Novel mechanism of C/EBPβ (NF-M) transcriptional control: activation through derepression

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Phosphorylation of transcription factors is regarded as a major mechanism to control their activity in regulation of gene expression. C/EBPβ is a transcription factor that becomes activated after phosphorylation to induce genes involved in inflammation, acute-phase response, cytokine expression, cell growth, and differentiation. The chicken homolog NF-M collaborates with Myb and various kinase oncogenes in normal myeloid differentiation as well as in the leukemic transformation of myelomonocytic cells. Here, we examined the structure of NF-M and its mechanism of activation. We show that NF-M is a repressed transcription factor with concealed activation potential. Derepressed NF-M exhibits enhanced transcriptional efficacy in reporter assays. More importantly, NF-M activates resident chromatin-embedded, myelomonocyte-specific target genes, even in heterologous cell types such as fibroblasts or erythroblasts. We identified two regions within NF-M that act to repress *trans*-activation. Repression is abolished by deletion of these regions, activation of signal transduction kinases including v-*erb*B, polyoma middle T, *ras* and *mil/raf*, or point mutation of a critical phosphorylation site for MAP kinases. We provide evidence that phosphorylation plays a unique role to derepress rather than to enhance the *trans*-activation domain as a novel mechanism to regulate gene expression by NF-M/C/EBPβ.

[*Key Words*: C/EBPβ; oncogenes; transcription; phosphorylation; gene activation]

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CAAT/enhancer-binding proteins (C/EBPs) are a family of transcription factors with highly similar carboxy-terminal DNA-binding domains (DBDs) and basic leucine zipper dimerization (LZ) domains. In contrast, the amino-terminal effector domains are not conserved. Whereas C/EBPα constitutively activates genes in cell cycle-arrested terminally differentiated hepatocytes or adipocytes (Christy et al. 1989; Friedman et al. 1989), C/EBPβ and C/EBPδ are implicated in signal transduction as nuclear targets for cytokine-induced gene expression in various dividing and nondividing cell types (Akira et al. 1990; Poli et al. 1990; Akira and Kishimoto 1992; Juan et al. 1993). The expression of C/EBPα, C/EBPβ, and C/EBPδ is regulated during cellular differentiation of adipoblasts or myeloblasts as well as during *Drosophila melanogaster* embryo development (Cao et al. 1991; Rorth and Montell 1992; Scott et al. 1992). Their expression kinetics together with the fact that C/EBPα antisense RNA inhibits terminal differentiation into adipocytes suggest important roles for C/EBP proteins during cellular differentiation (Samuelsson et al. 1991; Freytag and Geddes 1992; Lin and Lane 1992).

The cell type specificity of C/EBP-regulated gene expression is thought to result from combinatorial interactions with other transcription factors such as NF-Y (Milos and Zaret 1992), glucocorticoid receptor (Williams et al. 1991a), or the Myb (proto-) oncogene (Ness et al. 1993). Additionally, C/EBPβ in particular requires activation through kinases for efficient *trans*-activation of various reporter constructs. Cyclic AMP-dependent kinase (Metz and Ziff 1991), calcium-dependent kinases (Wegner et al. 1992), retrovirally derived oncogenic kinases (Sterneck et al. 1992b; Katz et al. 1993), mitogen-activated protein (MAP) kinase (Nakajima et al. 1993), or protein kinase C (PKC) (Trautwein et al. 1993) have all been shown to mediate phosphorylation of C/EBPβ at different sites and, consequently, enhance *trans*-activation efficacy.

In the hematopoietic system of mammals and the chicken (Scott et al. 1992; Katz et al. 1993), C/EBPβ/NF-M is solely expressed in the myelomonocytic lineage, the same cell type that also becomes transformed by retrovirally encoded v-*myb* genes. We have shown recently that in myelomonocytic cells of the chicken, the C/EBPβ homolog NF-M collaborates with Myb as well as with kinase (proto-) oncogenes to activate myeloid specific genes (Sterneck et al. 1992b; Ness et al. 1993). Intriguingly, ectopic expression of NF-M together with cellular or viral Myb activates the resident granulocyte-specific *mim-1* gene as well as the macrophage-specific lysozyme gene in cells such as erythroblasts and fibroblasts. Hence, NF-M and Myb act as a combinatorial

switch to induce myeloid-specific gene expression in heterologous cell types (Ness et al. 1993).

Whereas the function of NF-M in v-*myb*-induced myeloblast transformation has still to be elucidated, its role to mediate the activity of protein kinase-encoding oncogenes in v-*myb*- or v-*myc*-transformed myeloid cells has recently been established. In these cell types, NF-M is the critical target of activated kinase oncogenes to induce expression of the myelomonocytic growth factor (cMGF) gene (Adkins et al. 1984; Graf et al. 1986; Sterneck et al. 1992a,b). cMGF is required to sustain growth of v-*myb*- or v-*myc*-transformed leukemic cells in an autocrine fashion (Leutz et al. 1990; Metz et al. 1991). Similar to the differentiation-specific *mim-1* and lysozyme genes, cMGF is normally expressed in mature macrophages after activation with the bacterial cell wall product lipopolysaccharide (LPS). It therefore appears that NF-M mediates important myelomonocytic functions involved in cellular differentiation as well as in leukemic transformation.

Comparison of the NF-M amino acid sequence with its mammalian homologs (CEBPβ, NF–IL6, IL6–DBP, LAP, CRP2, and AGP/EBP (Akira et al. 1990; Chang et al. 1990; Descombes et al. 1990; Poli et al. 1990; Cao et al. 1991; Williams et al. 1991b) showed that the DNA-binding and dimerization motifs are contiguous and highly conserved with other C/EBP family members including C/EBPα, C/EBPβ, and C/EBPδ from various species. In contrast, comparison of the more amino-terminal sequence of NF-M with its mammalian homologs revealed seven small, discreetly conserved regions (CR1–CR7), interrupted by Ala-, Gly- and Pro-rich segments of variable length (Katz et al. 1993). Because the murine and rat C/EBPβ proteins display indistinguishable activity to NF-M in chicken cells (E. Kowenz-Leutz and A. Leutz, unpubl.), we hypothesized that these conserved regions could reflect functional domains of NF-M/C/EBPβ. Such regions may form a flexible "beads on a string" type structure connected by variable "spacer" sequences. On the basis of such a hypothetical arrangement of functional regions, we constructed a series of deletion mutants and examined their activity in reporter activation assays and their ability to activate chromatin-embedded endogenous genes.

Here, we show that NF-M is a repressed transcription factor with concealed activation potential that becomes unmasked by kinase oncogenes. Deinhibition of NF-M results in enhanced transcriptional efficacy of reporter constructs and, more significantly, of resident, myeloid-specific target genes in heterologous cell types. Inhibitory functions locate to two evolutionary conserved regions. Both regions contribute to silence the *trans*-activating domain. The inhibitory function can be neutralized by deletion. We show that one of the regions, CR7, can be neutralized by activation of the signal transduction cascade involving either a conditional v-*erb*B cell surface kinase, polyoma middle T (PMT), *ras*, or v-*mil*/*raf* oncogenes or by a point mutation of a phosphorylation site for mitogen-activated kinases (MAPK). Taken together, our data suggest that C/EBPβ is a target of kinase oncogenes and delineate a novel mechanism of C/EBPβ activation.

Results

Comparison of the NF-M amino acid sequence with its mammalian counterparts revealed seven discreetly conserved regions (CR1–CR7) in addition to the highly conserved DBD and LZ dimerization domains as shown in Figure 1. The regions between CR1 and CR7 are not conserved and are particulary rich in the amino acids Ala, Gly, and Pro (60%) when compared with total amino acid content (25%). The arrangement of conserved and nonconserved regions has been published (Katz et al. 1993) and is schematically depicted in Figure 1. The activation of reporter and resident genes by mammalian C/EBPβ in chicken cells is indistinguishable from that of NF-M (Ness et al. 1993; data not shown), suggesting a strong evolutionary conservation of those regions that determine the activity and/or specificity of the *trans*-activator proteins.

Inhibitory functions conceal the trans*-activation potential of NF-M and its ability to activate chromatin-embedded genes*

To test whether the conserved regions CR1–CR7 could reflect functional domains of NF-M/C/EBPβ, deletion mutants were constructed in expression plasmids and examined for their ability to activate reporter constructs as shown in Figure 2. The deletion endpoints were located in the divergent regions of the protein between Pro-Gly residues, thereby minimizing disruption of secondary structure. All resultant mutants were analyzed for RNA expression (data not shown), protein integrity, and ability to bind DNA (bandshift assay in Fig. 2A,B). By these means they were found to be highly similar (see also Fig. 4B,C, below, or relevant mutants). Using these constructs we asked which deletion mutants could activate reporter gene expression in fibroblasts. As seen in Figure 2A, sequential amino-terminal deletions of the first 140 amino acids gradually abolished NF-M-induced reporter expression. This indicates that

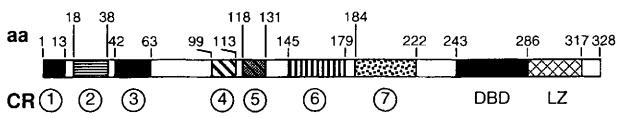


Figure 1. Schematic representation of NF-M. Amino acid numbers are at the *top*, conserved regions (CR) at the *bottom*. CRs are represented as shaded boxes; the Ala-, Gly-, Pro-rich sequences are indicated by white boxes. The coordinates of the conserved regions (CR) between NF-M (328 amino acids) and mammalian C/EBPβ (Katz et al. 1993) are CR1, amino acids 1–13; CR2, amino acids 18–38; CR3, amino acids 42–63; CR4, amino acids 99–113; CR5, amino acids 118–131; CR6, amino acids 145–179; CR7, amino acids 184–222; DBD and leucine zipper, amino acids 243–317.

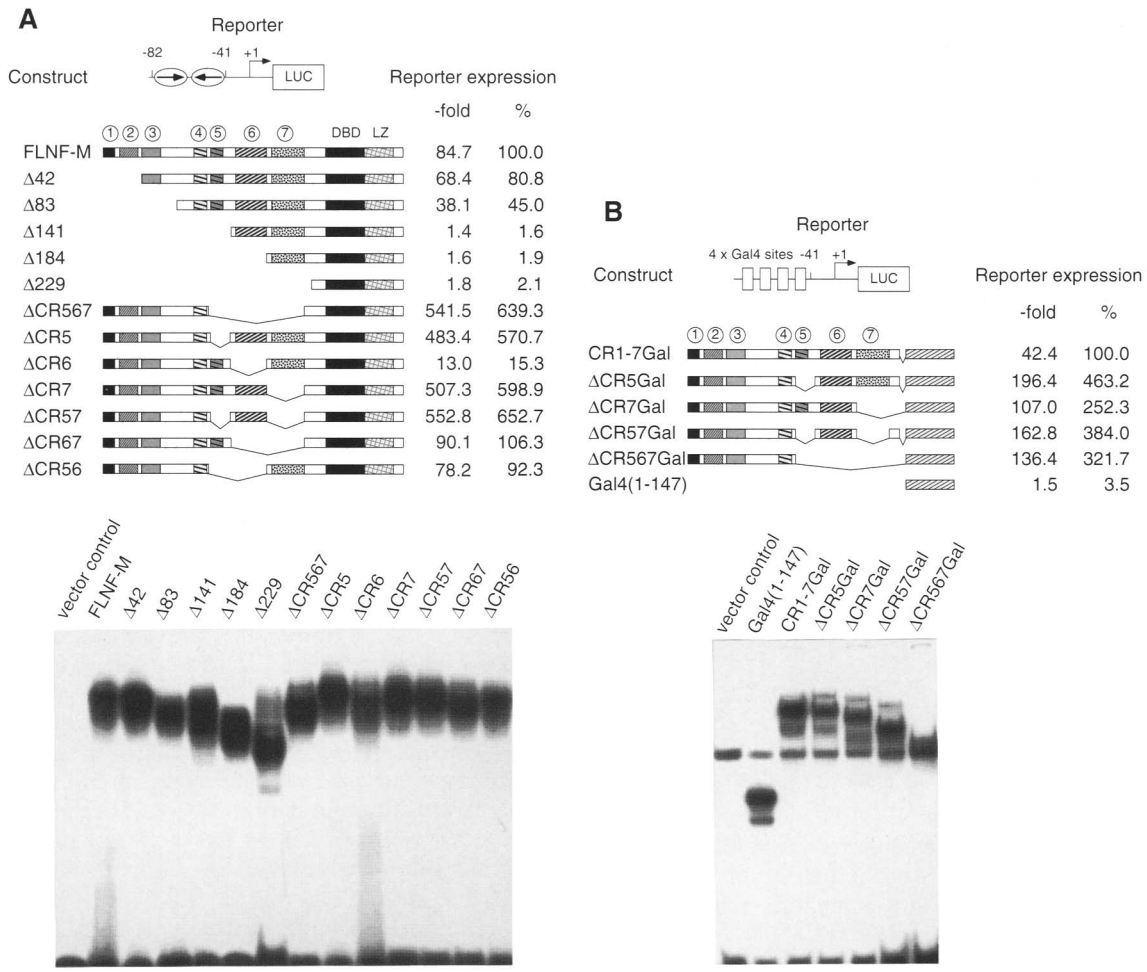


Figure 2. Functional analysis of NF-M reveals *trans*-activating and inhibitory domains. (*A*) NF-M deletions were constructed in the pCDM8 expression vector and transfected into QT6 fibroblasts together with the M82 cMGF promoter–luciferase reporter as shown at the *top*. The reporter contained both its NF-M-binding sites, which are arranged as an inverted repeat (Sterneck et al. 1992b). Forty-eight hours after transfection, cell extracts were prepared, normalized, and analyzed for reporter and bandshift activity (*lower*) as described (Sterneck et al. 1992a,b). (*B*) A modified version of the cMGF promoter containing 4× Gal4-binding sites replacing the NF-M-binding sites as indicated was transfected and analyzed as described above. The data represent means of triplicates; standard deviations were within the 10% range. Nuclear extracts were examined as in *A*.

several amino-terminal CRs contribute to the *trans*-activating function of the protein (a comprehensive analysis of the *trans*-activation region of NF-M will be published elsewhere). In contrast, internal deletion of amino acids 116–229 (CR5, CR6, and CR7) enhanced the *trans*-activation five- to sevenfold in comparison to full-length NF-M (FLNF-M). Thus, the region between amino acids 116 and 229 partially represses the activity of NF-M. Internal deletions within this region enabled a more detailed analysis of this possibility (Fig. 2A). Removal of CR5 or CR7 alone or in combination enhanced reporter expression five- to sevenfold over FLNF-M. Likewise, addition of CR5 or CR7 to a construct consisting of the *trans*-activating domain (CR1–CR4; amino acids 1–113) diminished reporter activity to wild-type level. Hence, two motifs, CR5 and CR7, repress the full *trans*-activation potential of NF-M. In contrast, CR6 appeared to partially antagonize the inhibitory effects of CR5 or CR7 because its deletion (ΔCR6) strongly reduced reporter expression, whereas its presence masks the negative effect of either single inhibitory region CR5 or CR7 alone (as in the constructs ΔCR5 and ΔCR7). Among other possibilities, it is tempting to speculate that CR6 is required to maintain a conformational state that is crucial for NF-M derepression.

Are the *trans*-activation and inhibitory domains independent modules of NF-M or are they intrinsically dependent on the carboxy-terminal portion of the protein? To address this question we exchanged the carboxy-terminal 96 amino acids of NF-M for the DNA-binding dimerization domain of the yeast transcription factor Gal4 [Gal4(1–147)] (Ptashne 1988; Sadowski and Ptashne 1989). Transient reporter expression (Fig. 2B) revealed that activatory and inhibitory domains functioned in a

similar way as in the original mutants. These results indicated that neither the activating nor the repressing functions of NF-M depended on the native DNA-binding or dimerization domains.

Reporter assays however do not prove conclusively that the inhibitory and activating domains of NF-M have similar functions on endogenous genes. Transient expression of NF-M alone is sufficient to activate a panel of myelomonocytic-specific genes in unrelated cell types (E. Kowenz-Leutz and A. Leutz, in prep.). This enabled us to examine whether the repression mediated by the inhibitory regions within amino acids 116–229 was also applicable to chromatin-embedded genes (see Fig. 3). Under normal conditions, fibroblasts do not express RNA for gene 126 [encoding a potential Ca^{2+}-binding protein that is specifically expressed in myelomonocytic cells (Nakano and Graf 1992)]. Transfection of the FLNF-M expression vector into QT6 fibroblasts already caused a weak induction of the 126 message. Here, too, removal of the inhibitory regions strongly enhanced 126 expression. Taken together, our data suggest that inhibitory functions within NF-M conceal its potential to activate reporter and resident genes.

Inhibition of NF-M is negatively regulated by kinase oncogenes

The fact that in fibroblasts NF-M is partially repressed suggests that its normal mode of activation in cells may be through derepression. *Trans*-activation of NF-M/C/EBPβ is enhanced by a calcium ionophore, phorbol ester treatment, bacterial lipopolysaccharide (LPS), by *ras*, or by various kinase oncogenes as found by us and other workers (Akira et al. 1990; Sterneck et al. 1992b; Wegner et al. 1992; Nakajima et al. 1993; Trautwein et al. 1993). Furthermore, activation of mammalian C/EBPβ by phorbol esters or oncogenic *ras* coincides with increase of phosphorylation at positions that are within inhibitory motifs CR5 and CR7 (Nakajima et al. 1993; Trautwein et al. 1993). To investigate whether an oncogenic receptor tyrosine kinase can enhance NF-M activity by neutralizing its inhibitory regions, we took advantage of an erythroid cell line (HD3) that harbors a temperature-sensitive version (*ts*) of the v-*erb*B oncogene (Graf et al. 1978; Beug et al. 1982). In contrast to QT6 fibroblasts, ectopically expressed FLNF-M induces #126 expression to a similar level as ΔCR7 in HD3 cells (Fig. 4A). This indicates that in HD3 cells, FLNF-M is already activated at the permissive temperature. That the activity of NF-M was dependent on the v-*erb*B kinase was confirmed by the finding that at the nonpermissive temperature where the conditional *tsv–erb*B kinase is inactivated (Beug and Hayman 1984), the expression of #126 by FLNF-M was completely abrogated. Importantly, both ΔCR5 or ΔCR7 induced #126 expression even in the absence of functional *tsv-erb*B. These results imply that *tsv-erb*B overcomes the inhibition of FLNF-M at the permissive temperature and that removal of the inhibitory regions circumvents the requirement for the kinase at the nonpermissive temperature. The observed differences were not the result of the particular gene (#126) examined, because another myelomonocyte-specific gene, #325 [encoding the chicken homolog of the granulocyte-specific goose-type lysozyme (Nakano and Graf 1992)], was activated in a similar fashion (Fig. 4A). Furthermore, protein levels were comparible between the wild-type and mutant proteins even at the nonpermissive temperature (Fig. 4B). Therefore, the differences were not because of altered mutant protein expression, intracellular localization, or changes in the ability of mutant proteins to bind to DNA, respectively, as shown by the controls (Fig. 4B,C).

We then examined whether or not kinase-induced activation of NF-M was dependent on protein synthesis. To do so, *tsv-erb*B kinase was reactivated by backshifting to the permissive temperature in the presence of cycloheximide as indicated in Figure 5. Under these conditions, *tsv-erb*B kinase induced #325 expression even when protein synthesis was blocked. These results suggest that the inhibitory function is mitigated by *tsv-erb*B or downstream mediators through a post-translational mechanism.

Finally, we asked whether oncogenes that act downstream of or in parallel to v-*erb*B in the signaling cascade can rescue FLNF-M activity. We transfected HD3 cells with expression vectors encoding EJ-*ras*, v-*mil*, or PMT oncogenes and incubated the cells at the nonpermissive temperature to inactivate the *tsv-erb*B kinase as shown in Figure 6a. In the presence of FLNF-M, each of the signaling oncogenes at least partially induced the resident #126 gene or #325 gene (data not shown) and thus complemented the inactive v-*erb*B kinase. In the absence of NF-M, none of the signaling oncogenes examined were capable of inducing #126 or #325 (data not shown; Fig. 6B). To rule out cell lineage-specific effects, we asked whether induction of the signaling cascade in fibroblasts could also activate NF-M. Figure 6B shows that

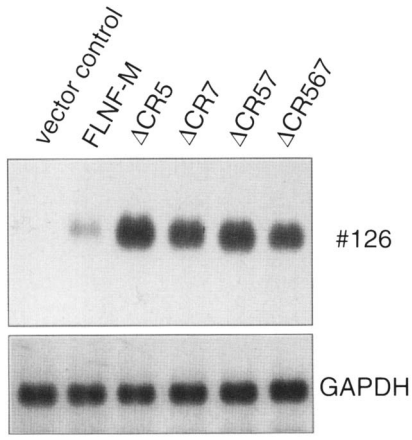


Figure 3. Inhibitory regions of NF-M conceal its ability to activate chromatin-embedded genes. Expression plasmids as indicated at the *top* were transfected in QT6 fibroblasts. After 36 hr, cells were lysed and their poly(A)$^+$ fraction examined by Northern analysis with #126 and GAPDH probes.

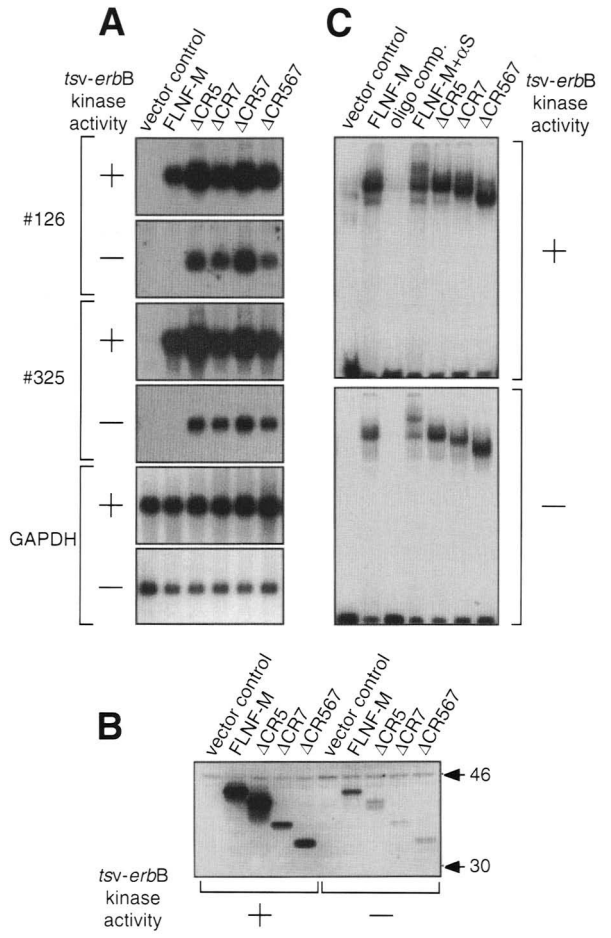


Figure 4. *Trans*-activation by NF-M depends on an activated receptor tyrosine kinase or removal of inhibitory regions. (*A*) HD3 cells harboring the conditional *tsv-erb*B oncogene (Beug and Hayman 1984) were transfected with expression constructs as indicated (cf. with Fig. 2a) and incubated for 24 hr at the permissive temperature (+, 36°C) or the nonpermissive temperature (−, 42°C, the normal body temperature of chicken). Activation of resident NF-M target genes and GAPDH controls were monitored by Northern analysis. (*B*) Total cellular protein extracts were prepared in parallel, equivalents of 5×10^5 cells were subjected to SDS–gel electrophoresis, blotted, and probed with an NF-M antiserum. (*C*) Nuclear protein extracts were prepared in parallel and examined for DNA binding by bandshift analysis as described (Sterneck et al. 1992b). NF-M shifts were controlled by competition analysis with a 100-fold excess of unlabeled oligonucleotide (oligo comp.) and supershift (+αS) using a 1:500 dilution of a specific NF-M antiserum (Katz et al. 1993).

coexpression of PMT together with NF-M induced #126 expression to a similar level as ΔCR7, whereas the activity of ΔCR7 was not further increased by PMT. PMT on its own did not induce #126 at all, confirming that *trans*-activation occurs through NF-M.

If phosphorylation of the inhibitory motifs is responsible for activation of NF-M, their removal should be accompanied by a reduction in phosphorylation of the mutant molecules. To examine this possibility, we compared specific phosphorylation levels of transiently expressed NF-M derivatives following metabolic labeling of HD3 cells as shown in Figure 7. To do so, NF-M constructs were modified to encode the FLAG-tag epitope at their carboxyl teminus to ensure equal antigenicity of the constructs. These constructs were then transfected into HD3 cells that were subsequently metabolically labeled with radioactive inorganic phosphate, lysed, immunoprecipitated with a FLAG-specific monoclonal antibody, and the ^{32}P label was quantified by PhosphorImage analysis. To determine specific ^{32}P incorporation into wild-type and mutant proteins quantitatively, the same blot was incubated with anti-FLAG followed by an antimouse ^{125}I Fab fragment and subsequently analyzed as above. The ratio of ^{32}P to ^{125}I label in wild-type NF-M was set to 1.0 and plotted as shown in Figure 7. Deletion of CR5, CR7, or both together diminished specific phosphate incorporation to 50%, 10%, and 5%, respectively. Thus, the inhibitory regions CR5 and CR7 provide the majority of phosphorylation sites in NF-M. It also appears that phosphorylation of CR5 and CR7 may to some extent be interdependent on each other. Importantly, ^{32}P incorporation was lowest in mutants with highest gene activation potential. It therefore appears that phospho-

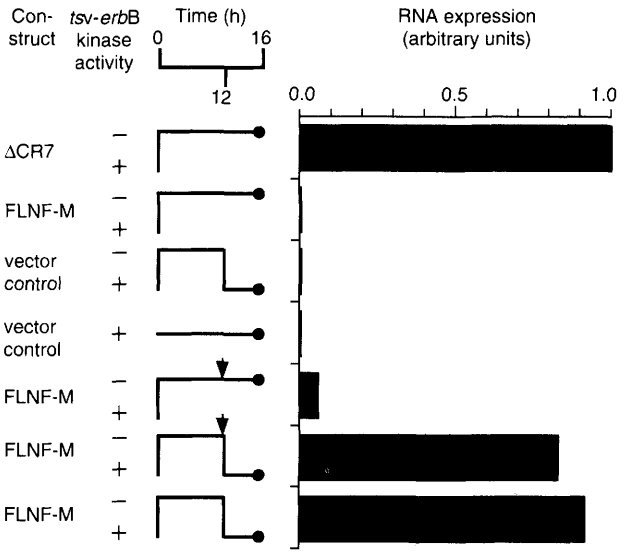


Figure 5. Kinase-mediated NF-M activation occurs independently of protein synthesis. HD3 cells were transfected with the constructs as indicated at *left* and #325 expression was analyzed 16 hr after transfection. Indicated is the time course of incubation temperatures, the state of activity of the *tsv-erb*B kinase (+, active; −, inactive), and the addition of cycloheximide by an arrow. Briefly, following transfection, cells were incubated at the nonpermissive temperature (with exception of one of the vector controls) for 16 hr or backshifted after 12 hr to the permissive temperature for 4 hr. Where indicated by an arrow, cycloheximide (at 50 μg/ml) was added 20 min before back shift to block protein synthesis. Northern blots were evaluated quantitatively by PhosphorImage analysis (Bas1000, Fuji) and normalized to the GAPDH control, and #325 expression induced by ΔCR7 was set to 1.0 (arbitrary units).

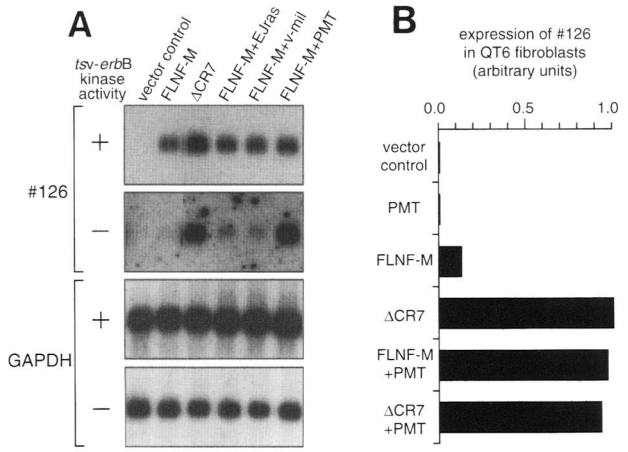


Figure 6. Deinhibition of NF-M occurs by signaling oncogenes. (*A*) Complementation of conditional *tsv-erbB* in HD3 erythroblasts. HD3 cells were transfected with expression constructs encoding NF-M derivatives, EJ-*ras*, v-*mil* (chicken *raf* oncogene), or PMT, as indicated, incubated at the permissive or nonpermissive temperature for 24 hr, and harvested; and the poly(A)$^+$ RNA fraction was analyzed by Northern blotting using #126 and GAPDH probes. (*B*) QT6 fibroblasts were transfected with expression constructs as indicated; and the level of resident #126 gene expression was evaluated quantitatively by PhosphorImaging analysis following Northern hybridization.

rylation of the inhibitory regions abolishes their ability to suppress *trans*-activation.

It appeared unlikely that the v-*erbB* kinase directly phosphorylates NF-M, as we failed to detect phosphorylated tyrosine residues on NF-M following metabolic labeling of HD3 cells (data not shown). On the other hand, we had pointed out previously that a consensus sequence for MAPK within CR7 (Pro-Gly-Thr-Pro) is conserved between avian and mammalian C/EBPβ (Katz et al. 1993). Subsequently, a peptide from NF-IL6 (human C/EBPβ) comprising this site was shown to be phosphorylated by purified MAPK and to be a target for the *ras* oncogene in P19 cells (Nakajima et al. 1993). Hence, we examined whether a point mutation at this site (thr218 to asp218) could also derepress NF-M. Figure 7B shows that mutation of the MAPK target in NF-M218D mitigated the requirement for v-*erbB* to activate the resident #126 and #325 genes. Thus, the inhibitory function could be overcome either by deletion of repressing regions, activation of signaling oncogenes, or by a point mutation at a MAPK phosphorylation site within the inhibitory CR7.

Inhibitory and trans*-activating regions of NF-M interact*

We decided to probe the mechanism by which NF-M inhibition is accomplished using the yeast "two-hybrid" interaction trap system. This approach allowed us to ascertain whether the inhibitory regions interact with the *trans*-activating region. To do so, we cloned various parts of NF-M into yeast expression vector pairs that express the peptides of interest as fusion proteins (Fields and Song 1989). Here, blue colonies appear on X-gal plates when two protein sequences interact to induce galactosidase expression. As shown in Table 1, we found that the *trans*-activating region CR1234 of NF-M (cloned into the activation hybrid vector, pGAD424) induced galactosidase activity when cotransferred with either CR5 or CR7 (cloned into the DNA-binding hybrid pGBT9). Moreover, CR7 domains can be seen to interact with each other under these conditions, an observation that is discussed later in this paper. The kinetics of color appearance in these experiments was similar to the color development observed in the positive control as shown in Table 1 (p53 protein interaction with SV40 large T antigen). Likewise, we examined the effect of the point mutation at amino acid 218 in the MAPK site. As shown in Table 1, CR7D218, which harbored the T218 to D218 mutation, was completely impaired in its interaction with CR1234. This was not because of the failure to express this mutant, because it still retained the ability to interact with CR7, as shown in the table. These results suggest that the inhibitory regions interact with the *trans*-activating

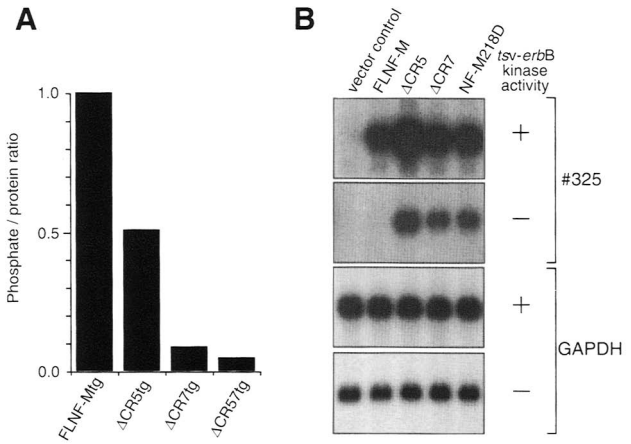


Figure 7. In vivo-labeling of NF-M mutants and mutagenesis of a critical phosphorylation site. (*A*) Specific phosphate contents of various NF-M constructs. To obtain equal antigenicity, NF-M constructs were tagged with the FLAG peptide (tg) fused in-frame to their carboxyl termini. These constructs were transfected into HD3 cells. After 16 hr, cells were labeled with [^{32}P]orthophosphate for 4 hr, lysed, and immunoprecipitated (Trautwein et al. 1993) with monoclonal anti-FLAG antibody, separated by SDS-PAGE, blotted to PVDF membrane, exposed to reveal ^{32}P label, and quantitatively evaluated by PhosphorImage analysis. To determine protein levels, the blot was subsequently incubated with anti-FLAG antibody followed by ^{125}I-labeled second antibody and quantitatively analyzed as above. The ratio between ^{32}P label (phosphate incorporation) and ^{125}I label (protein level) was calculated, normalized to 1.0 for FLNF-M, and plotted as shown. (*B*) HD3 erythroblasts were transfected with expression constructs encoding NF-M derivatives, incubated for 20 hr under permissive or nonpermissive conditions as shown, and analyzed for RNA expression by Northern analysis. NF-M218D is a derivative of the full-length protein, where threonine 218 was replaced with aspartic acid by site-directed mutagenesis.

Table 1. *Interaction between hybrid proteins in yeast*

DNA-binding hybrid (in pGBT9)	*Trans*-activation hybrid (in pGAD424)	Color of colonies[a]
GalDBD	GalAD	white
GalDBD–p53[b]	GalAD	white
GalDBD–p53	GalAD–CR1234	white
GalDBD–p53	GalAD–SV40largeT[c]	blue
GalDBD	GalAD–CR1234	white
GalDBD–CR5	GalAD	white
GalDBD–CR5	GalAD–CR1234	blue
GalDBD–CR7	GalAD	white
GalDBD–CR7	GalAD–CR1234	blue
GalDBD–CR7D218	GalAD	white
GalDBD–CR7D218	GalAD–CR1234	white
GalDBD–CR7	GalAD–CR7	blue
GalDBD–CR7D218	GalAD–CR7	blue
GalDBD	GalAD–CR7	white

[a]The number of colonies per plate was in the range of 50–250.
[b]Murine p53 amino acids 72–390.
[c]SV40 largeT amino acids 84–708.

region probably by masking its activation potential. The finding that interaction of CR7 with the *trans*-activating domain can be abrogated by mutation in the MAPK site lends support to the idea that deinhibition of NF-M occurs by kinase-induced unmasking of its *trans*-activation domain. Such a hypothetical mechanism is schematically shown in the model presented in Figure 8.

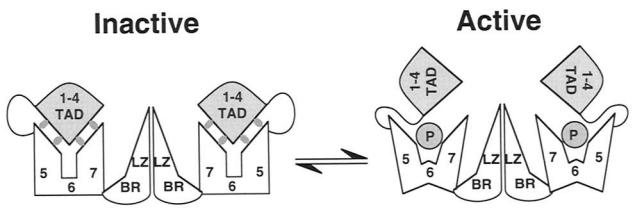
Discussion

Our data strongly favor a novel mechanism to control the activity of C/EBPβ. We suggest that activation of signaling pathways results in phosphorylation of the inhibitory domains of C/EBPβ, thereby eradicating the inhibition by exposing its *trans*-activating functions. Therefore, inactivation of inhibitory functions (negative regulation) rather than enhancement of the *trans*-activation domain (positive regulation) appears to be a major driving force of C/EBPβ-induced gene transcription.

The results obtained with reporter assays on defined promoter constructs demonstrate that activation of gene expression by deinhibition maps to the *cis*-regulatory elements known to bind NF-M or its derivatives. However, replacement of the carboxy-terminal one-third of the protein (containing the b-Zip motif and DNA-binding domain) with the DNA-binding domain of the yeast *trans*-activator Gal4 creates a fusion protein that could still be activated by the same deinhibition mechanism as the wild-type protein. This suggests that the highly conserved DBD–LZ region of NF-M is not essential for the activation mechanism. It does, however, not exclude that the DBD–LZ region participates in the regulation of NF-M/C/EBPβ or the activation of some genes, for example, in a cell type-dependent fashion as observed by others (Wegner et al. 1992).

We provide evidence that two inhibitory domains, CR5 and CR7, interact with the *trans*-activation domain of NF-M and silence its function. One of the pathways to activate C/EBPβ occurs through MAPKs that phosphorylate a highly conserved site in CR7 (Nakajima et al. 1993). We demonstrate that activation is achieved by deletion of the entire region that harbors the MAPK site or by a point mutation at the MAPK site that mimics the negative charge of a phosphate residue. Along these lines, a mutation that inhibits phosphorylation of the homologous site in NF-IL6 was shown by others to result in loss of activation by the *ras* oncogene (Nakajima et al. 1993). However, data presented in Figure 7A as well as peptide maps of in vivo-labeled NF-M mutants (data not shown) or human C/EBPβ (Nakajima et al. 1993) indicated several additional phosphorylation sites in the inhibitory regions with unknown function. At the moment we do not know the biological significance of these sites. However, the results obtained with the two-hybrid system indicates that the MAPK site is indeed a critical target of region CR7, because its mutation abrogates its interaction with the *trans*-activating domain of NF-M but not with CR7. The observation that the CR7 inhibitory regions interact with each other in the two-hybrid system also deserves attention. The major implication from this finding is that inhibition/activation may also be achieved by intermolecular interactions between NF-M molecules, as opposed to the intramolecular mechanism proposed for CR5 and CR7. Because NF-M/C/EBPβ readily forms heterodimers with other members of the C/EBP family, it will be interesting to investigate whether CR7 could also inhibit activation in *trans*. It would probably also help in understanding the function of the natural dominant-negative C/EBPβ that is translated from an internal start codon at the beginning of CR7 by a ribosomal scanning mechanism (Descombes and Schibler 1991).

The mechanism that targets the other inhibitory region, CR5, is less clear. Interestingly, PKC was suggested to mediate enhanced transcriptional efficacy through phosphorylation of a serine residue (Ser-105) in the rat C/EBPβ version (Trautwein et al. 1993). According to our amino acid alignment of C/EBP proteins (Katz et al. 1993), the equivalent position of rat C/EBPβ Ser105 would be at the amino-terminal border of CR5. Therefore, it appears that the apparent activation of rat C/EBPβ may also be occurring through disruption of CR5. However, this particular phosphorylation site in the rat protein is conserved neither in human nor in chicken C/EBPβ (Katz et al. 1993). It therefore appears possible that multiple ways exist to inactivate the inhibitory regions of C/EBPβ. Like rat and human C/EBPβ,



we also observe activation of NF-M by phorbol ester or LPS treatment. However, activation through both pathways is cell type-dependent. In myeloblasts that express large amounts of NF-M, neither LPS nor phorbol ester activate the NF-M-dependent cMGF promoter. On differentiation of myeloblasts into macrophages, however, NF-M-dependent cMGF expression and cMGF promoter activity is mediated by phorbol ester or LPS through the NF-M-binding sites (Beug et al. 1984). In contrast, kinase oncogenes activate the cMGF promoter through its NF-M sites irrespective of the differentiation status of myelomonocytic cells (Adkins et al. 1984; Graf et al. 1986; Sterneck et al. 1992a,b). This suggests that at least some of the pathways that mediate NF-M activation in myelomonocytic cells are sensitive to a differentiation program. Taken together, our data suggest that two regions within NF-M, CR5, and CR7 mask the *trans*-activation domain and prevent its interaction with the basic transcription machinery.

Many of the above data were obtained by exploiting the ability of NF-M to activate endogenous, chromatin-embedded target genes and by using the ability of a conditional oncogene to regulate the activity of NF-M. These experiments confirm the relevance of the deinhibition mechanism in vivo. Modulation of NF-M activity and, in particular, its ability to regulate resident genes is somewhat reminiscent of the myogenic helix–loop–helix proteins, where removing negative regulation of a single transcription factor can override constraints in the activation of cell lineage-specific genes (Weintraub 1993). Why is the *trans*-activation function of NF-M regulated by deinhibition? One advantage of this type of activation is that it could be achieved easily by many different means. They only need to have in common the ability to inactivate a specific protein function rather than to install one as it is the case with positive control mechanisms. We therefore anticipate that a range of signaling pathways, effector molecules, and protein–protein interactions might converge on the inhibitory regions of NF-M/C/EBPβ. Conceivably, a model approximating this mechanism is shown in Figure 8 and might involve neutralization of the inhibitory domain causing a structural change that in turn reveals the previously concealed activation domain. Of course, more complex mechanisms involving dimerization partners or ancillary proteins may also be possible and will be examined.

The derepression mechanism described in this paper provides an explanation why the cMGF gene (which contains binding sites for NF-M but not for Myb) becomes transcribed only after activation of oncogenic kinases in myelomonocytic cells that contain high amounts of NF-M (Sterneck et al. 1992a,b). In contrast, the *mim*-1 gene (which contains binding sites for both NF-M and Myb) becomes cooperatively activated by NF-M plus Myb in cells lacking an oncogenic kinase. How can this discrepancy be resolved? It is conceivable that on promoters that contain adjacent *cis*-acting elements for both NF-M and Myb, derepression might occur through interaction with adjacently bound Myb, omitting the kinase activation step. Activation of NF-M in this instance may occur through modulation of the same inhibitory regions by interaction with Myb. As mentioned earlier, it is possible that the local activation of NF-M by transcription factors binding to neighboring sites determines the cell type-restricted transcriptional activity of NF-M and other C/EBPs. The requirement of accessory transcription factor NF-Y in the case of C/EBPα-regulated albumin promoter activation has already been shown and supports this idea. Whether such a combinatorial site-restricted activation mechanism occurs between NF-M and Myb as well as how NF-M participates in v-*myb*-induced transformation are currently under investigation.

Materials and methods

Plasmid constructions

Expression plasmids were based on pCDM8 and maintained in the bacterial strain MC1061/P3 (Invitrogen). DNA cloning, restriction analysis, and sequencing were performed according to standard procedures (Ausubel et al. 1987; Sambrook et al. 1989). NF-M mutants and chimeric proteins were constructed by PCR approaches and sequenced following subcloning into pCDM8. NF-M constructs harboring their native DNA-binding and dimerization domains were generated from a 3′ primer (BX) containing *Bam*HI and *Xba*I cleavage sites. To construct NF-M 5′ deletion mutants, the following 5′ primers were obtained as synthetic oligonucleotides: NF-M HEN, GCAAGCTTGAATTCACCATGGAACGCCTGGTGGCCTGG; NF-MBX, GCTCTAGAGTGGATCCGCGCAGCGGGGCGAGGAAGCGAG; 5D43, CGGAATTCAAGCTTACCATGGCGGCCGGGGGCCGCTCC; 5D83, CGGAATTCAAGCTTACCATGGCAGCAGCAGCAGGGGGC; 5D141, CGGAATTCAAGCTTACCATGGGGGTGCTGCCGGGCTGC; 5D184, CGGAATTCAAGCTTACCATGGCCTCGCCCTACGGCAGC; 5D229, CGGAATTCAAGCTTACCATGGCCGCCGGCGCCGGGGGC. All 5′ primers provide optimized translation initiation sequences (Kozak 1984) and convenient restriction sites to ease subcloning.

Mutant constructions were done as follows: To 100 ng of linearized plasmid encoding wild-type NF-M, 100 pmoles of the respective primer pairs was added and cycled through eight rounds of PCR amplifications, cut with *Hin*dIII–*Xba*I, separated by agarose gel electrophoresis, and cloned into pCDM8. Internal deletions were constructed by choosing appropriate primer pairs between Pro-Gly residues in the intervening, nonconserved parts of NF-M as indicated in Figure 1. The internal deletion primers harbored *Sma*I–*Xma*I sites encoding Pro-Gly at the deletion endpoints and were used to amplify the respective parts of NF-M as described above. The following primers were used to construct internal deletions: ID881, ATCCCGGGGAGTCCTCCAAGTCGGCC; ID747, TACCCGGGCCCCGCTCCCCCTTCTTC; ID743, ATCCCGGGCCAGGACCGGGGGGCATG; ID645, TACCCGGGCAGCACCCCCGGCTTGTG; ID633, ATCCCGGGGTGCTGCCGGGCTGCTTC; ID561, TACCCGGGGCCGCCGCTGCCTTTATA. After amplification, products were cut with *Hin*dIII–*Xma*I and *Xma*I–*Xba*I, respectively, purified by DNA–agarose gel electrophoresis and joined by three-factor ligation into pCDM8. By a similar strategy, the translation initiation and multiple cloning site of Gal4 (1–147) in the plasmid pSG424 (Sadowski and Ptashne 1989) were modified to a *Nar*I site (which was in-frame with the NF-M *Nar*I site) at the 5′ end and an *Xba*I-translation stop

codon at the 3′ end (5′ primer sequence: GAL4DBSEH, GCACTAGTGAATTCAAGCTTACCATGGCGCTACTGTCTTCT; 3′ primer sequence, GAL4DBBXC, GCATCGATCTAGAGTGGATCCGATACAGTCAACTGTCT). Appropriate NF-M constructs were ligated as *Hin*dIII–*Nar*I fragments to the modified Gal4DBD. Point mutation of the NF-M MAPK site was achieved with the primer NF-MBX and

NF-M218D, CTCCGACCCCCCCGGCACCCCGAACCCCTCCGAGTCCTC. Amplification from wtNF-M template was performed as described above. The PCR product was digested with *Bgl*I and *Xba*I and ligated to the *Hin*dIII–*Bgl*I fragment of wtNF-M in pCDM8. This exchanged the threonine at position 218 to aspartic acid.

Two-hybrid system

The two-hybrid system, yeast strains, and hybrid plasmids pGBT9 and pGAD424 were purchased from Clonetech. Gal4–NFM chimeric proteins were constructed in two steps: NF-M fragments were first obtained by PCR and subsequently subcloned in-frame to Gal4 sequences as described above. The following primers were obtained as synthetic oligonucleotides and used to construct the fragments containing CR1234 (primers 1F and 4R), CR5 (primers 5F and 5R), CR7 (primers 7F and 7R), CR89 (primers 8F and 9R): 1F, GCGAATTCCCCGGGATGCAACGCCTGGTGGCC; 4R, GCGGATCCGCCGCCGCCGCTGCCTTTATAGTC; 5F, GCGAATTCCCCGGGGGCGGCAAGAAGCCCGACTAC; 5R, GCGGATCCCAGCACCCCCGGCTTGTGGCTCTG; 7F, GCGAATTCCCCGGGGGCATGTCCTCGCCCTAC; 7R, GCGGATCCTCACGGCCCCGAGTAGCCCCCGGC; 8F, GCGAATTCCCCGGGGCCGCCGGCGCCGGGGGCTAC; 9R, GCGGATCCTCAGCAGCGGGGCGAGGAAGCGAGCAG. All primers contain *Eco*RI or *Bam*HI restriction sites in-frame with the Gal moiety and cloning sites of the vectors. After PCR amplification, fragments were cut with *Eco*RI and *Bam*HI, separated by agarose gel electrophoresis, and ligated into appropriate vectors. Maxi-plasmid preparations were sequenced. All the experiments were performed as described in the Clontech kit, except that plasmids were transformed by the method developed by Gietz et al. (1992) using the yeast strain SFY526. Coloration of filter-transferred and lysed yeast colonies was analyzed in all cases after 6 hr at 30°C of incubation with *lacZ* buffer containing 0.35 mg/ml of X-gal. The ability of p53 to interact with large T was used as positive control, which was provided with the Clonetech kit. All experiments were repeated at least three times with identical results.

Cell culture, transfection, metabolic labeling, and immunoprecipitation

The quail fibroblast cell line QT6 and the chicken HD3 (erythroblast) line have been described (Beug et al. 1979). All cells were grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 8% fetal calf serum, 2% heat-inactivated chicken serum, 10 mM HEPES–NaOH (pH 7.2) plus penicillin, and streptomycin in a humidified incubator in 5% CO_2 at 36°C. Temperature shifts were done by incubating cells at 42.5°C to inactivate the *tsv-erb*B kinase. Cells were harvested 14–24 hr after the temperature shift. Backshift experiments were performed by incubating the cells for 12 hr at the nonpermissive temperature (42.5°C) followed by incubation for 4 hr at the permissive temperature (36°C). Where indicated, cycloheximide (50 μg/ml) was added 20 min before back shift to block protein synthesis. The transfection procedures using DEAE–dextran have been published in detail (Katz et al. 1993; Sterneck et al. 1992a,b). Briefly, for reporter assays, 2×10^6 cells were transfected for 30–45 min at 36°C with 200 ng of expression vector and 1 μg of reporter plasmid in 0.3 mg/ml of DEAE–dextran (Sigma) in STBS solution (Ausubel et al. 1987). Cells were pelleted, seeded in 4 ml of tissue culture medium, and harvested after 48 hr. For Northern analysis, transfection protocols and cell number were scaled up 10-fold. COS-7 cells were transfected with lipofectin (GIBCO-BRL) according to the manufacturer's instructions at 3×10^5 cells in 60-mm tissue culture dishes and harvested after 3 days. Twelve hours after transfection, metabolic labeling with [^{32}P]orthophosphate was performed for 4 hr. Cells were washed three times in phosphate-free DMEM (Sigma) and incubated with 0.5–1 mCi/ml of [^{32}P]orthophosphate (5000 Ci/mmole) in phosphate-free DMEM supplemented with 10% dialyzed fetal calf serum. Cells were washed twice in TBS (25 mM Tris-HCl at pH 7.5, 150 mM NaCl, 100 mM sodium orthovanadate, 1 mM DTT) and lysed in RIPA buffer (50 mM Tris at pH 8.0, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 10 mM DTT, 100 mM sodium orthovanadate, 10 mM aprotinin, 10 mM leupeptin). To equalize antigenicity of NF-M derivatives, the FLAG peptide (Kodak) tag was fused in-frame to the carboxyl terminus by the PCR method as described above. The sequence of the oligonucleotide encoding the FLAG tag was DTKDDDDK. NF-M–FLAG chimeras were immune-precipitated with anti-FLAG antibodies (Kodak) and washed three times with RIPA buffer. ^{32}P-Labeled immunoprecipitates were separated by SDS-PAGE, transferred to PVDF membranes (Millipore), and evaluated quantitatively by PhosphorImage analysis (Fuji-Bas 1000). Following exposure to determine ^{32}P contents, protein blots were blocked with dry-milk solution (6% in 25 mM Tris at pH 8.0, 150 mM NaCl), and probed with anti-FLAG M2 monoclonal antibody (Kodak). Quantitative evaluation of NF-M constructs were performed with ^{125}I-labeled second antibody and PhosphorImage analysis. The ratio of ^{32}P to ^{125}I-specific radioactivity was determined.

Reporter and resident gene activation assays

The cMGF reporter construct (M82) has been described (Sterneck et al. 1992b). The Gal4-responsive cMGF promoter was constructed by replacing the two NF-M-binding sites of the −82 cMGF promoter with four tandemerized binding sites for the Gal4 DBD. Transient expression assays, luciferase assays, and normalization were performed as described elsewhere (Sterneck et al. 1992a,b; Katz et al. 1993).

To monitor endogenous gene activation, total RNA was extracted from transfected cells using a guanidinium–isothiocyanate method (Chomczynski and Sacchi 1987). The polyadenylated RNA fraction was isolated using magnetic oligo(dT) beads (Dynal) as recommended by the manufacturer. RNA was separated on 1.2% formaldehyde–agarose gel electrophoresis and transferred by vacuum blotting to nylon membranes (Hybond, Amersham). Blots were probed in QuickHyb hybridization solution (Stratagene) at 65°C for 2 hr with appropriate random ^{32}P-labeled (Pharmacia, Amersham) DNA fragments as shown in Figures 3–7. Before exposure, filters were washed twice for 15 min with 2× SSC, 0.1% SDS at room temperature and twice for 15 min with 0.2× SSC, 0.1% SDS at 60°C and exposed to Kodak XAR5 X-ray film or to PhosphorImaging screens. PhosphorImage and quantitation analysis was performed using the manufacturer's software packages (Fuji or Molecular Dynamics). For reprobing, filters were stripped in 0.04× SSC, 0.1% SDS at 95°C for 2 min.

Nuclear extracts and DNA-binding assays

Nuclear extracts from HD3 or COS-7 cells were prepared as described by a mininuclear extract procedure (Schreiber et al. 1989) in the presence of protease inhibitors. The double-stranded oligonucleotide C/EBP-binding site (Ness et al. 1993) or Gal4-binding site was labeled with [α-^{32}P]dCTP by end-filling with Klenow polymerase. Mobility shifts were performed exactly as described previously (Sterneck et al. 1992b; Katz et al. 1993). Where indicated, polyclonal rabbit antiserum raised against bacterially expressed NF-M was diluted with PBS (1:500 final concentration) and added to the binding reaction 10 min after mixing nuclear extracts and DNA. Samples were incubated on ice for an additional 10 min before electrophoresis. Electrophoresis was performed on 5% polyacrylamide gels in 0.5× TBE at 25 mA at room temperature.

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