cell surface antigens by cytofluorometric analysis using

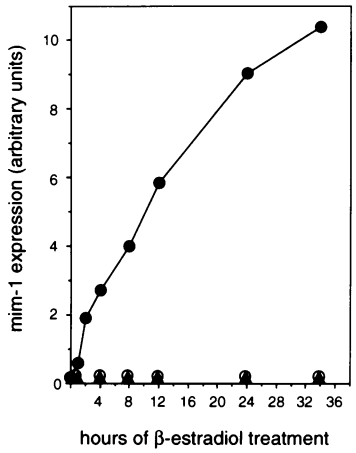

Fig. 3. Kinetics of *mim-1* expression after activation of the NF-M–ER chimera. Parental cells and NF-M–ER-expressing cells (cl.6) were treated or mock treated, respectively, with β-estradiol (1 μM) for different times as indicated. Northern analysis with ^{32}P-labeled DNA probes for *mim-1* and chicken GAPDH was performed and radioactivity was evaluated by PhosphorImager analysis normalized to GAPDH expression and plotted. Closed circles: NF-M–ER cl.6 plus β-estradiol; triangles: NF-M–ER cl.6 without hormone; open circles: parental cells plus β-estradiol.

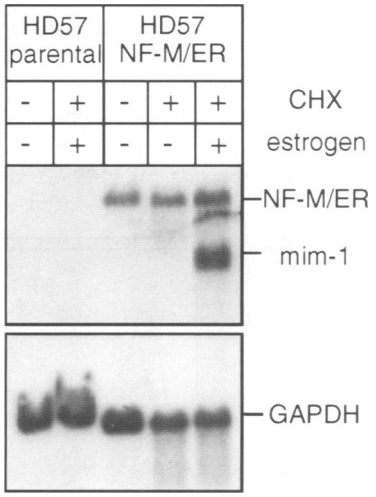

Fig. 4. Activation of myelomonocytic genes by NF-M–ER is protein synthesis independent. NF-M–ER-expressing cells (cl.6, 2×10^7 cells/lane) and parental HD57 MEP cells were treated with cycloheximide (50 μg/ml) or mock treated as indicated 30 min before addition of β-estradiol (1 μM). Cells were harvested 2.5 h later and poly(A)$^+$ RNA was subjected to a Northern analysis with ^{32}P-labeled DNA probes for NF-M, *mim-1* and GAPDH, as indicated.

specific monoclonal antibodies (Figure 5A). The expression of MEP21 and MEP26, two surface antigens which are characteristic for MEP cells and normal thrombocytes, was strongly down-regulated within 3 days after β-estradiol treatment (MEP26: from 92 to 17%; MEP21: from 94 to 38%). In parallel, the eosinophil-specific surface marker EOS47 was up-regulated in 40% of the population. In addition, 6% of the viable hormone-treated cells expressed eosinophil-specific peroxidase while no peroxidase-positive cells could be detected in the controls (data not shown). No changes in surface marker expression were observed after β-estradiol treatment of the parental cell line (data not shown). In another experiment, the time course of the altered expression of MEP21, MEP26 and EOS47 antigens was determined. As can be seen in Figure 5B, a significant decrease of the MEP antigens was already observed after 1 day of β-estradiol treatment, while the number of EOS47 antigen-positive cells increased only after 2 days. Finally, no increase in the expression of the myelomonocytic-specific cell surface antigens MYL51/2 and 4M12 could be detected even 4 days after hormone treatment of the cells. These data indicate that NF-M expression causes the down-regulation of MEP/thrombocyte-specific cell surface antigens and the up-regulation of eosinophil-specific markers.

NF-M is expressed in normal cells of the eosinophilic lineage

The results presented, together with the earlier observation that NF-M is expressed in Myb–Ets-transformed eosinophils (Kulessa *et al.*, 1995), suggest a role for NF-M in the differentiation of eosinophilic in addition to myelomonocytic cells. However, this could also represent an artefact of transformed cells lines. Therefore, to test whether normal eosinophils also express NF-M, we prepared chicken bone marrow and performed *in situ* peroxidase staining to reveal the eosinophils (Brune and Spitznagel, 1973; Graf *et al.*, 1992). Following the peroxidase reaction, cells were stained by indirect immunofluorescence with polyclonal NF-M antiserum and examined microscopically. All of the peroxidase-positive cells with an intact nucleus were also NF-M positive. In addition, a relatively high proportion of cells could be observed which were peroxidase negative but NF-M positive. Most of the latter probably represent myelomonocytic cells (Katz *et al.*, 1993) and possibly immature eosinophils. This is illustrated in Figure 6 which shows two NF-M-positive cells (Figure 6A), one of which exhibits characteristic peroxidase-positive granules (Figure 6B). This shows that NF-M is not only expressed in normal myelomonocytic cells but also in normal eosinophilic cells.

Activation of the NF-M–ER in HD57 MEP cells also induces apoptosis

During the course of the experiments described above, we observed that when NF-M–ER cells were treated for more than 2 days with β-estradiol, a large proportion of the cells died. Figure 7A and B show the kinetics of cell death, as determined by trypan blue exclusion for two NF-M–ER-expressing HD57 MEP clones following hormone addition. After 4 days of β-estradiol treatment, cell viability was reduced to ~10 and 30%, respectively. This effect was not due to a toxicity of the hormone since the parental cell line remained fully viable (Figure 7C). To determine whether this effect was due to the cryptic transactivation potential of the estrogen binding domain, the cells were treated with 4-hydroxytamoxifen (4OHT). This estrogen antagonist still dissociates the hormone receptor from the heat shock protein Hsp90 (Catelli *et al.*, 1985; Rebbe *et al.*, 1987; Picard *et al.*, 1988) whilst selectively blocking its cryptic transactivation potential (Berry *et al.*, 1990; Pham *et al.*, 1991). As shown in Figure 7A and B, 4OHT treatment exibited the same ability as β-estradiol to cause cell death. When the cells were seeded in semi-solid medium in the presence of β-estradiol and histidinol, a small number of colonies was obtained. However, of 14 such clones examined, none

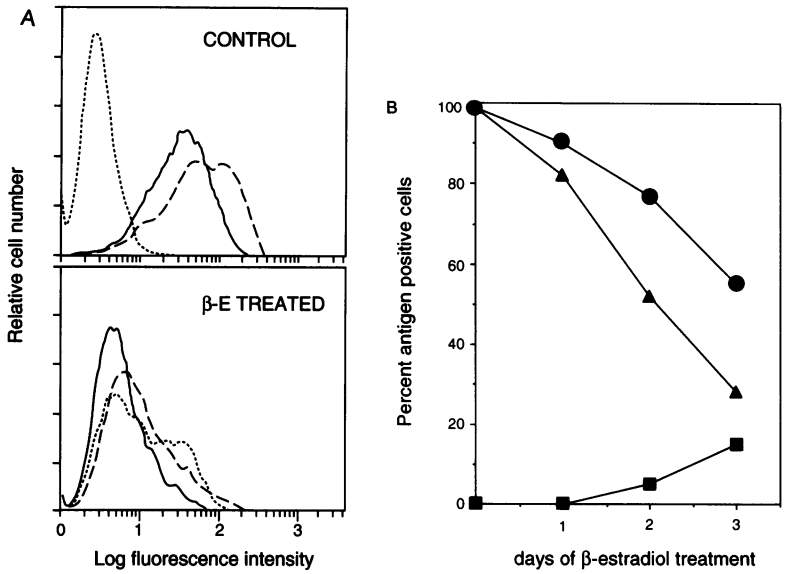

Fig. 5. Changes in the surface marker composition induced by the activated NF-M–ER. (**A**) Surface marker expression of NF-M–ER-expressing cells (cl.6) was determined by cytofluorometric analysis after treatment for 3 days with β-estradiol (150 nM, lower panel) or with solvent (upper panel) in the presence of histidinol (1 mg/ml) and recombinant cMGF. Flow cytometric profiles are shown using MEP21 (dashed line), MEP26 (solid line) and EOS47 (stippled line) monoclonal antibodies. (**B**) NF-M–ER-expressing cells (cl.6) were treated as in (A). Aliquots of cells were harvested daily and surface marker expression was determined by cytofluorometric analysis and plotted as percentage of antigen-expressing cells (circles: MEP21; triangles: MEP26; squares: EOS47).

expressed the NF-M–ER fusion protein as determined by Western blotting (data not shown). These data indicate that NF-M causes cell death and that selection for clones that have lost NF-M–ER expression protects them from estrogen-induced cell death.

Since hematopoietic differentiation is often accompanied by programed cell death, we examined whether NF-M induces apoptosis. First, the integrity of nuclear DNA was determined, as most apoptotic cells contain degraded chromosomal DNA by endonuclease digestion (Caron-Leslie *et al.*, 1991; Ucker *et al.*, 1992; Barry and Eastman, 1993; Peitsch *et al.*, 1993). This can be visualized by gel electrophoresis as a 'DNA ladder' with a typical 200 bp periodicity. We therefore examined, by gel electrophoresis, cellular DNA from two NF-M–ER-expressing clones and from the parental cell line before and after treatment with β-estradiol. The characteristic 'ladder' pattern of DNA (Figure 8A) was observed 24–36 h after estrogen activation of the NF-M–ER protein, indicating that death had occurred through apoptosis. Only traces of DNA degradation were found in untreated NF-M–ER-expressing cells or in the parental cell line before or after treatment with β-estradiol.

In a separate experiment, β-estradiol-treated cells were cytocentrifuged and tested in the 'TUNEL' assay. In this assay, DNA strand breaks are detected by treating the cells with terminal transferase and fluorescein isothiocyanate (FITC)-coupled dUTP which is subsequently visualized by fluorescence microscopy (Gavrieli *et al.*, 1992). As shown in Figure 8B and Table I, 60% of the NF-M–ER cells stained positive 24 h after addition of β-estradiol, while <0.5% positive cells were detected in the untreated samples or in the parental cells treated with β-estradiol. Finally, to test whether the β-estradiol-treated NF-M–ER cells also exhibit chromatin and nuclear condensations, they were centrifuged onto slides and the DNA was stained with 4,6-diamidin-2-indole dihydrochloride (DAPI). As

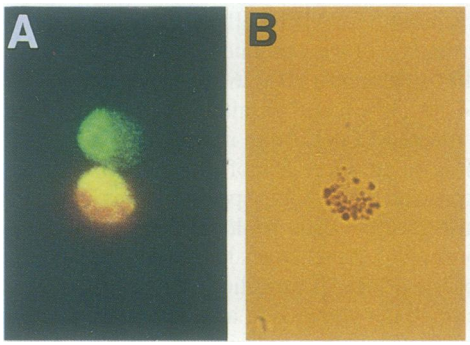

Fig. 6. Expression of NF-M in eosinophils. Bone marrow cells were fixed, and peroxidase staining and immunostaining using an NF-M-specific polyclonal antiserum were performed. The fluorescence micrograph (**A**) shows two cells expressing nuclear NF-M staining. The bright field micrograph of the same field (**B**) reveals one of them as an eosinophil.

shown in Figure 8C and Table I, 49% of the NF-M–ER-expressing cells had condensed nuclei 24 h after β-estradiol treatment. In contrast, <1% of the corresponding untreated cells or the parental cell line showed similar DNA condensations. Taken together, our data indicate that, in addition to its ability to induce eosinophil differentiation, NF-M is a potent inducer of programed cell death in HD57 MEP cells.

Discussion

To study the effects of NF-M (C/EBPβ) on hematopoietic differentiation, we used HD57 MEP cells expressing a conditional NF-M derivative. These cells possess differentiation potential towards myelomonocytic, eosinophilic, erythroid and thrombocytic lineages (Graf *et al.*, 1992; Frampton *et al.*, 1995; Kulessa *et al.* 1995) and lack NF-M expression (Katz *et al.*, 1993). In addition, phorbol ester-

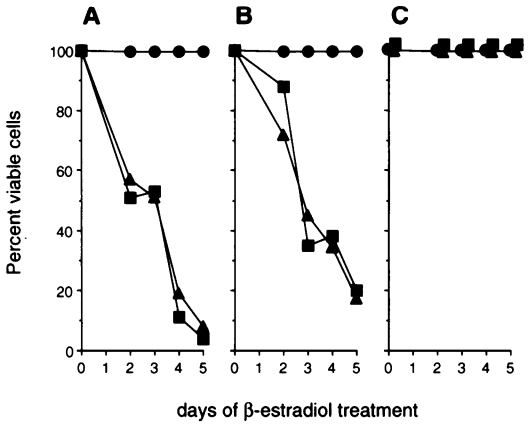

Fig. 7. Activation of NF-M–ER induces cell death. 1×10^5 cells from two NF-M–ER-expressing clones, cl.3 (**A**) and cl.14 (**B**), or from the parental cell line (**C**) were seeded in the presence of histidinol (1 mg/ml) and recombinant cMGF and treated with β-estradiol (1 μM) or 4-hydroxytamoxifen (4OHT, 1 μM) for 5 days. Aliquots were removed daily and the percentage of viable cells was determined by trypan blue staining as the mean of duplicates (circles: without hormone; triangles: plus β-estradiol; squares: plus 4OHT).

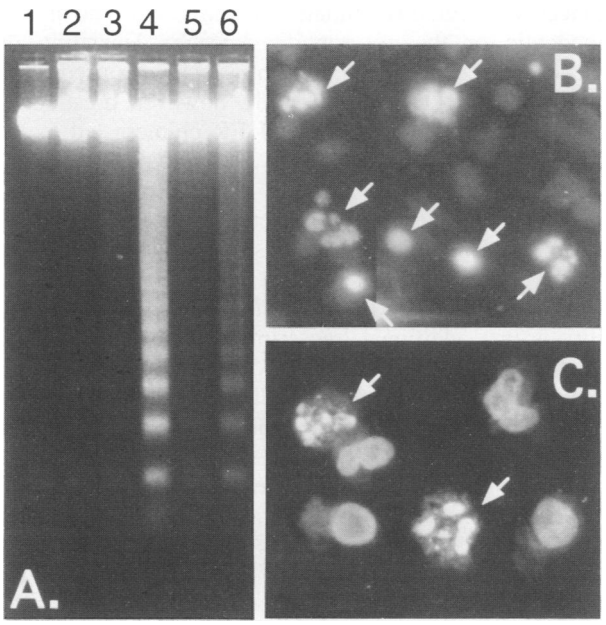

Fig. 8. Induction of apoptosis by NF-M. (**A**) Visualization of fragmented DNA by gel electrophoresis. NF-M–ER cl.3 and cl.6 DNA were prepared after 36 h of β-estradiol treatment (1 μM), separated on a 1.5% agarose gel and stained with ethidium bromide to visualize DNA (lane 1, parental cells, without hormone; lane 2, parental cells, plus β-estradiol; lane 3, NF-M–ER cl.3, without hormone; lane 4, NF-M–ER cl.3, plus β-estradiol; lane 5, NF-M–ER cl.6, without hormone; lane 6, NF-M–ER cl.6, plus β-estradiol). (**B**) Visualization of apoptotic cells by the TUNEL technique. NF-M–ER cl.3 cells were treated with β-estradiol (1 μM) for 24 h and pelleted by cytocentrifugation. After fixation and permeabilization, cells were labeled with fluorescein-coupled dUTP using terminal transferase (TUNEL reaction) and examined by fluorescence microscopy. Fluorescent nuclei indicative of apoptosis are marked by arrows. (**C**) Visualization of nuclear condensations by DAPI staining. NF-M–ER cl.3 cells were prepared as in (B), fixed and cellular DNA was stained with DAPI. The fluorescence micrograph shows nuclear condensations typical of apoptotic cells (indicated by arrows).

induced differentiation of MEP cells into the myeloid lineage correlated with the induction of NF-M expression (Katz *et al.*, 1993). Here we show that the *mim-1* gene is induced rapidly after β-estradiol activation of the NF-M–ER fusion protein in HD57 MEP cells. This gene is regulated directly by Myb and C/EBPβ in cells of the myelomonocytic and eosinophilic lineages (Ness *et al.*, 1989, 1993; Kulessa *et al.*, 1995). Furthermore, activation of the NF-M–ER chimera led to the expression of two eosinophilic differentiation markers, the surface antigen EOS47 and eosinophil peroxidase (Graf *et al.*, 1992). Apart from the activation of eosinophil-specific genes, NF-M also caused down-regulation of antigens characteristic for the early progenitor stage of MEP cells, a process which goes beyond the mere induction of target genes in heterologous cells. Taken together, these data suggest that NF-M activation causes eosinophilic lineage commitment of hematopoietic precursors.

Originally NF-M expression could only be detected in myelomonocytic cells of the hematopoietic system (Katz *et al.*, 1992; Scott *et al.*, 1992). Recently, however, NF-M was also found to be expressed in eosinophilic cells derived from a myeloblast cell line by ectopic expression of GATA-1 (Kulessa *et al.*, 1995), a finding which could be extended to normal eosinophils in the present study. Since eosinophils derived either from myelomonocytic cells or from MEP cells showed the concomitant expression of c-Myb, in addition to NF-M and GATA-1, while myelomonocytic cells lack GATA-1 (Kulessa *et al.*, 1995), it is tempting to speculate that the balance between these three transcription factors plays a decisive role in lineage determination. In agreement with this idea is the observation that activation of GATA-1 represses myelomonocytic gene expression (Kulessa *et al.*, 1995). We postulate, therefore, that the decision for lineage commitment of MEP cells (and possibly also of normal progenitor cells) is influenced by the presence or absence of NF-M (C/EBPβ) and GATA-1 on a background of Myb expression. If GATA-1 is expressed in the absence of NF-M, progenitors can differentiate into the thrombocytic and erythroid lineages. However, when NF-M is expressed in the absence of GATA-1, possibly in combination with a second factor (see below), myelomonocytic differentiation is favored. Furthermore, the combination of both factors, GATA-1 and NF-M, would allow differentiation towards the eosinophilic lineage. The decisions may also depend on the relative expression levels of the individual transcription factors.

In earlier studies, we have shown that phorbol ester

Table I. Apoptotic cells revealed by cytological staining

	HD57 MEP cells			
	Parental		NF-M–ER	
β-Estradiol[a]	–	+	–	+
Nuclear condensations by DAPI staining	2/612[b] (0.3)[c]	3/845 (0.4)	4/717 (0.6)	77/157 (49.0)
Terminal transferase (TUNEL) assay	16/868[b] (1.8)[c]	16/838 (1.9)	46/821 (5.6)	289/484 (59.7)

[a]Cells were treated with (+) or without (–) β-estradiol (1 μM) in the presence of histidinol (1 mM) and recombinant cMGF (200 U/ml).
[b]Positive cells versus total cell counts 24 h following estradiol treatment.
[c]Percentage of positive cells.

induces MEP cell differentiation towards either the myelomonocytic or the eosinophilic lineage, depending on whether high or low TPA concentrations were used (Graf *et al.*, 1992). In the present study, however, β-estradiol-induced activation of the NF-M–ER chimera led to the differentiation of eosinophils only. Failure to obtain myelomonocytic cells could indicate that C/EBPβ is not as efficient as other transcription factors of the C/EBP family such as C/EBPα, which is expressed in immature myelomonocytic cells but, in contrast to C/EBPβ, disappears during differentiation (Scott *et al.*, 1992). Another possibility is that additional transcription factors are missing which act in concert with NF-M or C/EBPα to sustain myelomonocytic differentiation. This possibility would be reminiscent of C/EBPα-induced adipocytic differentiation, which requires another adipocyte-specific factor, the lipid-activatable nuclear hormone receptor PPARγ2 (Tontonoz *et al.*, 1994). Since C/EBP proteins are expressed in differentiating cells of several tissues like fat, liver, intestine, skin and the hematopoietic system, synergizing partner proteins might always be required to control completion of the C/EBP-induced differentiation process in a tissue-specific manner.

Another possible explanation for the failure to detect myelomonocytic differentiation is that the cells die because suitable growth factors are missing (Williams *et al.*, 1990). We have previously shown that growth, survival and differentiation of chicken myeloblasts/promyelocytes depend on the G-CSF/Il-6-related chicken myelomonocytic growth factor, cMGF (Leutz *et al.*, 1989). Although the experiments were performed in the presence of cMGF, cell death could not be inhibited. This could reflect either the absence of cMGF receptor in estrogen-treated NF-M–ER-expressing cells or the requirement for yet another growth factor. We tried to circumvent the supposed factor dependence by ectopic expression of either the mammalian erythropoietin receptor or the G-CSF receptor, which are functional in chicken cells (Steinlein *et al.*, 1994; C.Müller, unpublished observations). However, neither erythropoietin nor G-CSF treatment of these cells prevented their hormone-induced death (data not shown). Finally, the inability of NF-M–ER cells to differentiate into myelomonocytic cells might also have more trivial reasons. Thus, we found that the NF-M–ER clones partially or completely lost their capacitiy to differentiate when treated with TPA (T.Graf, unpublished data). The fact that this is also seen with most HD57-derived subclones suggests that our culture conditions favor the outgrowth of cells that are unable to differentiate.

Several transcription factors normally involved in driving cells into proliferation have been shown to induce apoptosis when expressed inappropriately in resting cells. This has led to the suggestion that programed cell death occurs when quiescent cells receive an incomplete set of signals required for proliferation (Evan *et al.*, 1992; Wu and Levine, 1994). Our data provide an example for the converse situation, namely of a transcription factor involved in cell differentiation that can act as an inducer of apoptosis when activated in multiplying cells. This suggests that apoptosis in the hematopoietic system might be provoked not only by incomplete proliferation commands but by incomplete differentiation commands as well. Whether or not NF-M-induced differentiation and apoptosis represent alternative fates of MEP cells is an interesting question which remains to be studied.

Is the observed apoptotic activity of NF-M relevant for the hematopoietic differentiation process? Several observations support this possibility. Thus, phorbol ester-induced MEP differentiation is accompanied by the apoptopic death of a significant but variable proportion of the cell population (Graf *et al.*, 1992; T.Graf, preliminary results). Similarly, phorbol ester-induced differentiation of v-*myb*-transformed myeloblasts into macrophages also causes extensive cell death by apoptosis (Smarda and Lipsick, 1994). Finally, it has been shown that activation of protein kinase C (PKC) mediates phorbol ester-induced apoptosis in activated human macrophages, a process which seems to be independent of the presence of growth factors (Munn *et al.*, 1995). Since PKC activation strongly enhances C/EBPβ–NF-M transcription in myelomonocytic cells (Katz *et al.*, 1993; Trautwein *et al.*, 1993; Kowenz-Leutz *et al.*, 1994), PKC-mediated apoptosis in these cells might occur via activation of C/EBPβ–NF-M. On the other hand, the situation in HD57 MEP cells might be different, as apoptosis and gene activation induced by the chimeric NF-M–ER protein occur even in the absence of activated PKC. However, this discrepancy might be explained by failure of proper regulation of NF-M in the ER context following activation by β-estradiol. Since activation of NF-M by kinases is due to derepression, probably involving a conformational change of the protein (Kowenz-Leutz *et al.*, 1994), hormone binding to the chimeric protein might cause a similar structural alteration in NF-M.

As NF-M exhibits a highly modular structure, it would be interesting to know which domains are necessary for its capacity to induce apoptosis. Preliminary evidence based on mutational analysis indicates that apoptosis is critically dependent on its transactivation domains (Kowenz-Leutz *et al.*, 1994) implying that one or more genes have to be induced to mediate cell killing. It will be important to identify these genes which are likely to play a vital role in hematopoietic homeostasis. In addition, it will be interesting to see whether growth inhibition in other cell types following NF-M–CEBPβ activation is based on the same structural features of NF-M necessary for induction of apoptosis in myeloid cells, or whether these functions exhibit cell type specificity.

Materials and methods

Vector construction

An NF-M *Hin*dIII–*Bam*HI fragment which contains the complete coding region and translational initiation site (Katz *et al.*, 1993) was fused at its carboxyl-terminus in-frame to a *Bam*HI–*Bam*HI fragment which contained the hormone binding domain of the human estrogen receptor (amino acids 282–595) (Greene *et al.*, 1986) and the polyadenylation signal of the herpes virus thymidine kinase gene, both derived from the C/EBPα–ER expression vector (Umek *et al.*, 1991). The chimeric construct was cloned into the eukaryotic expression vector #321 (Buerstedde and Takeda, 1991), where it was transcibed from the chicken β-actin promoter. In addition, the vector carried a histidinol resistance gene driven by the Rous sarcoma virus long terminal repeat. Cloning was confirmed by DNA sequencing and the function and inducibility of the construct was tested by transient transfection experiments using cMGF promoter–luciferase reporter constructs (Sterneck *et al.*, 1992b).

Cells and tissue culture

Cells were incubated under tissue culture conditions in a humidified atmosphere with 5% CO_2 at 39°C. HD57 MEP cells were propagated

in phenol red-free medium-mix [350 ml of Dulbecco's modified Eagle's medium without phenol red (Gibco), 50 ml of bidest, 50 ml of fetal calf serum, 12.5 ml of chicken serum (not heat-treated), 13 ml of 7.5% $NaHCO_3$, 1.9 ml of 15 mg/ml conalbumin, 275 μl of 1.7 mg/ml insulin, 800 μl of 50 mM 2-mercaptoethanol, 9 ml of 20% glucose solution and the standard complement of antibiotics] (Graf *et al.*, 1992). In order to activate the NF-M–ER fusion protein, β-estradiol or 4-OHT was added to the medium in a final concentration of 1 μM and the medium was supplemented with 200 U/ml of recombinant cMGF (Leutz *et al.*, 1989). To inhibit protein biosynthesis, cycloheximide was added at 50 μg/ml final concentration.

Transient transfection

The erythroblast cell line HD3 (v-*erbA*, v-*erbB*-transformed; Beug *et al.*, 1979) was transfected by a DEAE–dextran procedure as described in detail by Sterneck *et al.* (1992b). The effector plasmid DNA concentration was 0.6 and 1 μg/ml with the luciferase reporters. Cells were transfected with 0.3 mg/ml DEAE–dextran in STBS solution (Ausubel *et al.*, 1987), treated with β-estradiol in a final concentration of 1 μM or with solvent, harvested after 40 h and lysed in 0.1 M potassium phosphate buffer, pH 7.8 by three freeze–thaw cycles. The extracts were cleared by centrifugation and 30 μl were assayed for luciferase activity as described (de Wet *et al.*, 1987; Sterneck *et al.*, 1992b). Assays were done in duplicate.

Stable transfection

Cells were washed twice with phosphate-buffered saline (PBS) and resuspended in PBS at 1×10^7 cells/45 μl. The plasmid DNA was purified twice on CsCl gradients and resuspended in PBS in a concentration of 1 μg/μl. For each electroporation, 5 μg of the DNA (5 μl) were mixed with 1×10^7 cells (45 μl) and transferred into an electroporation cuvette with an electrode gap of 0.4 cm (Bio-Rad). Electroporation was performed with a capacity of 250 μF and a voltage of 200 V which resulted in a time constant of 12–15 ms (Gene Pulser Apparatus, Bio-Rad). Electroporated cells were resuspended and seeded in 5 ml of medium-mix without phenol red. After 1–2 days, different concentrations of cells were seeded in semi-solid medium containing 2% methylcellulose (Methocel, Fulka) in phenol red-free medium-mix with a histidinol concentration of 3 mg/ml. Fourteen days later histidinol-resistant colonies were isolated and transferred to 500 μl of fresh medium-mix without phenol red which contained 1 mg/ml histidinol. Resistant clones were propagated and examined for NF-M–ER fusion protein expression by Western blotting.

Western blot analysis

Western blotting was performed with total cell lysates as indicated. Cells were lysed in 0.5 M NaOH, the lysate was then neutralized with 0.5 M HCl and mixed with 6× SDS loading buffer. An equivalent of 2.5×10^5 cells was subjected to reducing 10% SDS–PAGE and proteins were subsequently electrophoretically transferred onto a membrane (Millipore, PVDF membrane). Membranes were blocked with 5% non-fat dry milk in TBST (150 mM NaCl, 50 mM Tris–HCl, pH 7.4 and 0.02% v/v Tween-20) for 2 h and incubated for 2 h with a rabbit polyclonal antiserum raised against trpE–NF-M (Katz *et al.*, 1993) diluted 1:2000 in blocking solution. Filters were washed four times and incubated with a horseradish peroxidase-coupled second antibody (Bio-Rad) for 2 h. The blots were washed four times and immunoreactivity was detected with a chemoluminescence system (ECL; Amersham International).

Northern blot analysis

Total RNA was prepared using a guanidinium isothiocyanate method (Chomczynski and Sacchi, 1987). The poly(A)$^+$ fraction was isolated by oligo(dT) coupled to magnetic beads (Dynal). RNA was separated on a 1% formaldehyde agarose gel, transferred to Hybond membranes (Amersham), UV cross-linked and backed at 80°C for 2 h. The blots were probed in QuickHyb hybridization solution (Stratagene) at 65°C for 3 h with appropriate random ^{32}P-labeled (Pharmacia, Amersham) DNA fragments. The restriction fragments used as probes for GAPDH and *mim-1* have been described earlier (Ness *et al.*, 1989; Dugaiczyk *et al.*, 1983). Subsequently, the hybridization filters were washed twice for 15 min with 1× SSC, 0.1% SDS at room temperature and twice with 0.5× SSC, 0.1% SDS for 15 min at 60°C and exposed to Kodak X- OMAT-AR X-ray films or processed by PhosphorImager analysis (Fuji).

Cytofluorometric analysis

The MEP-specific monoclonal antibodies MEP21 and MEP26 and the eosinophilic-specific monoclonal antibody EOS47 have been described earlier (McNagny *et al.*, 1992). Cytofluorometric analysis was performed using FITC-conjugated goat anti-mouse antibodies. The second antibody alone was used as a negative control. Antigen-positive cells, negative for propidium iodide uptake, were evaluated using the Becton Dickinson FACScan cytometer.

For the detection of NF-M expression, cells were fixed with 3.7% paraformaldehyde in PBS, lysed with 0.05% NP-40 and stained with a polyclonal NF-M-specific antiserum. As second antibodies, FITC-conjugated goat anti-rabbit antibodies were used.

Peroxidase staining

Cells were suspended in 50 μl of medium to which 20 μl of peroxidase reagent was added for 30 min at room temperature. Peroxidase-positive cells stained dark brown.

Determination of cell viability

Cells (1×10^5) were seeded in 5 ml of phenol red-free medium-mix containing 1 mg/ml histidinol and 200 U of recombinant cMGF. β-Estradiol (1 μM) or the solvent alone in control experiments was added as indicated. Aliquots (50 μl) from the cultures were taken daily and mixed with 50 μl of concentrated trypan blue stain solution (Gibco). The total cell number and the number of dead cells were determined by microscopic evaluation using a Neubauer counting chamber.

Alternatively, cells were immobilized on a microscopy glass slide by cytocentrifugation, incubated with DAPI solution (100 μg DAPI/ml methanol) for 10 min at room temperature and analyzed by fluorescence microscopy.

To perform the TUNEL assay, cells were immobilized by cytocentrifugation and fixed with 3.7% paraformaldehyde solution in PBS for 30 min at room temperature. They were then washed once with PBS, permeabilized in 0.1% Triton X-100, 0.1% sodium citrate for 2 min at 4°C and washed twice with PBS. The TUNEL reaction was performed by incubation in a moist chamber with 0.3 nmol of FITC–12-dUTP (Boehringer Mannheim) 3 nmol of dATP, 2 μl of 25 mM $CoCl_2$, 25 U of terminal transferase (Boehringer Mannheim) and TdT buffer (30 mM Tris pH 7.2, 140 mM sodium cacodylate) in a total volume of 50 μl for 1 h at 37°C. The reaction was stopped by adding 2 μl of 0.5 M EDTA. Slides were washed twice in PBS and analyzed by fluorescence microscopy.

DNA ladder assay

Cells (2×10^6) were harvested, washed twice in 140 mM sodium chloride, 20 mM EDTA and incubated in lysis buffer (200 mM Tris pH 8.3, 100 mM EDTA, 50 μg/ml proteinase K, 1% SDS) at 37°C for 4 h. The DNA was extracted by adding an equal volume of phenol followed by centrifugation. The aqueous phase was dialyzed overnight against 10 mM Tris pH 7.5, 1 mM EDTA. Then EDTA was added to a final concentration of 25 mM and the DNA solution was incubated with RNase A (50 μg/ml) for 3 h at 37°C followed by incubation with proteinase K (120 μg/ml) for 3 h at 37°C. After phenol–chloroform extraction and ethanol precipitation, the DNA pellet was gently resuspended in 50 μl of TE and 3 μg of DNA were separated on a 1.5% agarose gel containing EtBr.

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