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The NF-M transcription factor is related to C/EBPβ and plays a role in signal transduction, differentiation and leukemogenesis of avian myelomonocytic cells

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Retroviral oncogenes encode nuclear regulators of gene expression or signal transduction molecules, such as protein kinases, which stimulate the activity of cellular transcription factors. Here we describe the cloning of NF-M, a myeloid-specific transcription factor related to C/EBPβ, which is a target of activated protein kinases. NF-M stimulates the expression of the gene encoding cMGF, a myeloid cell-specific growth factor, creating an autocrine growth loop crucial to oncogene transformation of myeloid cells. The NF-M protein bound directly to the cMGF gene promoter and activated its transcription, even in erythroid cells where the promoter is usually inactive. In addition, a truncated, dominant-negative form of NF-M inhibited cMGF expression in macrophages, indicating that NF-M is required for the normal activation of the gene. When multipotent hematopoietic progenitor cells were stimulated to differentiate, NF-M expression was induced at a very early stage, suggesting that the transcription factor plays a role in lineage commitment. The stimulation of transformed myelomonocytic cells or of normal peripheral blood macrophages with kinases or LPS or TPA respectively, led to the rapid redistribution of NF-M protein from the cell bodies to the nucleus, consistent with the notion that NF-M was directly affected by such treatments. Our data indicate that NF-M plays a key role in myelomonocytic differentiation, in signal transduction during macrophage activation and in the development of myelogenous leukemia.

Key words: C/EBP/leukemia/NF-M/oncogenes/signal transduction

Introduction

We first identified NF-M as a critical transcription factor required for the expression of chicken myelomonocytic growth factor (cMGF), which is distantly related to the mammalian hematopoietic growth factors G-CSF and IL-6 (Leutz *et al.*, 1989; Sterneck *et al.*, 1992b). cMGF is required for the outgrowth of chicken bone marrow-derived macrophage, granulocyte and mixed granulocyte/macrophage colonies (Leutz *et al.*, 1984, 1989). Similarly, myelomonocytic cells transformed by various natural and recombinant retrovirus strains encoding v-*myc* or v-*myb* oncogenes are dependent on cMGF for both survival and proliferation (Adkins *et al.*, 1984; Leutz *et al.*, 1984).

The expression of retrovirally-encoded, activated forms of serine/threonine or tyrosine kinases in v-*myc* or v-*myb*-transformed myelomonocytic cells induces the production and release of cMGF, resulting in an autocrine growth stimulation (Adkins *et al.*, 1984). A paradigm for such a collaboration between kinase- and nuclear protein-encoding oncogenes is the natural leukemia virus isolate MH2. This virus contains two oncogenes: v-*myc*, which encodes a transforming version of a transcription factor, and v-*mil* or chicken *raf*, which encodes an activated serine/threonine kinase that stimulates the autocrine growth loop (Graf *et al.*, 1986). The production of cMGF can also be induced by agents that 'activate' macrophages, like bacterial lipopolysaccharide (LPS) or *O*-tetradecanoyl phorbol 13-acetate [phorbol esters, TPA; (Leutz *et al.*, 1989)], suggesting that activation of the gene is a normal and necessary event in macrophage function. We showed recently that the growth promoting effect of a kinase oncogene could be fully replaced by the cMGF cDNA expressed from a recombinant retrovirus (Metz *et al.*, 1991). This suggests that it is the cMGF expression and not the kinase activity *per se* that contributes to leukemia induction in myeloid cells (Adkins *et al.*, 1984; Metz *et al.*, 1991).

Within the hematopoietic system, cMGF protein and mRNA are found only in monocytic cells (Leutz at al., 1989). Nuclear run-on analysis showed that cMGF expression is regulated at the level of gene transcription (Sterneck *et al.*, 1992a). In co-transfection assays, cMGF promoter–reporter constructs are highly expressed in stimulated myelomonocytic cells, but not in cells of other hematopoietic lineages (Sterneck *et al.*, 1992a). The cMGF promoter contains a single binding site for the AP-1 transactivator complex and two binding sites for a novel nuclear factor termed NF-M (Sterneck *et al.*, 1992b). The latter are absolutely required for both the kinase inducibility and tissue specificity of the cMGF promoter. Interestingly, NF-M proteins are found in chicken and in human myelomonocytic cells, but not in cells of other hematopoietic lineages. Furthermore, the addition of synthetic NF-M binding sites to a heterologous promoter confers a *cis*-activation in chicken and in human myelomonocytic cells that express NF-M-like proteins, but not in erythroid or lymphoid cells of either species (Haas *et al.*, 1992; Sterneck *et al.*, 1992b). This indicates that NF-M is a lineage-specific transcriptional activator required for cMGF gene activation, which has been conserved in species as diverse as humans and birds.

We set out to characterize NF-M in order to understand better its roles both as an activator of cMGF gene expression and also as a mediator of the activity of protein kinase-encoding oncogenes. We have isolated a cDNA clone

encoding NF-M, which is a chicken bZip phosphoprotein related to mammalian C/EBPβ. Constitutive expression of the NF-M cDNA transactivates the cMGF promoter–reporter gene constructs in both myeloid and non-myeloid cell types, indicating that the cDNA encodes a fully functional form of NF-M, which can relieve the tissue specificity of the cMGF promoter. Analysis of the NF-M protein suggests that its cytoplasmic/nuclear translocation is coupled to various kinase signalling pathways. We conclude that NF-M plays a key role in linking extracellular signals to changes in macrophage gene expression, including those that contribute to myelogenous leukemias.

Results

Chick NF-M is related to C/EBPβ

The cMGF promoter contains two binding sites for the nuclear factor(s) NF-M arranged as a tandem, inverted repeat, both of which are required for myelomonocyte-specific cMGF gene expression (Sterneck *et al.*, 1992b). These binding sites are related to those that bind the CCAAT/enhancer binding protein C/EBP and synthetic oligonucleotides containing *bona fide* C/EBP binding sites compete with NF-M binding to the cMGF promoter. In addition, synthetic leucine zipper peptides from C/EBP-related proteins can disrupt NF-M binding to DNA, suggesting that NF-M and C/EBP have related protein dimerization domains (Sterneck *et al.*, 1992b). We therefore assumed that NF-M and C/EBP would be structurally related and we used rat C/EBPα and C/EBPβ probes to screen a cDNA library derived from the chick myeloblast, avian myeloblastosis virus (AMV)-transformed, BM-2 cell line. From 1.5×10^5 plaques, 36 hybridized to both probes. The positive plaques were isolated, purified and their inserts were subcloned into Bluescript plasmid vectors, then the plasmids were used as templates for *in vitro* transcription/translation assays using T7 and T3 RNA polymerases and reticulocyte lysates. The ability of the resulting proteins to bind the NF-

A

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   1  ggatggtccgcagagagccgccagctccagccgcagctccgggagcgggagagccccgcc   60

  61  gcgccgcacggactgagacgcccgggcgggcagcgcagcgggccgtgccagcgccgggct  120

 121  cgggagacccccacctgacgacggccgctcgatcccgtgccgggggggsscccagcgccg  180

 181  tcttctcctccccgcatccccctttgctttcatgcaacgcctggtggcctgggacgcagc  240
   1                                  M  Q  R  L  V  A  W  D  A  A   10

 241  atgcctccccattcagccgcccgcctttaaatccatggaagtggctaatttctattacga  300
  11   C  L  P  I  Q  P  P  A  F  K  S  M  E  V  A  N  F  Y  Y  E   30

 301  ggcggactgtttggctgctctcaacaagctgcacccgcgggcggccgggggccgctccat  360
  31   A  D  C  L  A  A  L  N  K  L  H  P  R  A  A  G  G  R  S  M   50

 361  gaccgaacttaccgtaggggaccacgagagagccattgacttcagcccctatctggaccc  420
  51   T  E  L  T  V  G  D  H  E  R  A  I  D  F  S  P  Y  L  D  P   70

 421  cttagcagcatcccagcagccggcgcagccgccgcctcccgcagcagcagcaggggcaa  480
  71   L  A  A  S  Q  Q  P  A  Q  P  P  P  P  A  A  A  A  G  G  N   90

 481  ctttgagcctgcctgcagtagcggcggccaagatttcctttccgatctcttcgccgagga  540
  91   F  E  P  A  C  S  S  G  G  Q  D  F  L  S  D  L  F  A  E  D  110

 541  ctataaaggcagcggcggcggcaagaagcccgactacacctacatcagcctcacccggca  600
 111   Y  K  G  S  G  G  G  K  K  P  D  Y  T  Y  I  S  L  T  R  H  130

 601  cggccacccgtgcggcagccagagccacaagccgggggtgctgccgggctgcttcccgcc  660
 131   G  H  P  C  G  S  Q  S  H  K  P  G  V  L  P  G  C  F  P  P  150

 661  ccagatcgtggagaccaaagtggagccggtcttcgagaccctggactcttgcaaagggcc  720
 151   Q  I  V  E  T  K  V  E  P  V  F  E  T  L  D  S  C  K  G  P  170

 721  ccgtaaggaagaaggggagcggggccaggaccgggggcatgtcctcgccctacggcag  780
 171   R  K  E  E  G  G  A  G  P  G  P  G  G  M  S  S  P  Y  G  S  190

 781  caccgtgcgctcctacctgggctaccagtcggtgccgagcggcagcagcgggaacctgtc  840
 191   T  V  R  S  Y  L  G  Y  Q  S  V  P  S  G  S  S  G  N  L  S  210

 841  cacctcgtcctcctccagccccccggcaccccgaacccctccgagtcctccaagtcggc  900
 211   T  S  S  S  S  S  P  P  G  T  P  N  P  S  E  S  S  K  S  A  230

 901  cgccggcgccgggggctactcggggccgccggcgggcaagaacaagcccaagaagtgcgt  960
 231   A  G  A  G  G  Y  S  G  P  P  A  G  K  N  K  P  K  K  C  V  250

 961  ggacaagcacagcgacgagtacaagctgcgccgggagcggaacaacatcgctgtgcgcaa 1020
 251   D  K  H  S  D  E  Y  K  L  R  R  E  R  N  N  I  A  V  R  K  270

1021  gagccgcgacaaagccaaaatgcgcaacctggagacgcagcacaaagtcttggaactgac 1080
 271   S  R  D  K  A  K  M  R  N  L  E  T  Q  H  K  V  L  E  L  T  290

1081  ggccgagaacgagaggctgcagaagaaggtggagcagctctctcgggagctgagcaccct 1140
 291   A  E  N  E  R  L  Q  K  K  V  E  Q  L  S  R  E  L  S  T  L  310

1141  caggaacttgttcaaacagctgcccgagcccctgctcgcttcctcgccccgctgctgacc 1200
 311   R  N  L  F  K  Q  L  P  E  P  L  L  A  S  S  P  R  C       328

1201  cccgcgccggccgcgcgagctgagcgctgccccccacgccctcctcccggggccggccg 1260

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1321  gtctctaccg 1330
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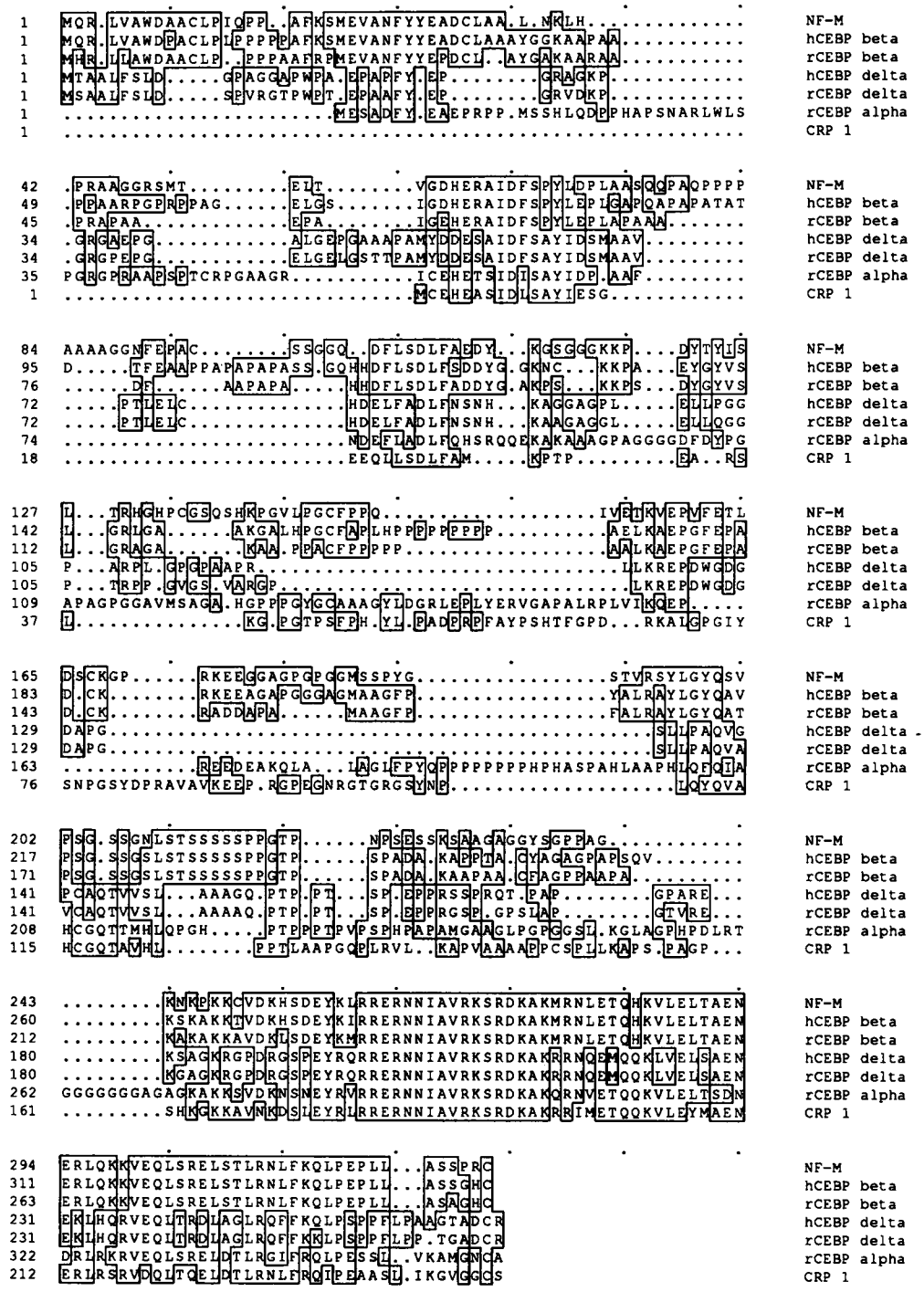

Fig. 1. Sequence of NF-M cDNA and relationship to mammalian C/EBP homologs. **A.** cDNA sequence of NF-M. The leucine residues of the predicted 'leucine zipper' are indicated by circles. **B.** Comparison of various C/EBP homologues. Identical amino acids are boxed. Gaps are indicated by dots.

M binding sites from the cMGF promoter was tested using a band-shift assay. Of the first 12 phage inserts examined, six encoded proteins that bound intact but not mutated forms of the NF-M binding sites (data not shown). Three inserts were characterized further, by restriction enzyme mapping and DNA sequencing, and were found to partially overlap (data not shown and Figure 1A). One insert of 1330 nucleotides (# 16.3) contained an open reading frame of 984 nucleotides corresponding to 328 amino acids (Figure 1A), which appeared to encode a full-length NF-M protein.

The inferred amino acid sequence of NF-M corresponds to a protein with a calculated molecular weight of 35 kDa, containing a basic region and a leucine zipper at its C-terminus (Figure 1A and B). The overall amino acid sequence of NF-M is highly similar to the bZip transcriptional activators NF-IL6 (Akira *et al.*, 1990), IL-6 DBP (Poli *et al.*, 1990), LAP (Descombes *et al.*, 1990), AGP-EBP (Chang *et al.*, 1990), C/EBPβ (Cao *et al.*, 1991) and CRP-2 (Williams *et al.*, 1991). The C-terminal ends of all these proteins are nearly identical, despite the fact that they come from species as divergent as chickens and man.

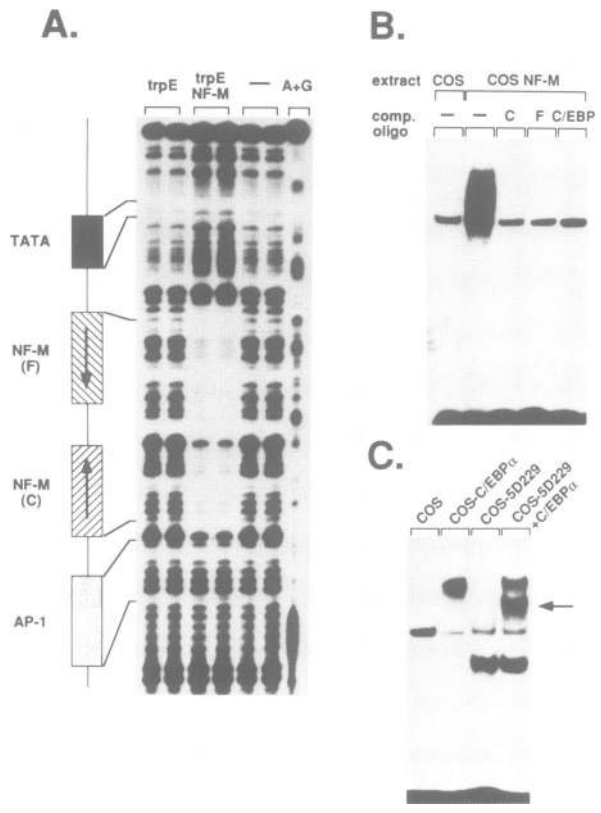

Fig. 2. Binding specificity of recombinant NF-M. **A.** Footprint analysis of the cMGF promoter region using bacterially-expressed NF-M. On the left, the scheme shows the relevant features of the cMGF promoter (Sterneck *et al.*, 1992b) indicating the two NF-M binding sites and the corresponding oligonucleotides C and F. trpE, trpE protein of vector, trpE−NF-M, fusion protein between trpE and NF-M; A + G, Sequence of the cMGF promotor showing A and G nucleotides. **B.** Bandshift analysis with COS cell nuclear extracts. Nuclear extracts from COS-7 cells transfected with plasmid pCDM8 (COS) or with pCDM8-NF-M were analysed in a gel mobility shift assay using ^{32}P-labelled oligonucleotide F (probe) and NF-M binding sites C, F and C/EBP binding sites as competitors, each at a 200-fold molar excess. **C.** Heterodimer formation between NF-M and rat C/EBPα. pCDM8 expression vectors encoding rat C/EBPα or the N-terminal-truncated NF-M 5D229 mutant were expressed in COS cells and nuclear extracts were examined by band-shift analysis using the C/EBP oligonucleotide as a probe before and after mixing of extracts. Arrow on the right indicates the new, intermediate size heterodimer that forms after mixing of extracts from C/EBPα and 5D229 transfected cells.

An alignment of the NF-M sequence with other C/EBP-related proteins is shown in Figure 1B (see also Materials and methods). The alignment suggests that the proteins share a number of structural elements in addition to the highly conserved b-Zip region. Some of these regions are separated by peptides rich in alanine, glycine and proline, which could link functional domains.

We transferred the cDNA clone from phage # 12.1, which starts at Ala72, into a bacterial expression vector and expressed the resulting trpE−NF-M fusion protein in bacteria. As shown in Figure 2A, the recombinant, bacterially-expressed NF-M bound to both NF-M binding sites on the cMGF promoter with high affinity (Figure 2A), confirming that it encodes a functional, DNA-binding version of NF-M. The full-length cDNA from phage # 16.3 was also transferred into the eukaryotic expression vector pCDM8 and the resulting plasmid was transfected into COS-7 cells. Nuclear extracts of the cells were examined for DNA binding activity in a band-shift analysis, as shown in Figure 2B. The extracts from cells transfected with the NF-M-expressing plasmid, but not with the control vector alone, contained a nuclear protein that specifically bound to both the distal and proximal C- and F-sites of the cMGF promoter (Figure 2B). The complexes formed by the recombinant NF-M co-migrated with the NF-M complexes formed by nuclear extracts from myeloid cells (not shown). Also, the binding was specifically and efficiently competed by synthetic oligonucleotides containing either the C- or F-sites or by a C/EBP binding site (Figure 2B), but not by a number of related oligonucleotides that are unable to bind the macrophage-derived protein [(Sterneck *et al.*, 1992b) and data not shown]. Finally, when synthetic, leucine zipper-containing peptides were added to the band-shift assays, the peptides derived from the rat proteins C/EBPα and NF-IL6 disrupted the NF-M binding, but the peptide derived from the CREB protein did not (data not shown). These results are identical to those obtained previously with macrophage-derived NF-M protein (Sterneck *et al.*, 1992b), showing that the cDNA clone in fact encodes chicken NF-M.

The experiments with leucine zipper peptides suggested that NF-M may form dimers with other C/EBP-like proteins. To test this directly, we constructed an N-terminal deletion mutant of NF-M in the pCDM8 vector, termed 5D229, and compared extracts from COS cells expressing either it or rat C/EBPα by band-shift analysis. As shown in Figure 2B and C, the untransfected COS cells were found to contain a protein species that binds the C/EBP binding site probe, but extracts from cells transfected with the rat C/EBPα expression vector also formed an additional, more slowly-migrating complex. The 5D229 deletion mutant of NF-M formed the fastest migrating complex, consistent with the protein's smaller molecular weight. Finally, when extracts of the cells transfected with the rat C/EBPα or 5D229 expression vectors were mixed, an additional complex formed, presumably composed of C/EBPα-5D229 heterodimers, with an intermediate rate of migration. We conclude that rat C/EBPα and chicken NF-M 5D229 can readily form both homo- and heterodimers capable of binding to C/EBP binding sites. Thus, NF-M is most likely a leucine zipper protein that binds the cMGF promoter at two sites, either as a homodimer or as a complex with other, as yet unidentified leucine zipper-containing proteins.

NF-M activates transcription from the cMGF promoter

We used transient co-transfection assays to determine whether our NF-M cDNA could transactivate the cMGF promoter. As a model system, we introduced the eukaryotic NF-M expression vector into the erythroid cell line HD3, along with a suitable cMGF promoter−reporter gene construct. We had shown previously that such reporter constructs could be transactivated in HD3 cells, which normally express neither NF-M nor cMGF, when co-transfected with a plasmid encoding rat C/EBPα (Sterneck *et al.*, 1992b). Figure 3 shows that introduction of the NF-M expression vector, but not the pCDM8 control plasmid, greatly induced the expression of the cMGF promoter. Similarly, when synthetic NF-M binding sites were introduced upstream of the minimal TK promoter (Sterneck *et al.*, 1992b) they conferred NF-M inducibility to the heterologous construct (data not shown). Finally, mutated cMGF promoter−reporter constructs that failed to bind NF-M were not stimulated in this assay [(Sterneck *et al.*, 1992b)

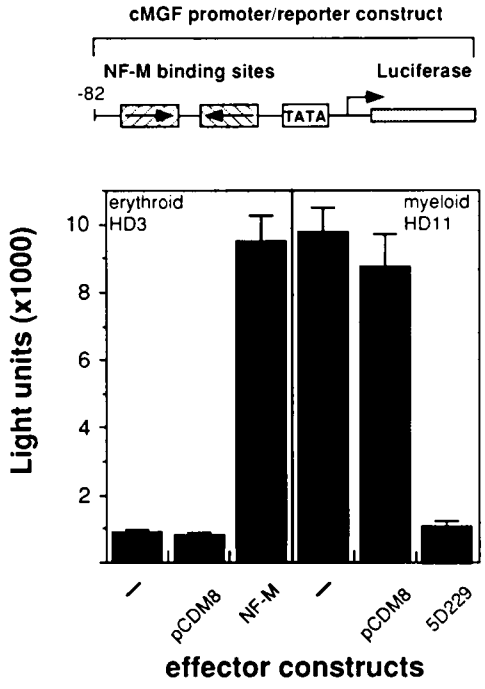

Fig. 3. Transient transfection assay of NF-M expression constructs. Effector constructs were transfected into erythroid (HD3) or macrophage (HD11) cell lines together with the luciferase reporter construct using the pM82 cMGF promoter as indicated on the top (Sterneck *et al.*, 1992a). Luciferase activity was determined 48 h after transfection. −, no effector; pCDM8, vector control; NF-M, pCDM8-NF-M; 5D229, pCDM8-5D229.

and data not shown]. These results demonstrate that NF-M is a transcriptional regulator that activates reporter expression through its binding sites in the cMGF promoter. When the 5D229 expression vector was introduced into erythroid HD3 cells, no activation of the cMGF promoter was observed (not shown). However, when this construct was introduced along with the cMGF promoter−reporter constructs into HD11 macrophage cells, the expression from the reporter construct was almost completely inhibited (Figure 3). These cells already express NF-M and the cMGF promoter is usually quite active. This effect was not observed with control plasmids like pCDM8 (Figure 3) or with other promoters that are independent of NF-M for their activity (Ness,S.A., Kowenz-Leutz,E., Cassini,T., Graf,T. and Leutz,A., submitted). Since the 5D229 deletion removed the putative transactivation domain without affecting the DNA binding and the leucine zipper dimerization domain, this suggests that 5D229 inhibits cMGF promoter expression by competing for binding sites with the endogenous, wild-type NF-M protein. Alternatively, it might form inactive heterodimers with the full-length transcription factor. We conclude that NF-M is a DNA-binding transcriptional regulator that activates expression of the cMGF gene by binding to specific sites in its promoter.

NF-M is specifically expressed in myelomonocytic cells

To characterize the native NF-M protein in more detail, we raised an antiserum to the bacterially-expressed trpE−NF-M fusion protein. First, we used an immunoprecipitation assay to examine the NF-M protein made by transformed macrophages. As shown in Figure 4A, the antiserum

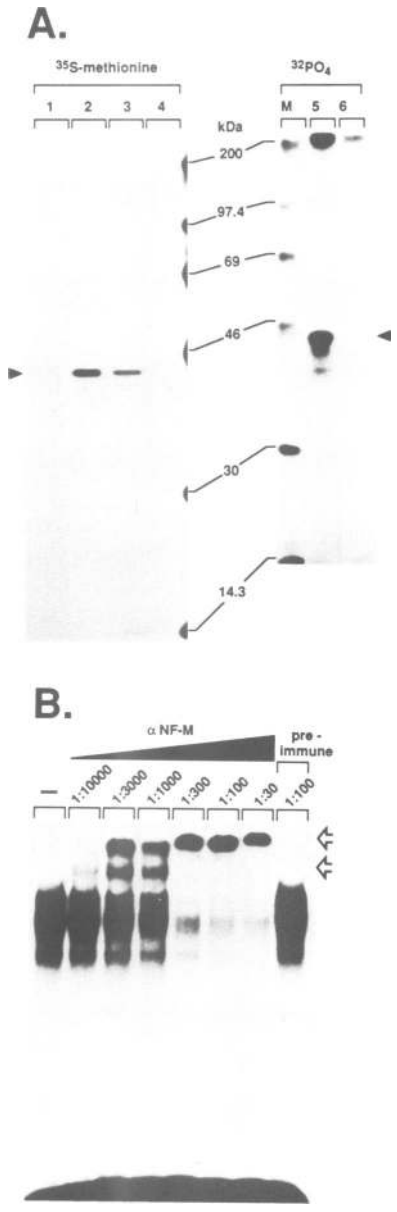

Fig. 4. Immunoprecipitation and immunoshift using an NF-M antiserum. **A.** Immunoprecipitation. COS cells transfected with pCDM8 (lane 1) or pCDM8-NF-M (lane 2) or HD11 macrophages (lanes 3−6) were labelled with either [^{35}S]methionine or ^{32}P and extracts were subjected to immunoprecipitation using rabbit antiserum raised against trpE−NF-M (lanes 1−3 and 5) or using preimmune serum (lanes 4 and 6). The left autoradiograph shows results obtained with [^{35}S]methionine, the right shows results obtained with ^{32}P-labelled cells. Arrow heads on the left and right indicate bands corresponding to metabolically-labelled NF-M. **B.** Immunoshift. 10 μg of nuclear protein from HD11 macrophages was incubated with ^{32}P-labelled oligonucleotide F for 15 min on ice in binding buffer. Antiserum or preimmune serum was then added for another 15 min before loading samples on a gel. Open arrows on the right indicate bands corresponding to supershift.

precipitated a 37−40 kDa protein from both HD11 cells and COS cells transfected with an NF-M expression plasmid. No such protein was precipitated from mock-transfected COS cells or when preimmune serum was used. The NF-M antiserum also failed to detect rat C/EBPα (data not shown) or NF-M 5D229, suggesting that the epitope(s) it recognized are not within the DNA binding and dimerization domains. Metabolic labelling with [^{32}P]orthophosphate showed that

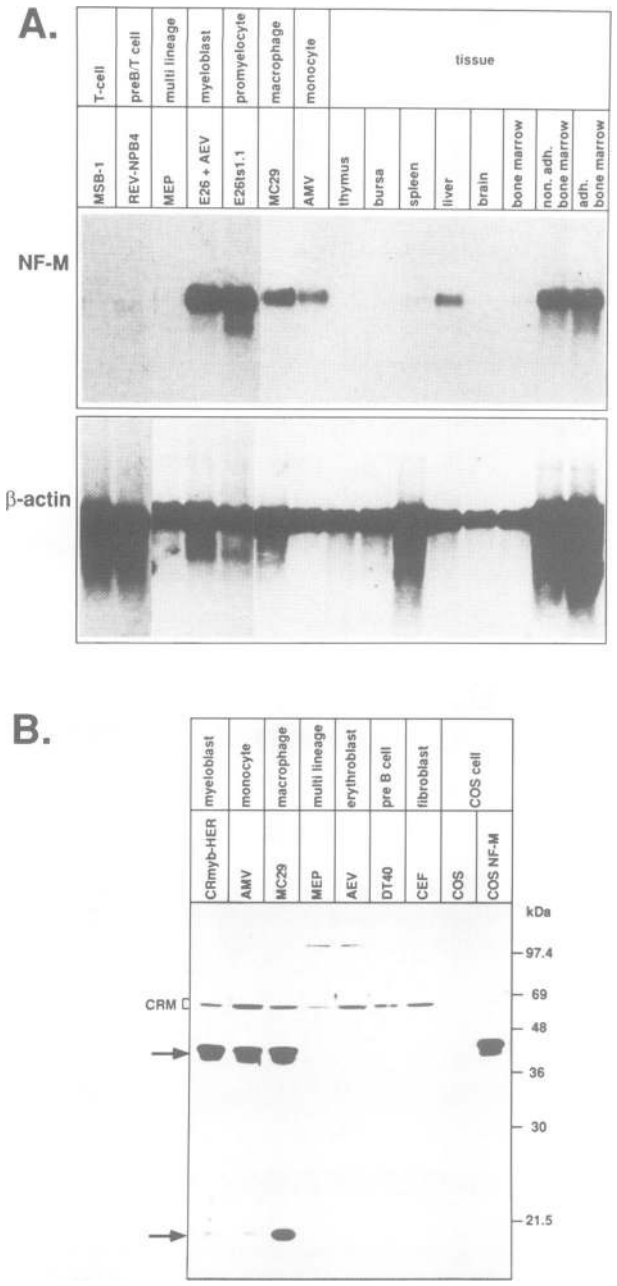

Fig. 5. Tissue distribution of NF-M RNA and protein. **A.** Northern analysis of various hematopoietic cell types and tissues. The probes used are indicated on the left. Cell types: MSB-1, REV-NPB4, T-lymphoid and pre B/pre T lymphoblasts, respectively. MEP, E26 virus-transfected multipotent cells, myeloblasts and promyelocytes respectively; E26 + AEV, E26-transformed myeloblasts that were superinfected with the AEV virus; E26ts1.1, myeloblasts transformed by the temperature-sensitive ts1.1 E26 virus; MC29, virus-transformed primary macrophages; AMV, AMV-transformed cell line BM2 C2; bone marrow, RNA from the nonadherent and the adherent fraction of bone marrow cells (obtained from a 7 day-old chick) 48 h after plating. **B.** Western analysis of various cell types. Total extracts were prepared and immunoblotted with NF-M antiserum. CRmyb−HER, myeloblasts/promyelocytes transformed by a recombinant retrovirus expressing both Myb and the human EGF receptor; AMV, AMV-transformed myeloblast cell line BM-2; MC29, MC29-transformed HD11 macrophage cell line; AEV, tsAEV-transformed erythroblast cell line HD 3; DT40, pre-B cell line; CEF, chicken embryo fibroblasts; COS, COS-7 cells transfected with vector DNA; COS-NF-M, COS-7 cells transfected with pCDM8-NF-M. Arrows indicate the position of NF-M (37−40 kDa) and a NF-M fragment of 18−20 kDa. CRM: cross reactive material found in all chicken cell lines examined.

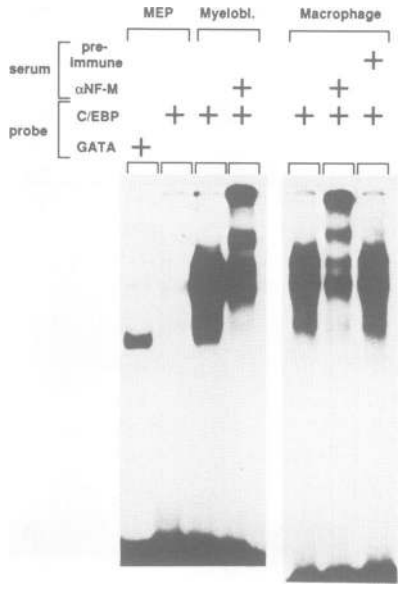

Fig. 6. NF-M expression and myelomonocytic lineage commitment. Nuclear extracts of E26-transformed multipotent progenitor cells were examined for the expression of NF-M before (MEP) and after (5 days) induction of differentiation towards myeloblasts by TPA (Myelobl.). Mobility shift assays with labelled oligonucleotides as indicated (probe) were performed in the presence of pre-immune serum or NF-M antiserum. HD11 cell (Macrophage) nuclear extracts were used as a control.

the macrophage-derived protein is phosphorylated (Figure 4A, right).

As shown in Figure 4B, when sufficient NF-M antiserum was added to a macrophage extract in a band-shift assay, >90% of the resulting NF-M−DNA complex could be 'super-shifted' to a slower migrating form, presumably because the interaction with NF-M antibodies increased the apparent molecular weight of the complex. There was no effect of the preimmune serum or a rat C/EBPα peptide antiserum (data not shown) in this assay. Thus, NF-M is a component of essentially all the nuclear protein complexes in macrophage nuclear extracts that bind to the NF-M binding sites. We cannot rule out the possibility that some or all of the NF-M is involved in heterodimeric complexes with other C/EBP-like, leucine zipper-containing proteins and that these complexes react with the antiserum in our assay.

Using band-shift assays, we showed previously that NF-M DNA binding activity was only detectable in myelomonocytic cells (Haas *et al.*, 1992; Sterneck *et al.*, 1992b). The availability of an NF-M cDNA clone made it possible to confirm these findings by Northern analysis. As shown in Figure 5, NF-M mRNA was detected in all retrovirally-transformed myelomonocytic cell lines, but not in transformed B-cells, preB/T-cells or erythroblasts. Of the organs examined, only the liver contained detectable levels of NF-M RNA while thymus and bursa were negative. Traces of NF-M mRNA could also be detected on prolonged exposures in spleen and bone marrow. Bone marrow cells, which had been cultured for several days to select specifically for myelomonocytic cells, expressed NF-M mRNA at high levels. No NF-M mRNA was found in brain (Figure 5), kidney, muscle or heart, although lung tissue contained traces of NF-M mRNA (data not shown).

Because the expression of rat C/EBPβ (LAP) mRNA does

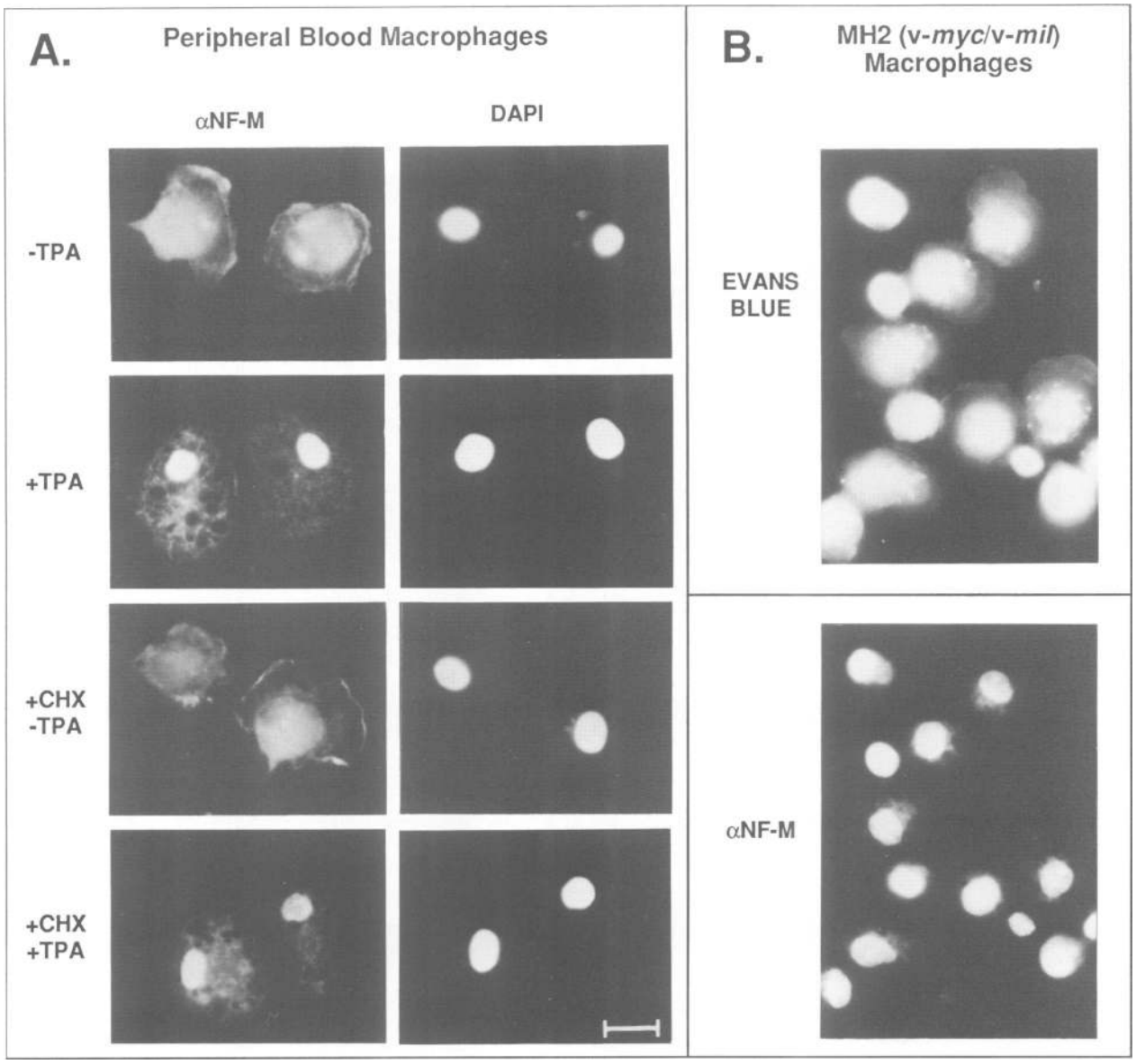

Fig. 7. Subcellular distribution of NF-M in normal and MH2-transformed macrophages. **A.** Macrophages were isolated from peripheral blood by Ficoll centrifugation and grown on cover slips for 3 days. TPA was added for 30 min to cell cultures. Where indicated, cycloheximide CHX (final concentration 50 μg/ml) was added 10 min before TPA. Cells were fixed and stained with NF-M antiserum and DAPI as indicated and photographed with appropriate filters. **B.** Cytospins of MH2 leukemia virus-transformed macrophages. As indicated, MH2 macrophages express the v-*myc* and the v-*mil* (the chicken raf homologue) oncogenes. The v-*mil* kinase induces cMGF secretion and autocrine growth stimulation of MH2 macrophages. Cells were stained with Evans blue in addition to NF-M antiserum. Bar corresponds to 15 μm.

not always reflect the production of the corresponding protein (Descombes *et al.*, 1990), we used Western blotting to determine the level of NF-M protein expression in various hematopoietic cells. As shown in Figure 5B, all myelomonocytic cells examined that express NF-M RNA also express NF-M protein. Interestingly, using a gel with a higher percentage of polyacrylamide, an additional immunoreactive band was observed at 20 kDa. We do not know whether this band corresponds to the LIP homologue (Descombes and Schibler, 1991) of NF-M, a cross-reacting protein or a NF-M degradation product. NF-M could not be found in multipotent precursor cells (Graf *et al.*, 1992), in erythroid or in lymphoid cells. We were unable to detect other potential NF-M-related proteins like chicken C/EBPα (not shown), suggesting that if such proteins are expressed in chicken cells, they must be immunologically distinct from NF-M. We conclude from these experiments that within the hematopoietic system, NF-M expression is restricted to the myelomonocytic lineage.

Recently, it has been shown that the avian leukemia virus E26, which encodes a *gag−myb-ets* fusion oncogene, transforms cells that have multipotential stem cell or precursor cell-like properties (Graf *et al.*, 1992). These so-called MEP cells can be induced to differentiate into the myelomonocytic lineage by treatment with TPA. To investigate whether myelomonocytic differentiation is accompanied by expression of NF-M, we examined nuclear extracts of MEP cells before and after induction of myelomonocytic differentiation. As shown in Figure 6, uninduced MEP cells expressed GATA-1 but not NF-M (see also Figure 5). However, when the MEP cells were induced to differentiate along the myelomonocytic lineage the

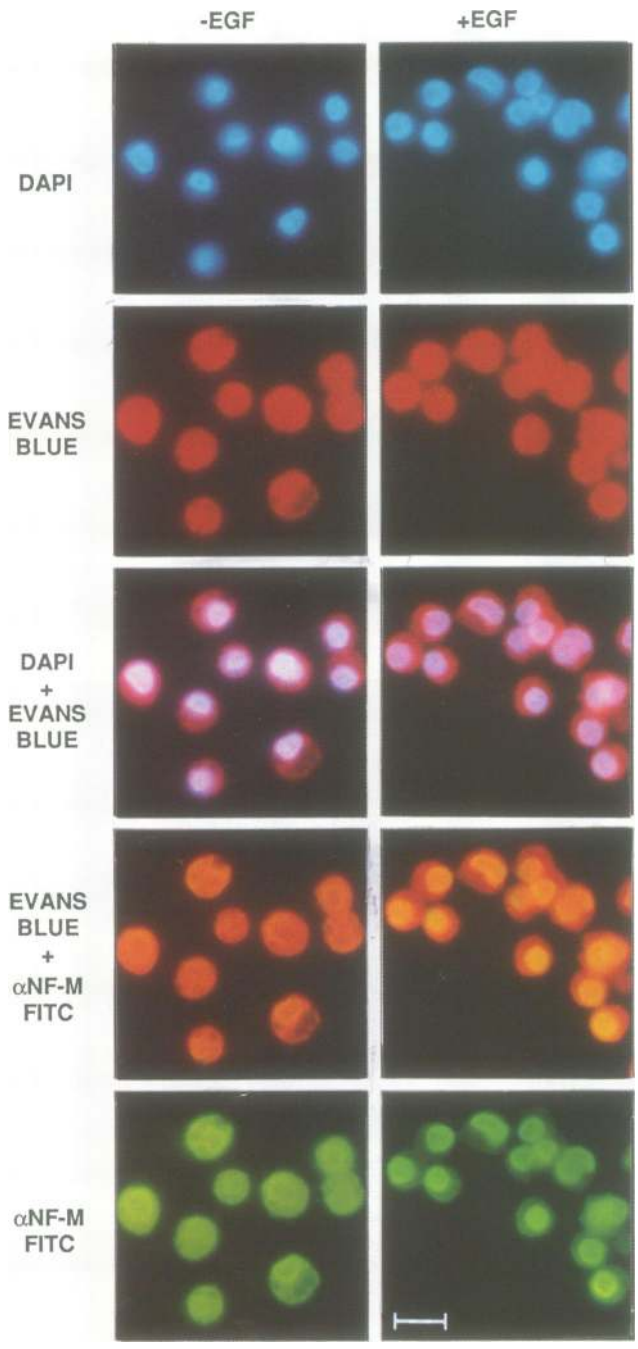

Fig. 8. Change in subcellular distribution of NF-M following membrane tyrosine kinase activation. CRmyb−HER-transformed myeloblasts were grown in the presence of cMGF. As indicated on the top, cells were treated with EGF (20 ng/ml) for 1 h, cytocentrifuged, fixed and stained with DAPI, Evans blue, and NF-M antiserum. Exposures and double exposures using appropriate filters were performed as indicated on the left. Bar corresponds to 10 μm.

resulting myeloblasts strongly expressed NF-M. We conclude that NF-M expression is turned on during myelomonocytic differentiation, probably at some point prior to the myeloblast stage.

NF-M protein translocates from the cytoplasm to the nucleus

It has been shown that C/EBPβ in PC12 cells is subject to post-translational modification and cytoplasmic/nuclear

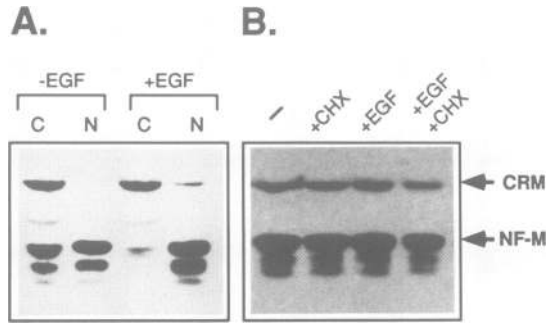

Figure 9. Western blot analysis of CRmyb−HER-transformed myeloblasts. **A.** Myeloblasts were not treated or were treated with EGF (20 ng/ml) for 1 h, washed in ice cold PBS, nuclear and cytoplasmic extracts were prepared as described by Sterneck *et al.* (1992b). The protein concentrations of the cytoplasmic and nuclear fractions were determined and 50 μg of protein were loaded per lane. Following electrophoresis, proteins were blotted to PVDF membranes and stained for NF-M immunoreactivity as described in Materials and methods. **B.** Cells were grown as in A. Where indicated, cycloheximide (50 μg/ml) was added 10 min before addition of EGF. Extracts were prepared from whole cells by boiling the washed cells in SDS gel sample buffer. Equivalents of 2.5×10^5 cells were loaded per lane and analysed as in panel A.

translocation (Metz and Ziff, 1991b). To determine whether in myeloid cells the localization of NF-M is affected by LPS or TPA treatment, we examined the subcellular distribution of NF-M in normal, peripheral macrophages by immunofluorescence. Figure 7A shows that NF-M was ubiquitously expressed in the cytoplasm and in the nucleus of normal macrophages. In some cells, a trabecular and sometimes spotted rather than a diffuse staining was found in the cytoplasm. The staining pattern changed drastically upon TPA (Figure 7A) or LPS (data not shown) treatment. Most of the immunoreactive NF-M concentrated in the nucleus after addition of TPA, while the cytoplasmic staining became faint. The increase in nuclear fluorescence and concomitant loss of cytoplasmic fluorescence was also observed when cells were retreated with cycloheximide to block protein synthesis before TPA (Figure 7A) or LPS (data not shown) was added, while cycloheximide on its own had no effect on subcellular NF-M distribution. These results suggest that stimulation of macrophages induces pre-existing NF-M protein to migrate to the nucleus and that activation of NF-M in this way may occur, at least in part, through post-translational modifications.

A number of activated kinases have been shown to induce cMGF expression and autocrine growth stimulation in transformed myelomonocytic cells (Adkins *et al.*, 1984). Most notably, the v-*mil* kinase oncogene of the leukemia virus MH2 transcriptionally activates cMGF gene expression (Sterneck *et al.*, 1992a,b) and growth factor secretion (Adkins *et al.*, 1984), which is a key step in the development of a monocytic leukemia (Graf *et al.*, 1986; Metz *et al.*, 1991). It was therefore interesting to examine where NF-M is localized in MH2-transformed macrophages. As shown in Figure 7B, MH2-transformed macrophages were stained with Evans blue to visualize whole cell bodies and with antiserum to reveal NF-M immunoreactivity. It is evident that in MH2-transformed monocytes, NF-M is solely expressed in the nucleus and in contrast to normal macrophages virtually absent in the cytoplasm.

Since protein kinase C activation and c-*mil* activity are

normally coupled to membrane receptors, we examined whether a ligand-activated membrane receptor tyrosine kinase would induce cytoplasmic/nuclear NF-M translocation. We took advantage of the recombinant retrovirus CRmyb−HER, which encodes a v-*myb* oncogene and the human epidermal growth factor receptor (HER) cDNA (Metz *et al.*, 1991). We have previously shown that in CRmyb−HER-transformed myeloblasts EGF activates the cMGF promoter through its NF-M binding sites (Sterneck *et al.*, 1992b) and induces cMGF expression and autocrine growth (Metz *et al.*, 1991). The effect of EGF treatment of CRmyb−HER myeloblasts is shown in Figure 8. Cells were stained before and after EGF treatment with Evans blue, DAPI and the NF-M antiserum to distinguish clearly whole cell bodies, nuclei and NF-M immunoreactivity respectively, of the small round blast cells. Initially, NF-M is ubiquitously distributed throughout CRmyb−HER myeloblasts. However, after addition of EGF the NF-M immunostaining concentrates in the nucleus. As already shown for normal peripheral blood macrophages stimulated with TPA (Figure 7), nuclear accumulation of NF-M immunoreactivity occurred also in the presence of cycloheximide (data not shown).

These data suggest that in CRmyb−HER myeloblast NF-M translocates from the cytoplasm to the nucleus after activation of the EGF receptor. However, we cannot rule out that at the same time, a minor proportion of cytoplasmic immunoreactivity becomes proteolytically degraded. Western blot analysis of cytoplasmic and nuclear fractions (Figure 9A), as well as of total cellular protein (Figure 9B), confirmed the change in subcellular distribution of NF-M observed after EGF treatment and that within the time limits examined, neither EGF nor cycloheximide significantly affect the overall NF-M concentration in myeloblasts. Several of the experiments described above have been repeated with a monoclonal antibody that we have raised against NF-M with virtually identical results.

Our data show that kinase induction of cMGF promoter activity and the altered localization of NF-M immunoreactivity correlate. Every inducer of cMGF expression that we tested also induces the transport of NF-M from the cytoplasm to the nucleus. The results raise the possibility that nuclear translocation of NF-M is coupled to various kinase inducible pathways in macrophages, implicating NF-M as a common target in signalling pathways involving cell surface receptor tyrosine kinases, the cytoplasmic protein kinase C and the serine/threonine kinase encoded by v-*mil*.

Discussion

We have isolated and characterized a molecular clone of NF-M, a C/EBP-related bZip protein that binds as a dimer to two sites in the cMGF promoter. NF-M is likely to be the chicken homologue of C/EBPβ, a transcription factor implicated in the regulation of the IL-6 gene in hematopoietic cells (Akira *et al.*, 1990) and of acute phase genes in the liver (Poli *et al.*, 1990). Within the hematopoietic system, NF-M is expressed only in the myelomonocytic lineage and appears early during lineage commitment and differentiation. We found that stimulation of mature macrophages with LPS or TPA induces NF-M to translocate from the cytoplasm to the nucleus. Interestingly, the same type of translocation was induced by a membrane receptor tyrosine kinase, although the NF-M protein was always nuclear in monocytes that expressed a constitutive *v-mil* oncogene. Our results suggest that NF-M (or C/EBPβ) plays a novel and essential role in the transmission of signals from the cell surface to the nucleus of myelomonocytes and in the expression of myelomonocyte-specific genes.

Structure of NF-M

The fact that chick NF-M is so closely related to rat C/EBPβ suggests that the proteins might have important and conserved functions. In fact, we found strong hybridization signals under high stringency conditions on Southern blots from *Drosophila*, slime mold and *Arabidopsis* DNA (data not shown) suggesting that C/EBP-related transactivators are probably found in many species, where they are likely to play important roles in regulating gene expression, differentiation and development.

An interesting aspect that emerges from our sequence alignment is that the similarity of NF-M with C/EBPβ- and other C/EBP-related proteins is not evenly distributed throughout the protein, but rather falls into distinct short regions, often separated from each other by sequences having a high alanine, glycine and/or proline content. It is tempting to speculate that some of the conserved regions might be protein contact sites that mediate common as well as specific functions during regulation of gene transcription. The Ala-, Gly-, Pro-rich regions may serve as flexible hinges. We speculate that the conserved regions form interfaces for the assembly of interacting transcription factors at certain promoter/enhancer elements according to the recently proposed 'jigsaw puzzle' concept (Lamb and McKnight, 1991). Such peptide domains might aid or permit assembly of specific complexes between interspersed *cis*-regulatory sites and integrate gene transcription depending on the promoter architecture, the pool of other *trans*-activators available, the developmental stage as well as on signals received from the outside. Although this notion is speculative, it can be tested using 'domain swap' and/or site-directed mutagenesis experiments.

Role of NF-M in differentiation and signal transduction

Of the various chicken tissues examined, NF-M RNA was remarkably only found in liver with traces in lung, spleen and bone marrow, all organs that are particularly rich in hematopoietic cells of the monocytic lineage. Within a panel of normal and retrovirally-transformed hematopoietic cells examined, NF-M mRNA was highly expressed in myelomonocytic cells, but not expressed in erythroid or lymphoid cells. These results agree with our previous studies, where we found NF-M binding activity only in myelomonocytic cells, whether from chicken or human origin, but disagree with the published results concerning rat C/EBPβ mRNA, which has been detected in many tissues. This discrepancy is difficult to rationalize, since the results are so different. It is possible that NF-M represents a different, C/EBP-related protein that is not the functional homologue of C/EBPβ. Immunhistochemical analysis will be required to reveal whether cells different from the myelomonocytic lineage express NF-M. Furthermore, it remains to be seen whether the expression of other C/EBP-related proteins is also restricted to particular hematopoietic lineages.

It is remarkable that the multipotential MEP cells express NF-M only after induction of myelomonocyte differentiation. This invites the speculation that NF-M plays a decisive role during the early commitment of myelomonocytic cells. We are currently addressing this question by attempting to ectopically express NF-M in MEP cells, with the hope of inducing such cells to 'switch' to the myelomonocytic lineage. In any case our data give support to the notion that C/EBPs are at the centre of various commitment and/or differentiation processes.

One of our most interesting findings concerns the expression of NF-M in mature macrophages and its change in localization following treatments that activate the signal transduction cascade. Activation of macrophages results in differential expression of many gene products involved in various defence mechanisms and it is possible that NF-M participates in their regulation. One such protein whose synthesis and release is triggered by LPS and probably also in response to infections is the NF-M-regulated cMGF gene (Sterneck *et al.*, 1992a). Release of cMGF might in turn lead to an increase in the number of mature and functional myelomonocytic cells. Such a view is supported by our previous finding that myeloblasts transformed by the conditional ts21 mutant of E26 virus gain the ability to respond to LPS and to express cMGF as they differentiate into macrophages (Beug *et al.*, 1984). Thus, cMGF expression can be induced both by the process of differentiation and the activation of protein kinases. The fact that the latter in turn activate the transcription factors AP-1 and NF-M (Sterneck *et al.*, 1992b) suggests that the regulation of these factors plays a decisive role in macrophage proliferation, differentiation and function.

It seems possible that various protein kinases induce NF-M translocation by a common mechanism. Recently, it has been found that C/EBPβ translocates from cytoplasm to the nucleus in PC12 cells after cAMP stimulation and that this occurs concomitantly with the phosphorylation of the protein (Metz and Ziff, 1991b). In our hands cAMP also induces cMGF promoter reporter expression, albeit much less efficiently than the other kinase pathways tested [(Sterneck *et al.*, 1992b) and data not shown]. It is therefore possible that in different cell types, C/EBPβ activation is coupled to different signal transduction mechanisms. In this context it is noteworthy that a potential MAP kinase phosphorylation site (amino acids 218–221, sequence: PGTP) is conserved between NF-M and its mammalian homologues, although the proposed PKA phosphorylation site (Metz and Ziff, 1991a,b) is not. Whatever the exact mechanism that triggers translocation, our data suggest that NF-M function is directly coupled to various signalling pathways. It appears that the machinery to respond to such signals is acquired during myelomonocytic differentiation and that kinase oncogenes utilize the same pathways to induce hematopoietic lineage commitment and autocrine growth stimulation. The elucidation of these pathways should answer important questions about lineage commitment, differentiation, transcription factor activation and leukemogenesis.

Materials and methods

Cells and tissue culture

Cells were propagated in Dulbecco's modified Eagle's medium (Gibco) plus 8% fetal calf serum, 2% chicken serum and 10 mM Hepes, pH 7.4. Growth factor-dependent myelomonocytic cell cultures were supplemented with 20 units/ml of recombinant cMGF (Leutz *et al.*, 1989). To obtain retrovirally-transformed cells, 20×10^6 bone marrow cells from 1–7 day-old Spafas chicks were infected with virus (sterile filtered medium from virus producing cells) in liquid medium containing 2 mg/ml polybrene (Graf *et al.*, 1977a,b). Transformed cells were cultured in the presence of recombinant cMGF and rapidly proliferating transformed cells were purified by multiple passages.

Cell lines, retroviruses and oncogenes

The HD3 cell line is an AEV ES4-transformed non-producer erythroblast line expressing the viral v-*erbA* and ts v-*erbB* oncogenes (Beug *et al.*, 1982). HD11 is a MC29-transformed macrophage cell line expressing the v-*myc* oncogene (Beug *et al.*, 1979). BM-2 is a AMV-transformed, v-*myb*-expressing myeloblast cell line (Moscovici and Gazzolo, 1982). The MH2 retrovirus expresses *myc* and *gag–mil* (Coll *et al.*, 1983). E26 expresses *gag–myb-ets*. CRmyb–HER contains an AMV/E26 recombinant *myb* and the human EGF receptor (Metz *et al.*, 1991). The lymphoid cell lines MSB-1, RPL 12 and REV-NPB4 are described in (Beug *et al.*, 1981). MEP cells were obtained from T.Graf (Graf *et al.*, 1992). Chicken embryo fibroblasts were prepared from 11 day-old SPAFAS chicken.

cDNA library screening and isolation of NF-M clones

gt11 plaque filter replicas of a BM-2 library (1.5×10^5 plaques) on nitrocellulose membranes (Schleicher and Schuell) were prehybridized and then hybridized to C/EBP (full-length coding region) and rat NF-IL6 DBP [(Poli *et al.*, 1990); *Sma*I fragment] probes at 60°C for 16 h. Membranes were briefly washed twice at room temperature and then twice at 60°C in 1×SSC for 20 min. 36 positive plaques were found, 16 of which hybridized to both probes. All plaques were plaque-purified and inserts were subcloned into Bluescript (Stratagene). Recombinant plasmids were transcribed using T3 and T7 polymerase, the RNAs were translated in a reticulolysate essentially as described by the manufacturer (Promega) and the programmed reticulolysates were subjected to band-shift analyses.

Expression constructs

The inserts of phage clone 12.1 was subcloned into the pATH-2 vector and trpE–NF-M expression was induced in M9 medium by tryptophan starvation. Bacteria were harvested and proteins were extracted as described by Desplan *et al.* (1985) and Leutz *et al.* (1989). trpE–NF-M was renatured from 8 M urea by dialysis or by dilution (1:50) into ice-cold 20 mM HEPES, 50 mM NaCl, 1 mM DTT, 10% glycerol solution and subsequently concentration by centricon (Amicon) centrifugation. For eukaryotic expression, a NF-M *Hin*dIII–*Xba*I fragment from phage 16.3 insert previously subcloned into Bluescript (Stratagene) was cloned into pCDM8 (Seed, 1987).

Transient expression assays

Hematopoietic cells were transfected by a DEAE-dextran procedure as described in detail by Sterneck *et al.* (1992b). Plasmids were purified twice on CsCl gradients. The plasmid DNA concentration was 0.1 mg/ml with pCDM8 and derivatives to avoid promoter squelching effects and 0.5 mg/ml with luciferase reporters. Briefly, cells were transfected with 0.3 mg/ml DEAE dextran in STBS solution (Ausubel *et al.*, 1987), harvested after 40–48 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 5–50 μl were assayed for luciferase activity as described (de Wet *et al.*, 1987; Sterneck *et al.*, 1992b). Protein content was determined with the Bio-Rad protein assay kit using chicken lysozyme as a standard. Internal normalization against a co-transfected β-galactosidase reporter was omitted because several promoters examined were affected by C/EBP-related proteins. Instead assays were done in triplicates and repeated three times using different plasmid preparations.

COS-7 cells were transfected with lipofectin (BRL) according to manufacturer's instructions or with DEAE-dextran (Levesque *et al.*, 1991).

Antibodies

Antisera were prepared from rabbits after immunization with bacterially derived trpE–NF-M fusion protein and monoclonal antibodies were prepared following standard procedures (Harlow and Lane, 1988).

Electrophoretic mobility shift assay

Nuclear extracts from hematopoietic cells or COS-7 cells were prepared as described by Sterneck *et al.* (1992b) or by a mini nuclear extract procedure (Schreiber *et al.*, 1989). Bandshift and competition analysis with oligonucleotides were performed exactly as described by Sterneck *et al.* (1992b). For immunomobility shift assays immune or pre-immune rabbit polyclonal antisera were diluted with PBS as indicated and incubated with nuclear extract for 10 min after addition of labelled DNA for 15 min on

ice. Oligonucleotides C, F and C/EBP are the same as in Sterneck *et al.* (1992b). The GATA-1 oligonucleotide was described by Graf *et al.* (1992).

DNase I footprinting

DNase I footprints were performed according to Lee *et al.* (1987) using either bacterially-expressed trpE or trpE−NF-M protein, respectively. Purified DNA was separated on a 6% sequencing gel. Sequence markers were prepared by subjecting end-labelled fragment to G + A-specific reactions (Ausubel *et al.*, 1987). Unspecific competitor DNA was omitted. The binding reaction was performed for 15 min on ice in 10 mM HEPES (pH 7.9), 60 mM KCl, 4 mM $MgCl_2$, 4 mM spermidine, 10% glycerol and 0.1 mM EDTA. Protein binding was assayed on an end-labelled fragment that contained the cMGF genomic *Pvu*II−*Sph*I fragment (−12 to −95; Sterneck *et al.*, 1992a) blunt-end cloned into the Bluescript (Stratagene) *Sma*I site and excised with *Xba*I−*Xho*I.

DNA, RNA and protein analysis

DNA sequencing by the chain termination method (Sanger *et al.*, 1977) was carried out on single-stranded templates on both NF-M strands using custom prepared oligonucleotides and Sequenase sequencing kit (USB) with dGTP and dITP reactions run in parallel.

Compilation and analysis of sequence data was performed by use of the University of Wisconsin Genetics Computer Group Package of computer programs. Initially, programs for multiple sequence alignment were used to align various C/EBP-related proteins. However, even various changes of alignment parameters could not fully cover particular sequence similarities observed between the selected C/EBP-related proteins. Therefore, final adjustments were done manually.

RNA was extracted (Chomczynski and Sacchi, 1987) and polyadenylated RNA was obtained by oligo(dT)-cellulose chromatography. RNA was fractionated on 1% formaldehyde−agarose gels, transferred to gene-screen membranes (Du Pont), UV-crosslinked and baked at 80°C for 2 h. DNA probes were prepared from purified restriction fragments and labelled by random priming (Stratagene) to a specific activity of $2-6\times10^8$ c.p.m./μg DNA. The NF-M DNA probe was prepared from the *Eco*RI fragment of phage 16.3. Hybridization of filters with DNA probes was performed with QuikHyb (Stratagene) at 68° C for 2 h. Subsequently, the filters were washed twice with 0.2×SSC, 0.1% SDS at 65°C and exposed to Kodak X-ray film.

Western blotting was performed with total cell lysates as indicated. Cells were lysed in boiling SDS gel sample buffer. An equivalent of 2.5×10^5 cells was subjected to reducing 12.5% 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 1 h and incubated for 2 h with the rabbit polyclonal antiserum raised against trpE−NF-M diluted 1:1500 in blocking solution. Filters were washed three times and incubated with a horseradish peroxidase coupled second antibody (Bio-Rad) for 1 h. The blots were washed three times and immunoreactivity was detected with a chemoluminescence system (ECL; Amersham International).

Metabolic labelling of proteins was performed in DMEM without methionine (Gibco-BRL) for 2 h with 1.5×10^7 cells in the presence of 250 mCi/ml of [^{35}S]methionine (Amersham). Cells were lysed in RIPA buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.5% DOC, 1% Triton-X 100 and 0.1% SDS) or in a boiling solution of 1% SDS, 10 mM Tris pH 7.0, 1 mM EDTA for 3 min, and then diluted 10-fold with RIPA buffer without SDS. Lysates were centrifuged at 4°C for 20 min at 20 000 *g* to remove insoluble material. Incorporated radioactivity was determined by spotting 10 μl of cleared lysate on glass fibre filters (Whatman GF-C) that were air dried, extracted in 20% trichloracetic acid solution for 20 min at room temperature and washed three times for 5 min in 100% acetone. Insoluble radioactivity was determined by liquid scintillation counting and 1×10^7 c.p.m. were used per immunoprecipitation. Immunoprecipitation was performed according to standard methods using rabbit antiserum raised against trpE−NF-M or preimmune serum (Harlow and Lane, 1988) and protein A−Sepharose beads (Pharmacia).

^{32}P labelling was performed for 4 h in phosphate-free medium (MEM, Gibco). Equal amounts of lysate (10%) were used per immunoprecipitation. Immunoprecipitations were performed as described above.

Immunocytological staining

Peripheral blood cells were grown on cover slips or microscope slides (Nunc Lab Tek) for 3 days. Where indicated cells were treated with cycloheximide (50 μg/ml) for 10 min before stimulation. Cells were treated with TPA (200 mM), LPS (5 μg/ml final) or EGF (20 ng/ml) for 1 h. After removal of the medium, cells were washed with PBS, fixed for 20 min in 3.7% paraformaldehyde, followed by a brief incubation at −20°C in ethanol. Fixed cells were exposed for 1 h to NF-M rabbit antiserum (diluted 1:200) or monoclonal antibody in 0.5% BSA and 0.1% gelatine in PBS. Cells were then washed with PBS and incubated for 30 min with 200-fold dilution of fluoresceinisothiocyanate (FITC)-labelled second antibody (Jackson Immunoresearch). Where indicated, cells were stained with DAPI (50 ng/ml) for 5 min, washed and stained with Evans blue (100 ng/ml) for 2 min. Cover slips were embedded with aquatex (Merck) and analysed by fluorescence microscopy (Leitz-Axioplan).

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Note added in proof

The nucleotide sequence data reported in this paper will appear in the EMBL, GenBank and DDBJ nucleotide sequence databases under the accession number Z21646.