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# Multiple regions of p45 NF-E2 are required for β-globin gene expression in erythroid cells

**Teresa L. Bean and Paul A. Ney***

Department of Biochemistry, Saint Jude Children's Research Hospital, 332 North Lauderdale, Memphis, TN 38101, USA

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## ABSTRACT

**Regulated expression of genes in the β-globin cluster depends upon sequences located between 5 and 20 kb upstream of the ε gene, known as the locus control region (LCR). β-Globin expression in murine erythroleukemia (MEL) cells depends on NF-E2, a transcription factor which binds to enhancer sequences in the LCR. To gain insight into the mechanism of globin gene activation by NF-E2, an NF-E2 null MEL cell line was used to map regions of NF-E2 required for β-globin expression. Within the transactivation domain, two discrete proline-rich regions were required for rescue of β-globin expression. The first was located at the N-terminus of NF-E2, while the second was located N-terminal of the cap 'n collar (CNC) domain. Other proline-rich sequences were dispensable, indicating that proline content *per se* does not determine NF-E2 activity. Mutations within the conserved CNC domain markedly diminished rescue of β-globin expression. This domain was required, in addition to the basic leucine zipper domain, for DNA binding activity. The requirement for discrete proline-rich sequences within the transactivation domain suggests that globin gene expression in MEL cells depends on specific interactions between NF-E2 and downstream effector molecules.**

## INTRODUCTION

High level expression from the β-like globin genes depends upon sequences located 5–20 kb upstream of the ε gene, known as the locus control region (LCR) (1–3). The β-globin LCR contains four core enhancers, of 200–300 bp each, which are marked by erythroid-specific DNase I hypersensitive sites (HS1–HS4). Individual core enhancers do not retain the activity of the full LCR, but are sufficient to confer high level, tissue-specific expression on a linked β-globin gene in transgenic mice (4–7). These core enhancers contain a high density of binding sites for erythroid-specific and ubiquitous transcription factors, including GATA, NF-E2 and GGTGG/CACC motifs. In contrast to other binding motifs, the distribution of NF-E2 binding sites in globin regulatory sequences is limited to core enhancers in the β-globin LCR and the α-globin positive regulatory element (PRE). This distribution, along with the evolutionary conservation of these sites (8–12), suggests an integral role for NF-E2 sites in LCR function.

The importance of tandem NF-E2 sites for activity of the HS2 core enhancer is well established (13–16). Activity of the α-globin PRE also depends upon intact NF-E2 sites in transient transfection experiments (17). Full activity of these sequences correlates with binding by NF-E2 (5,18,19). Recently, it has been shown that ablation of individual core enhancers HS2 and HS3 is associated with a 30% decrease in β-globin expression (20,21). However, the mild defect seen in these experiments does not account for the cumulative effect of NF-E2 sites present in each of the three most active core enhancers (HS2–HS4).

NF-E2 is a heterodimeric member of the basic leucine zipper (bZIP) transcription factor family, composed of 45 and 18 kDa subunits (p45 and p18 NF-E2). Recent experiments have shown that the *Fli-2* locus, a common site of Friend murine leukemia virus (F-MuLV) integration, is p45 NF-E2 (22). Lu *et al.* have described an NF-E2 null MEL cell line, CB3, which resulted from disruption of one p45 NF-E2 allele by F-MuLV coupled with loss or inactivation of the other allele. CB3 cells do not express p45 NF-E2 nor do they express α- or β-globin. Rescue of α- and β-globin expression by p45 NF-E2 demonstrated that globin gene expression is dependent on NF-E2 in these cells. Similar results have been obtained in MEL cells by expression of a dominant negative form of p18 NF-E2 (23). We have utilized MEL cells as a model to study the mechanism of globin gene activation by NF-E2.

NF-E2 has been purified from murine and human erythroleukemia cells and both subunits have been cloned (24–27). The smaller subunit, p18 NF-E2, is widely expressed and is related to the small Maf proto-oncogene family in chicken (MafF, MafG and MafK). p18 NF-E2, the murine homolog of MafK, does not possess a canonical transactivation domain. Consistent with this, over-expression of p18 NF-E2 has been shown to repress transcription through NF-E2 binding sites (28). The larger subunit, p45 NF-E2, is expressed primarily in hematopoietic cells. p45 NF-E2, hereafter referred to as NF-E2, is a member of the CNC family of bZIP proteins. These proteins, named for the prototype cap 'n collar in *Drosophila*, are distinguished by a conserved region of 43 amino acids which lies immediately N-terminal of the bZIP domain. Besides NF-E2, CNC family members in humans include NF-E2-related factors-1 and -2 (Nrf-1/LCR-F1 and Nrf-2) (19,29,30).

*To whom correspondence should be addressed. Tel: +1 901 495 2049; Fax: +1 901 525 8025; Email: paul.ney@stjude.org

Different mechanisms have been proposed for the activation of globin gene expression by NF-E2. NF-E2 has chromatin opening properties which may be necessary for hypersensitive site formation and accessibility of other factors to binding sites in the LCR (31,32). NF-E2 also functions as a transcriptional activator (19), suggesting that it may interact with co-activators or components of the transcription initiation complex. The N-terminal half of NF-E2 is required for globin gene expression in MEL cells (23). This region is rich in proline and acidic residues and contains two small conserved motifs, but otherwise lacks salient features. To localize the regions of NF-E2 required for globin gene expression, mutants of NF-E2 have been transfected into CB3 cells. Stable clones have been studied for expression of NF-E2 and β-globin. These experiments demonstrate that sequences within the transactivation domain of NF-E2, as well as the conserved CNC domain, are required for endogenous β-globin expression. Additional experiments address the function of the CNC domain.

## MATERIALS AND METHODS

### DNA constructs

Deletion mutants of NF-E2 (M1–M7 and M5A–D) were made by restriction digestion of NF-E2 followed by ligation with oligonucleotides. This resulted in in-frame deletion of intervening sequence (M1, *Pst*I–*Sst*I; M2, *Sst*I–*Stu*I; M3, *Stu*I–*Alw*NI; M4, *Alw*NI–*Bsa*AI; M5, *Bsa*AI–*Avr*II; M6, *Avr*II–*Hpa*I; M7, *Hpa*I–*Sal*I). Oligonucleotide sequences are available upon request. The precise amino acids deleted by each mutant are shown in Figures 1A and 3.

Point mutations were introduced into the CNC domain (C1–C3) by subcloning oligonucleotides into the M7 deletion mutant of NF-E2. Conservative base pair changes were incorporated into the M7 oligonucleotide creating unique *Nhe*I and *Spe*I sites on either side of the M7 deletion. To confirm that the base pair changes were not detrimental to NF-E2 function, wild-type sequence was cloned into the *Nhe*I and *Spe*I sites. This construct had the same activity as wild-type NF-E2. The M7 oligonucleotide was AACTTGCCG-GTAGATGACTTTAATGAGTT*GCTAGC*A↓*CTAGT*TCGGGA-CATCCG**A** (with *Nhe*I and *Spe*I sites italicized, base pair changes in bold and location of the deletion shown by a downward arrow). Subcloning oligonucleotides into the *Nhe*I and *Spe*I sites effectively recreated the wild-type NF-E2 molecule except for the desired mutations. Base pair changes were made which optimized codon usage in murine cells.

Mutants N1–N3 were made by PCR, sequenced and subcloned into the *Pst*I site of NF-E2. Wild-type and mutant NF-E2 cDNAs (*BH*I–*Xho*I) were subcloned into the eukaryotic expression vector pEF1α-neo (*BH*I–*Sal*I) (33; a gift of S.Orkin). The *in vitro* transcription/translation vector was made by subcloning a PCR-generated fragment of NF-E2, from the translational start site to *Pst*I, into pCITE4a (Novagen), in-frame with the translational enhancer. Wild-type and mutant versions of NF-E2 (*Pst*I–*Xho*I) were subcloned into this vector.

### Tissue culture and cell transfections

CB3 cells were obtained from the laboratory of S.Orkin (with permission from Y.Ben-David; 22). CB3 cells were cultured in DMEM with low glucose (Life Technologies, catalogue no. 11885-084) supplemented with 10% fetal bovine serum, 4 mM glutamine and Pen-Strep. For cell transfections, CB3 cells were washed once and resuspended in HEBS buffer (20 mM HEPES, pH 7.4, 137 mM NaCl, 5 mM KCl, 5 mM dextrose). Samples of $1\times 10^7$ CB3 cells were transfected with 10 µg *Pvu*I-linearized plasmid DNA using a BioRad Gene Pulser apparatus (0.23 kV, 960 µF). Forty eight hours following transfection, CB3 cells were plated in 96-well plates in medium containing 1 mg/ml active G418 (Life Technologies). After 10 days selection, individual clones were picked and expanded for further analysis. Erythroid differentiation was induced by supplementing the medium with 1.8% DMSO for 72 h.

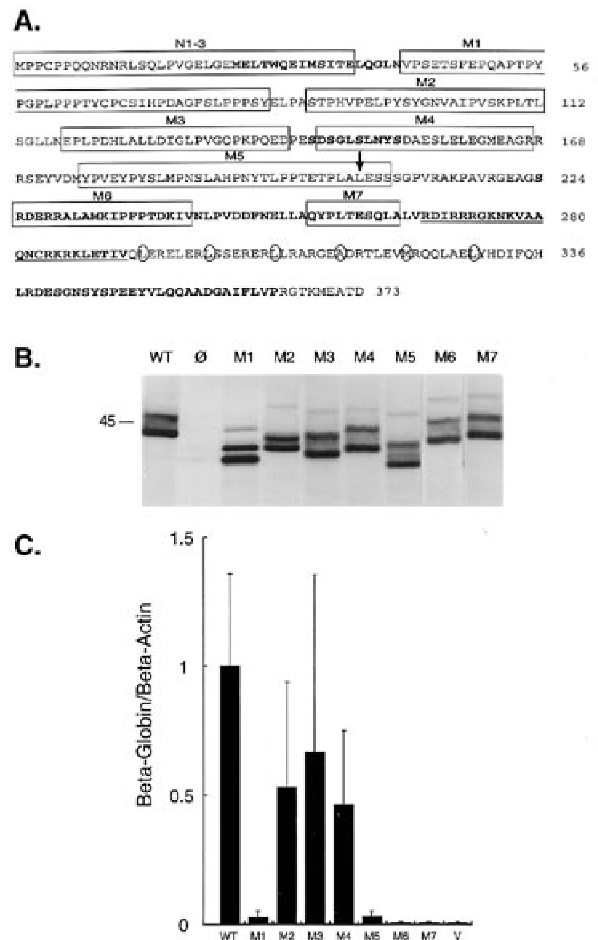


**Figure 1.** Internal deletion mutants of NF-E2. (**A**) Amino acid sequence of NF-E2. In-frame deletions are indicated by boxes, along with the corresponding mutant number. Sequences conserved in Nrf-1 and Nrf-2 are shown in bold. The basic DNA binding domain is underlined. Circles denote the heptad repeat of the leucine zipper. The arrow marks an N-terminal deletion defining a domain required for globin gene expression in CB3 cells (23). (**B**) *In vitro* translation of deletion mutants. The doublet of NF-E2 polypeptides of $M_r$ ~45 000 has been previously reported (24). We have found that wild-type p45 NF-E2 migrates as a doublet of 46 and 44 kDa. Labels are as in (A) (WT, wild-type; Ø, unprogramed reticulocyte lysate). The molecular weight (kDa) is shown on the left. (**C**) Quantitation of β-globin expression. β-Globin expression was divided by β-actin expression for each sample and the mean ± SD was determined. Mean values were expressed relative to wild-type NF-E2. Mutants of NF-E2 were compared with wild-type using Student's *t*-test [M1, $P < 0.001$ ($n = 9$); M2, $P = 0.038$ ($n = 7$); M3, not significant ($n = 10$); M4, $P = 0.004$ ($n = 10$); M5, $P < 0.001$ ($n = 9$); M6, $P < 0.001$ ($n = 12$); M7, $P < 0.001$ ($n = 11$); V, $P < 0.001$ ($n = 12$)]. Labels are as in (A) (WT, wild-type; V, vector).

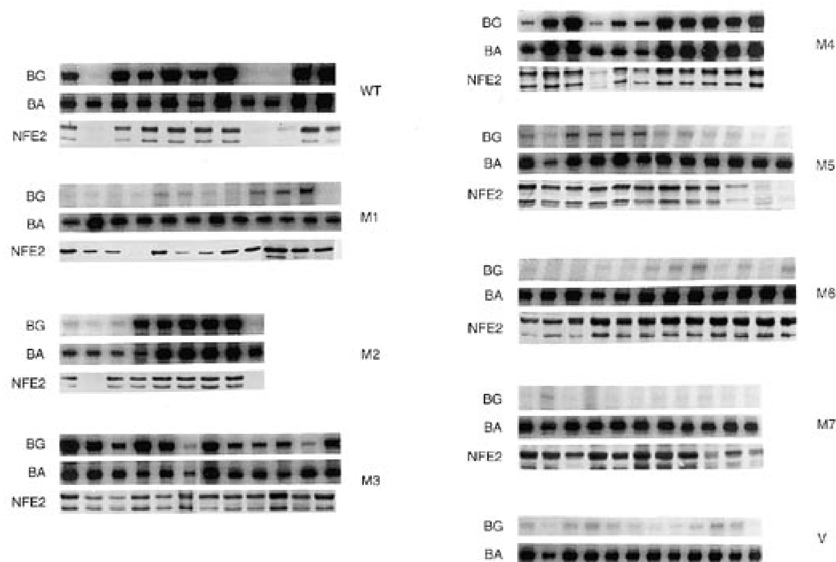


**Figure 2.** Rescue of β-globin expression by internal deletion mutants. Results are for internal deletion mutants shown in Figure 1A (M1–M7; WT, wild-type; V, vector). For each set of three panels, the upper panel shows murine β-globin expression (BG) and the middle panel shows β-actin (BA) expression, determined by RNase protection assay. The β-globin and β-actin riboprobes protected 245 and 150 bp transcripts respectively. The lower panel shows expression of NF-E2 protein. The order of the clones is the same in all panels. The NF-E2 doublet is thought to result from utilization of an alternative translation intitiation site. Consistent with this, deletions at or near the N-terminus of NF-E2 (e.g. M1) resulted in a single band on Western blots.

## RNA analysis

RNA was prepared by the method of Chomczynski and Sacchi (RNazol B, Tel-Test) (34). Murine β-globin and β-actin riboprobes were made by PCR (β-globin, ACTCCGATGAAGTTGGTGGTG, GGATCCACATGCAGCTTGTCA; β-actin, AACGAGCGGTTC-CGATG, ACCAGACAGCACTGTGTTGG) and cloned into pCR-Script (Stratagene). Both riboprobes were made with the MAXIscript kit (Ambion), using T3 RNA polymerase. The β-globin and β-actin riboprobes protected 245 and 150 bp transcripts respectively. RNase protection assays were performed with an RPA II kit (Ambion) using 10–15 µg total RNA and analyzed on a 6% denaturing polyacrylamide gel. Linearity of the assay was demonstrated with a standard curve using 0.1–120 µg RNA from a NF-E2-transfected CB3 clone which expressed β-globin. β-Globin expression was quantitated using a phosphorimager and normalized to β-actin.

## Protein assays

CB3 clones were expanded in 6-well plates, centrifuged and resuspended in SDS–PAGE loading buffer (~$4 \times 10^6$ cells in 0.3 ml loading buffer, 95°C) (35). Samples were boiled for 8 min and sheared 10 times through a 25 gauge needle. Twenty microliters of sample were loaded onto a 10% SDS–PAGE gel, resolved and transferred to nitrocellulose overnight. Filters were processed using an ECL kit (Amersham). The anti-NF-E2 rabbit polyclonal antiserum was raised using a synthetic peptide (NVPSETSFEP-QAPTPY) coupled to keyhole limpet hemocyanin (Rockland Inc., Boyertown, PA). This epitope is lacking from mutant M1, consequently a polyclonal antiserum (amino acids 1–348 of NF-E2) was used to characterize expression in those clones (24). *In vitro* transcription and translation was done using TNT Retic lysate (Promega) and [$^{35}$S]methionine in accordance with the manufacturer's instructions.

## Expression analysis

The ratio of β-globin to β-actin expression was calculated and the mean and standard deviation determined for each group. Each mutant construct was compared with wild-type NF-E2 using Student's *t*-test. NF-E2 non-expressing clones, determined by Western blot analysis, were omitted from the analysis (typically 2–4 clones/construct).

## DNA binding assay

Gel mobility shift experiments (GMSA) were performed as previously described (18). For one or more clones from each mutant, nuclear extracts were made from $1 \times 10^8$ CB3 cells. The DNA sequence of the wild-type NF-E2 probe was GGAACCT-G<u>TGCTGAGTCAC</u>TGGAGG. The sequence of the mutant NF-E2 probe was GGAACCTG<u>T**T**CTGAGTCAC</u>TGGAGG (36). Gel shift probes were labeled with dCTP and Klenow enzyme (Boehringer Mannheim). NF-E2 protein was demonstrated in nuclear extracts by Western blot analysis, as described above.

# RESULTS

## Regions of NF-E2 required for β-globin gene expression

CB3 cells are an NF-E2 null MEL cell line which do not express α- or β-globin in culture and cannot be induced to express globin in the absence of NF-E2 (22). Previous experiments have shown that endogenous globin gene expression is restored in CB3 cells following stable transfection with NF-E2. However, transfection of CB3 cells with an N-terminal truncated form of NF-E2 failed to rescue globin gene expression (23). Based on this result, seven in-frame internal deletions (M1–M7) of NF-E2 were made (Fig. 1A). For each of these mutants, the presence of an intact open reading frame was confirmed by *in vitro* translation (Fig. 1B). Mutants M1–M5 spanned the proline-rich transactivation domain of NF-E2. Mutants M6 and M7 had deletions in the conserved

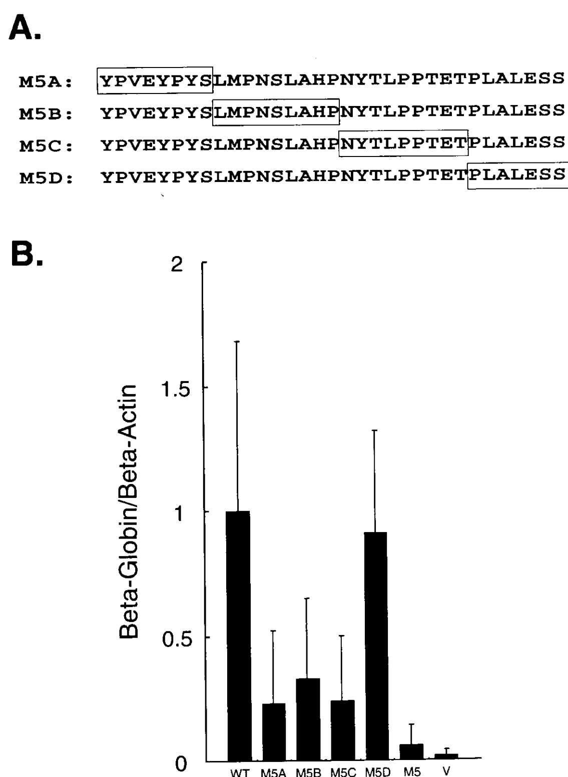


**Figure 3.** Deletion mutants of the M5 region. (**A**) Amino acid sequence of the M5 region. In-frame deletions are indicated by boxes, along with the corresponding mutant number. (**B**) Quantitation of β-globin expression. β-Globin expression was analyzed as described in Figure 1 [M5A, $P = 0.024$ ($n = 10$); M5B, $P = 0.044$ ($n = 8$); M5C, $P = 0.026$ ($n = 7$); M5D, not sigificant ($n = 10$); M5, $P = 0.010$ ($n = 9$); V, $P = 0.009$ ($n = 11$)]. Labels are as in (A) (WT, wild-type; V, vector).

CNC domain. In addition, three deletion mutants of the N-terminus were made (N1–N3).

Mutants of NF-E2 were subcloned into the eukaryotic expression vector pEF1α-neo (33). Following transfection into CB3 cells and selection, ~12 clones/construct were studied for expression of β-globin and β-actin RNA and NF-E2 protein. CB3 cells transfected with wild-type NF-E2 showed rescue of endogenous β-globin expression (Figs 1C and 2, WT compared with V). Rescue of β-globin expression correlated well with expression of NF-E2 protein. Deletion of proline-rich sequences from amino acids 42–83 (M1), diminished β-globin expression 40-fold. Deletion of amino acids 88–111, 118–141 or 145–167 (M2–M4) had relatively little effect on β-globin expression. Deletion of amino acids 176–209 (M5), adjacent to the CNC domain, diminished β-globin expression 30-fold. Similarly, deletions within the CNC domain, amino acids 225–243 or 256–265 (M6 and M7), had a marked effect on rescue of β-globin expression by NF-E2.

The M5 region was scanned with smaller deletions to further delineate active sequences (Fig. 3). Activity of these mutants (M5A–D) was greater than that of mutant M5. In addition, activity was evenly distributed over a region of 26 amino acids. Thus, activity is not highly localized within the M5 region.

The N-terminal half of NF-E2 is generally divergent from Nrf-1 and Nrf-2. The exceptions to this are two conserved motifs, one near the N-terminus and a second which is mostly deleted in the M4 mutant (Fig. 1A). As previously noted, deletion of the second motif had little effect on β-globin expression. Deletion of the motif near the N-terminus caused a 2.5- to 5-fold decrease in mean β-globin expression (N1 and N2) (Fig. 4). Activity of N2 was significantly less than wild-type NF-E2. The functional difference between N1 and N2, which lacks a potential alternative translation initiation site, was not significant. A larger deletion of the N-terminus (N3) resulted in further loss of β-globin expression, demonstrating the presence of additional active sequences.

### Function of the CNC domain

The effect of internal deletions on DNA binding activity was determined in GMSA. CB3 cells expressing wild-type NF-E2 showed specific DNA binding activity (Fig. 5). This activity was preserved for deletion mutants in the first 209 amino acids of NF-E2 (N1–3 and M1–5). In contrast, DNA binding activity was greatly diminished or eliminated by deletions within the CNC domain (M6 and M7). Thus, the CNC domain is required, in addition to the bZIP domain, for DNA binding by NF-E2.

The effect of subtle mutations on CNC domain function was analyzed by subcloning oligonucleotides into deletion mutant M7. As described in Materials and Methods, this effectively introduced point mutations into the CNC domain of full-length NF-E2 (Fig. 6A). Introduction of wild-type sequence restored full activity (data not shown). The effect of CNC mutations on β-globin rescue is shown in Figures 6B and 7. The effect of these mutations on DNA binding activity is shown in Figure 6C. Mutation of potential phosphorylation sites in the CNC domain (C1) had no effect on rescue of β-globin expression, despite diminished DNA binding activity. In contrast, mutation of conserved leucine residues (C2A and C2B) disrupted DNA binding and prevented rescue of β-globin expression. Mutation of conserved glutamine and glutamic acid residues (C3A and C3B) also prevented rescue of β-globin expression, but compared with the leucine mutations had little effect on DNA binding.

## DISCUSSION

By analogy with other transcription factors, the ability of NF-E2 to activate globin gene expression is likely to depend on discrete domains that mediate DNA binding and transcriptional activation (37). The N-terminal half of NF-E2 activates transcription from the β-globin promoter in transiently transfected K562 erythroleukemia cells (19). The same region contains sequences that are required for β-globin expression (23). Whether these sequences are distributed throughout the N-terminal half of NF-E2 or whether they are localized to discrete domains has not been previously established. Therefore, one objective of these studies was to localize these sequences.

Two regions in the proline-rich transactivation domain were required for β-globin expression. The first was localized to the N-terminus of the molecule. Within this region, amino acids 2–36 and 42–83 contained sequences essential for β-globin expression. Amino acids 24–36, which are conserved in Nrf-1 and Nrf-2, accounted for some of the activity. However, most of the activity appeared to reside in proline-rich sequences. Two PXXP motifs were found within this region. Short proline-rich sequences containing this motif have been shown to form a left-handed type II polyproline (PPII) helix (38). These structures commonly occur on the surface of globular proteins and are capable of mediating protein interactions (39).

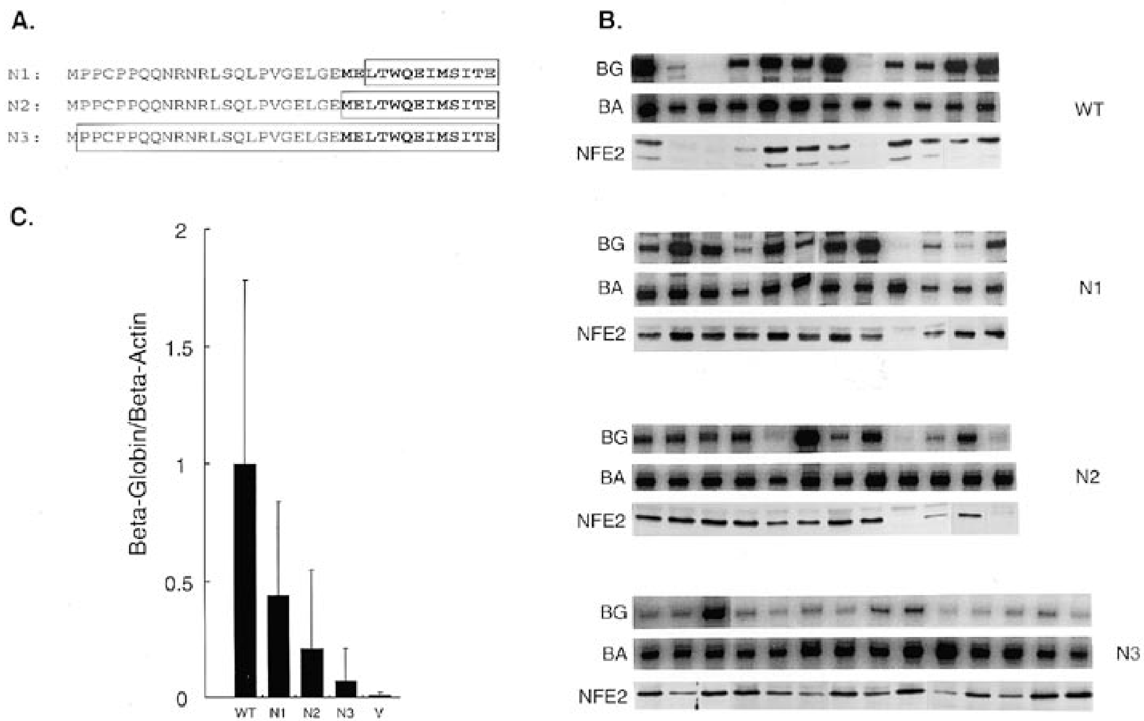


**Figure 4.** Deletion mutants of the N-terminus. (**A**) Amino acid sequence of the N-terminus. In-frame deletions are indicated by boxes, along with the corresponding mutant number. Sequences conserved with Nrf-1 and Nrf-2 are shown in bold. (**B**) Rescue of β-globin expression. Panels are organized as in Figure 2. (**C**) Quantitation of β-globin expression. β-Globin expression was analyzed as described in Figure 1 [N1, not significant ($n = 11$); N2, $P = 0.026$ ($n = 9$); N3, $P = 0.012$ ($n = 16$)]. Labels are as in (A) (WT, wild-type; V, vector).

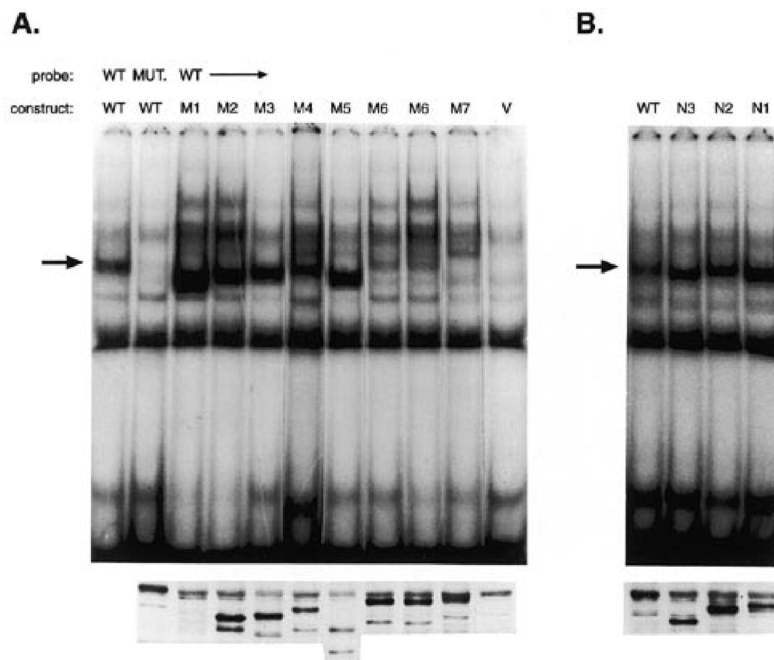


**Figure 5.** DNA binding activity of NF-E2 mutants. GMSA were performed using a wild-type NF-E2 site probe (WT) derived from the PBGD promoter or a mutant probe (MUT.) which selectively ablates NF-E2 binding (36). Nuclear extracts were made from one or more of the stable clones from each construct and studied by GMSA (top) and immunoblot assay (bottom). Wild-type and mutant NF-E2 constructs are indicated at the top: (**A**) M1–M7; (**B**) N1–N3. The arrows indicate the position of NF-E2 binding activity. Slower migrating bands represent sequence-specific binding by other proteins and include members of the AP-1 family (4,18). Migration of NF-E2 varied depending on the size of the deletion. The exposure for lane M4 is slightly darker to demonstrate NF-E2 binding activity. In the immunoblot assays, a 45 kDa background band was present which was readily distinguished from immunoreactive bands. Migration of NF-E2 protein in the immunoblot also varied depending on the size of the deletion, but the pattern was quite similar to that seen with *in vitro* translated protein (Fig. 1B). Mutant M1, which lacks the immunoreactive epitope, was not recognized by the antibody, but showed gel shift activity.

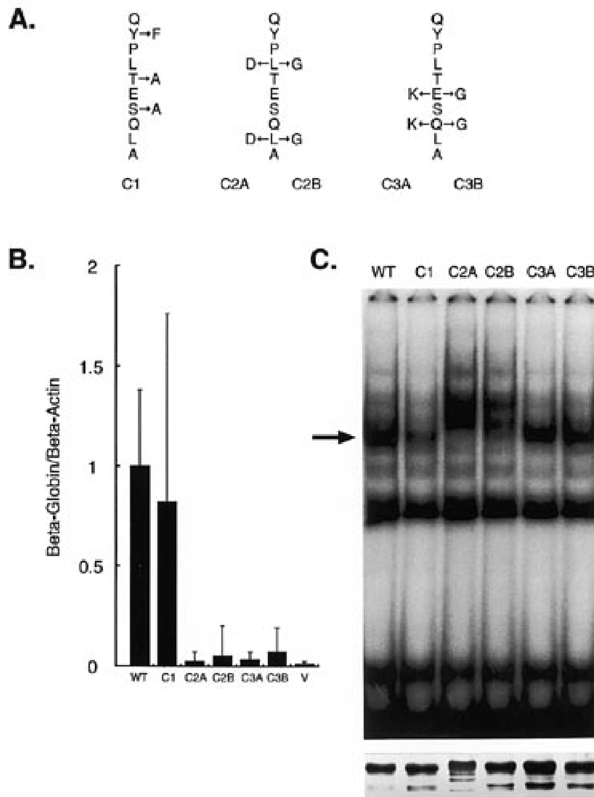


**Figure 6.** Point mutants in the CNC domain. (**A**) Wild-type sequence is shown vertically with point mutants on either side. Further detail on the mutants is provided in the text. (**B**) Quantitation of β-globin expression. β-Globin expression was analyzed as described in Figure 1 [C1, not significant ($n = 11$); C2A, $P < 0.001$ ($n = 11$); C2B, $P < 0.001$ ($n = 11$); C3A, $P < 0.001$ ($n = 11$); C3B, $P < 0.001$ ($n = 5$); V, $P < 0.001$ ($n = 10$)]. Labels are as in (A) (WT, wild-type; V, vector). (**C**) GMSA was performed using a wild-type NF-E2 probe. Nuclear extracts were made from one stable clone from each construct and studied by GMSA (top) and immunoblot assay (bottom). Wild-type and mutant NF-E2 constructs are indicated at the top (C1, C2A+B and C3A+B). The arrow indicates the position of NF-E2 binding activity. Slower mobility bands in lanes with mutants C2A and C2B do not represent novel p45 NF-E2-containing complexes, but a general increase in background bands (data not shown).

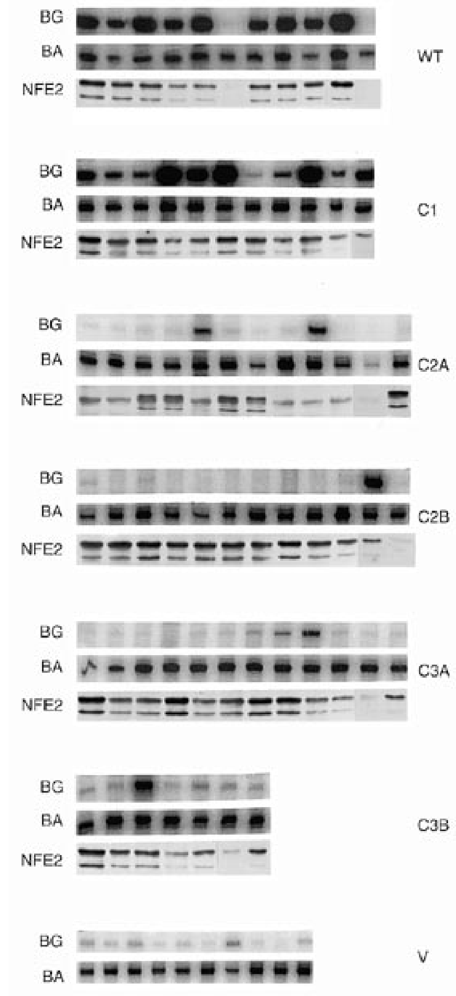


**Figure 7.** Rescue of β-globin expression by CNC domain mutants. Results are for point mutants shown in Figure 6A. Panels are organized as in Figure 2.

The second region was located N-terminal of the CNC domain. Activity was distributed throughout this proline-rich region, consistent with the presence of secondary structure. Notably, deletion of the C-terminal eight amino acids, which had no effect on activity, resulted in importation of two proline residues. Deletion of other proline-rich and acidic sequences from the N-terminal half of NF-E2 had relatively little effect on β-globin expression, indicating that proline content *per se* does not determine activity.

The second objective of these studies was to determine the function of the CNC domain. This conserved region of 43 amino acids lies immediately N-terminal of the bZIP domain. Murine NF-E2-related proteins Nrf-1 and Nrf-2 are 61 and 68% identical respectively to NF-E2 in this domain. Consistent with this degree of conservation, deletions within the CNC domain were very detrimental to NF-E2 function. This loss of function was due, at least in part, to a loss of NF-E2 binding activity. Despite the presence of an intact bZIP domain, NF-E2 mutants with deletions in the CNC domain were unable to bind DNA. Experiments with Skn-1, a member of the CNC family in *Caenorhabditis elegans*, suggest that the CNC domain may function by orienting the basic DNA binding domain in the major groove (40). The CNC domain of Skn-1 is only 23% identical to that of NF-E2. In addition, Skn-1 lacks a leucine zipper and binds DNA as a monomer. Despite these differences, our experiments indicate that this domain is generally required for DNA binding by members of the CNC family.

Increased NF-E2 binding activity during terminal MEL cell differentiation depends on protein kinase A (41). To determine the potential effect of phosphorylation on DNA binding by NF-E2, conservative mutations were introduced into the CNC domain. The modest effect of these mutations indicates that phosphorylation of sites within the CNC domain is not essential for NF-E2 binding activity or function. Mutation of conserved glutamine and glutamic acid residues, while detrimental to β-globin expression, had little effect on gel shift activity, further suggesting that CNC domain function may not be limited to a role in DNA binding.

Experiments in genetically modified mice illustrate the complexity of globin gene regulation by NF-E2. Disruption of either subunit

of NF-E2 has little or no effect on globin gene expression in mice (42,43,44). For p18 NF-E2, the lack of a phenotype can be attributed to the presence of redundant factors. For p45 NF-E2, the role of redundant factors is less clear. Nrf-1 or Nrf-2 may activate globin gene expression in the absence of NF-E2. Nrf-2 dimerizes with p18 NF-E2 and binds specifically to NF-E2 sites (unpublished results). Targeted disruption of Nrf-2 has no apparent effect on globin gene expression (45; unpublished results), while the effect of combined NF-E2 and Nrf-2 deficiency is under investigation.

The reason for the difference between primary erythroblasts and MEL cells is not known. Nonetheless, MEL cells provide a useful model to study the activation of globin gene expression by NF-E2. These experiments have mapped the regions of NF-E2 required for β-globin expression. The requirement for discrete proline-rich sequences within the transactivation domain suggests that globin gene expression in MEL cells depends on specific interactions between NF-E2 and downstream effector molecules. Support for this hypothesis comes from the recent demonstration that p45 NF-E2 interacts with the co-activator CREB binding protein (46). Future experiments will be directed towards identifying these molecules.

## ACKNOWLEDGEMENTS

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