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# Evidence for Distinct DNA Binding Forms of the Erythroid-Specific Transcription Factor NF-E2<sup>†</sup>

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Received August 10, 1995; Revised Manuscript Received October 6, 1995<sup>‡</sup>

**ABSTRACT:** The transcriptional activity of the  $\beta$ -globin genes is regulated by a complex genetic element, the locus control region (LCR), at the 5'-end of the  $\beta$ -globin locus. Tandem binding sites for the erythroid-specific transcription factor NF-E2 are important for the transcriptional activation function of the LCR. We discovered that vanadate strongly stimulates the DNA binding activity of NF-E2 in crude and fractionated nuclear extracts. The other oxyanions, molybdate and tungstate, do not affect NF-E2 DNA binding. Quantitative DNA binding experiments indicated that vanadate stimulates NF-E2 DNA binding by increasing the number of NF-E2 molecules that are competent to bind to DNA, rather than influencing the affinity of binding. Gel filtration analysis revealed a similar Stokes' radius for NF-E2, in the absence or presence of vanadate, inconsistent with a role for vanadate in stabilizing the heteromeric NF-E2 complex. Distinct NF-E2 forms, which were either weakly or strongly induced by vanadate, were resolved by cation and anion exchange chromatography. A model is proposed in which two conformers of NF-E2 share an identical subunit composition, but differ in DNA binding activity. Vanadate may interact directly with one of the conformers to generate the high-affinity DNA binding state. The presence of a non-DNA binding pool of NF-E2 suggests that the formation of an active NF-E2 heteromer may be a regulated step in the cell.

At least two mechanisms are responsible for establishing tissue-specific patterns of gene expression. First, the correct complement of tissue-specific and ubiquitous transcription factors must be present in a cell. Second, the DNA template must be organized into an appropriate chromatin structure. The human  $\beta$ -globin gene cluster on chromosome 11 has been a very useful model system for investigating how these two mechanisms are integrated.

The transcriptional activity of the  $\beta$ -globin genes ( $\epsilon$ , embryonic;  $G\gamma$  and  $A\gamma$ , fetal;  $\delta$  and  $\beta$ , adult) is regulated by a complex genetic element, the locus control region (LCR),<sup>1</sup> at the 5'-end of the globin locus (Forrester *et al.*, 1986; Grosveld *et al.*, 1987). The LCR maintains the  $\beta$ -globin cluster in an "active" chromatin conformation and is necessary for early replication of the globin domain, during the S phase of the cell cycle (Forrester *et al.*, 1990; Kim *et al.*, 1992). As the LCR can activate multiple promoters on a single chromosome (Bresnick & Felsenfeld, 1994a), it may represent a class of genetic elements distinct from enhancers. Typically, enhancers are committed to activating a single promoter (Ptashne & Gann, 1990). Our working model is that the LCR can prevent the folding of the globin domain

into a higher-order chromatin structure, thus increasing the accessibility of the globin promoters to trans-acting factors. Quantitative and qualitative changes in the trans-acting factors would determine which globin gene is active at a particular developmental stage.

The human  $\beta$ -globin LCR consists of four erythroid-specific DNase I hypersensitive sites, HS1–HS4 (Tuan *et al.*, 1985; Forrester *et al.*, 1986). Each hss contains recognition sequences for a variety of ubiquitous and tissue-specific transcription factors (Talbot *et al.*, 1990; Caterina *et al.*, 1991; Bresnick & Felsenfeld, 1993). Mutations of sites that bind NF-E2, GATA-1, SP-1, and USF proteins all have strong inhibitory effects on the transcriptional activation of a linked globin gene (Caterina *et al.*, 1994a), suggesting that multiple proteins are required for optimal activity of the LCR. The tandem NF-E2 sites, in particular, are crucial for high-level expression of a linked globin gene in transfection assays (Tuan *et al.*, 1989; Ney *et al.*, 1990) and in transgenic mice (Talbot & Grosveld, 1991).

It is known that multiple proteins can interact with the NF-E2 consensus DNA binding site (T/C)GCTGA(G/C)-TCA(C/T) (Andrews *et al.*, 1993). These include the ubiquitous factors AP-1 (Ney *et al.*, 1990), LCR-F1 (Caterina *et al.*, 1994b), Nrf-1 (Chan *et al.*, 1993), and Nrf-2 (Moi *et al.*, 1994) and the erythroid-specific factor NF-E2 (Mignotte *et al.*, 1989; Andrews *et al.*, 1993). As the transcriptional activation property of the LCR is high only in erythroid cells, it is likely that NF-E2, and/or an unknown component, is required for LCR function.

<sup>†</sup> Supported by grants from the American Cancer Society (IRG-35-36-4) and the American Heart Association (95-GS-63), by a faculty development award from the Pharmaceutical Manufacturer's Association, and by grants from the University of Wisconsin Comprehensive Cancer Center and the Cremer Foundation.

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<sup>‡</sup> Abstract published in *Advance ACS Abstracts*, November 15, 1995.

<sup>1</sup> Abbreviations: BSA, bovine serum albumin; DTT, dithiothreitol; EMSA, electrophoretic mobility shift assay; HMBA, hexamethylenebisacetamide; HS1, HS2, HS3, and HS4, hypersensitive sites 1–4 of the human  $\beta$ -globin locus; hss, hypersensitive site; LCR, locus control region; MEL, mouse erythroleukemia cells; PAGE, polyacrylamide gel electrophoresis; R, Stokes' radius; USF, upstream stimulatory factor.

NF-E2 was initially purified, and its cDNA was cloned from MEL cells (Andrews *et al.*, 1993). The cDNA encoded a 45-kDa protein, whose expression was restricted to cells of the erythroid and megakaryocytic lineages. The p45 protein is a member of the b-zip family of transcription

factors, which are characterized by a conserved basic domain and a carboxy-terminal leucine zipper (Baxevanis & Vinson, 1993). The b-zip proteins typically exist as homodimers or heterodimers.

A protein of 18 kDa copurified with human p45 (Ney *et al.*, 1993). It was suggested that p18 forms a heterodimer with p45. The cDNA for p18 was cloned, and p18 was shown to be a ubiquitous leucine zipper-containing protein that heterodimerizes with p45 to form the DNA binding activity, NF-E2 (Andrews *et al.*, 1994). Additional cDNAs with strong homology to p18 have since been isolated (Igarashi *et al.*, 1995). As these proteins are related to the Maf oncogene (Nishizawa *et al.*, 1989), they are referred to as Maf proteins. MafK appears to be identical to the p18 component of NF-E2 (Igarashi *et al.*, 1995). It is not known if the p45–p18 heterodimer is the major species in the cell, or if monomers or homodimers of p45 and p18 exist, or even higher-order oligomers.

During the course of experiments to examine the role of protein–protein interactions in the assembly of a nucleoprotein complex on the HS2 region of the LCR, we discovered that vanadate strongly increased NF-E2 DNA binding activity. Vanadate is a commonly used inhibitor of tyrosine phosphatases (Gresser & Tracey, 1990), which should be active under the DNA binding conditions. Thus, we hypothesized that NF-E2 might require phosphorylation for high-affinity DNA binding. The DNA binding activity of NF-E2 would be negatively regulated by a phosphatase, and vanadate would increase NF-E2 DNA binding by inhibiting the phosphatase.

In this paper, we describe the positive effect of vanadate on NF-E2 DNA binding and provide evidence that the stimulation is not dependent upon inhibition of a phosphatase. A model is proposed in which NF-E2 exists in nuclear extracts in two distinct forms. Vanadate interacts directly with one of these forms to facilitate the formation of an active NF-E2 complex. As a significant amount of NF-E2 is incompetent to bind to DNA in the absence of vanadate, there appears to be a pool of functionally inactive NF-E2.

## EXPERIMENTAL PROCEDURES

**Cell Culture.** The human erythroleukemia cell line K562 was propagated in IMEM medium (Biofluids), containing 10% fetal calf serum (Gibco-BRL), 2 mM glutamine, and gentamycin (25 µg/mL). The murine erythroleukemia cell line MEL was propagated in DMEM medium (Biofluids), containing 10% calf serum (Gibco-BRL), 4 mM glutamine, penicillin (100 units/mL), and streptomycin (100 µg/mL). Both cell lines were grown in a humidified incubator at 37 °C, in the presence of 5% carbon dioxide. In certain experiments, K562 cells were induced with 20 µM hemin (Sigma) for 72 h. MEL cells were always induced with 1.5% dimethyl sulfoxide for 72 h. K562 cells were used for the initial experiments. After determining that NF-E2 from K562 and MEL cells behaves similarly, MEL cells were used for the remaining experiments, as they are less expensive to propagate.

**Nuclear Isolation and Preparation of Nuclear Extracts.** Nuclear extracts were prepared from K562 and MEL cells as described previously (Bresnick & Felsenfeld, 1993). Cells were harvested by centrifugation for 10 min at 150g. Cells were washed once in 50 volumes of ice-cold phosphate-

buffered saline and resuspended in 2 volumes of 10 mM Tris-HCl (pH 7.5), 10 mM NaCl, 3 mM MgCl<sub>2</sub>, 0.2% Nonidet P-40, and 10 mM DTT. Cells were lysed with 10 gentle strokes of a type B Dounce homogenizer. Nuclei were collected by centrifugation for 5 min at 600g. Nuclei were washed by gently resuspending in 2 volumes of 10 mM Tris-HCl (pH 7.5), 10 mM NaCl, 3 mM MgCl<sub>2</sub>, and 10 mM DTT and then collected by centrifugation for 3 min at 600g. Nuclei were immediately resuspended in an equal volume of 20 mM HEPES (pH 7.5), 25% glycerol, 20 mM KCl, 0.2 mM EDTA, and 10 mM DTT, and 1.33 volumes of the same buffer containing 1.2 M KCl was added dropwise. Nuclei were extracted for 30 min at 4 °C with constant rotation. The suspension was then centrifuged for 30 min at 150000g. The supernatant was dialyzed against 500 mL of 20 mM HEPES (pH 7.5), 20% glycerol, 100 mM KCl, 0.2 mM EDTA, and 10 mM DTT for 1 h at 4 °C. Aliquots were frozen on dry ice and stored at -70 °C. The protein concentration as measured by the Bradford assay, with γ-globulin as a standard, ranged from 4 to 12 mg/mL. Leupeptin (0.02 mg/mL) and phenylmethanesulfonyl fluoride (0.2 mM) were included in all buffers.

A previously unthawed aliquot of extract was used in each experiment. It was found that repeated rounds of freeze–thawing can result in degradation of NF-E2 and low binding activity.

**Electrophoretic Mobility Shift Assay.** DNA binding reactions were performed as described previously (Bresnick & Felsenfeld, 1993, 1994b). Aliquots of unfractionated or fractionated nuclear extract were incubated in 10 mM HEPES (pH 7.8), 60 mM KCl, 10% glycerol, 1 mM MgCl<sub>2</sub>, 6 mM DTT, 2 µg of poly(dI-dC), 1 µg of BSA, and 20 fmol of end-labeled, double-stranded oligonucleotide, in a final volume of 15 µL for 20 min at 25 °C. For the experiment of Figure 3, the pH of the DNA binding buffer was varied. Samples were resolved on 6.5% nondenaturing polyacrylamide gels in 0.75× Tris-acetate/EDTA running buffer (30 mM Tris-acetate, 0.75 mM EDTA, pH 8.0) at 200 V for 1.5–2 h at 4 °C. Gels were preelectrophoresed for at least 10 min at 4 °C. Similar results were obtained by resolving protein–DNA complexes on nondenaturing MetaPhor XR (FMC BioProducts) agarose gels. DNA binding activity was quantitated by analyzing gels with a PhosphorImager (Molecular Dynamics).

In the quantitative DNA binding analysis of Figure 6, variable concentrations of radiolabeled DNA were used to estimate equilibrium binding constants, as described previously (Bresnick & Felsenfeld, 1993). The amount of protein–DNA complex formed was plotted as a function of the DNA concentration. The hyperbolic binding isotherms were subjected to nonlinear regression analysis with the KaleidaGraph program (Synergy Software) to estimate  $K_D$  and  $B_M$  values.

**Chromatographic Fractionation of NF-E2.** A Superdex 200 HR 10/30 column (Pharmacia) was calibrated several times by applying protein standards (5 µL each of a 10 mg/mL solution, diluted to 200 µL with equilibration buffer) and eluting with equilibration buffer. Standard proteins were detected by measuring the absorbance at 280 nm with an on-line absorbance detector. The void volume ( $V_0$ ) was determined by measuring the eluted volume ( $V_e$ ) with blue dextran. The  $V_e$  for protein standards and the  $V_0$  were used to calculate  $K_{av}$  using the equation  $K_{av} = (V_e - V_0)/(V_t -$

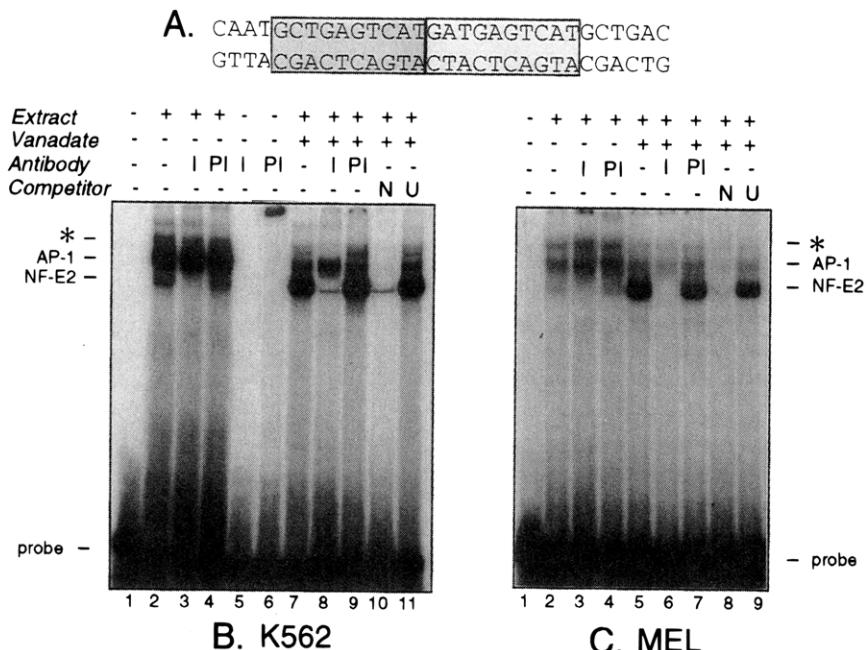


FIGURE 1: Activation of NF-E2 DNA binding by vanadate. Nuclear extracts from K562 or MEL cells were incubated with or without vanadate, followed by either buffer or preimmune (PI) or anti-NF-E2 antibodies (I) for 10 min at 25 °C. DNA binding was assayed by EMSA with 20 fmol of a <sup>32</sup>P-labeled oligonucleotide (panel A), containing tandem NF-E2 sites for 20 min at 25 °C. A 50-fold molar excess of unlabeled NF-E2 (N) or USF (U) oligonucleotide was included in reactions to assess the specificity of binding. Protein–DNA complexes were resolved on a 6.5% nondenaturing polyacrylamide gel. (B) EMSA with K562 nuclear extract. (C) EMSA with MEL nuclear extract. The positions of unbound probe and complexes formed by NF-E2 and AP-1 binding are indicated. The asterisk indicates a higher-order complex formed by occupancy of both NF-E2 sites.

$V_0$ ).  $K_{av}$  values were plotted against the appropriate Stokes' radii ( $R_s$ ) to obtain a linear calibration plot that was used to determine the  $R_s$  for NF-E2.

MEL nuclear extract (200  $\mu$ L) was subjected to centrifugation for 1 min at 18700g at 4 °C. The clarified extract was fractionated on the Superdex 200 HR column, equilibrated in 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 5% glycerol, 0.2 mM EDTA, and 5 mM DTT at 4 °C. In the experiment of Figure 7B, vanadate (10 mM) was included in equilibration and elution buffers. Proteins were eluted at a flow rate of 0.5 mL/min, and 0.5 mL fractions were collected. Aliquots of fractions (5  $\mu$ L) were assayed for NF-E2 activity by EMSA with an end-labeled oligonucleotide, containing a single NF-E2 binding site.

Ion exchange chromatography was also used to fractionate the MEL nuclear extract. The extract was subjected to centrifugation for 1 min at 18700g at 4 °C. The clarified extract (800  $\mu$ L) was fractionated on Resource-S (1 mL) cation exchange or Q-Sepharose fast-flow anion exchange (2 mL) columns (Pharmacia). The Resource-S column was equilibrated in 20 mM HEPES (pH 7.2), 50 mM NaCl, 5% glycerol, 0.2 mM EDTA, and 5 mM DTT. The Q-Sepharose column was equilibrated in 20 mM Tris (pH 7.5), 50 mM NaCl, 5% glycerol, 0.2 mM EDTA, and 5 mM DTT. Proteins were resolved with a 50–450 mM NaCl gradient in equilibration buffer at a flow rate of 1 mL/min, and 0.4 mL fractions were collected. Aliquots of fractions (3  $\mu$ L) were assayed for NF-E2 activity by EMSA as described above.

## RESULTS

*Formation of an NF-E2–DNA Complex Is Enhanced by Vanadate.* During the course of experiments to examine the assembly of protein–DNA complexes on HS2, we discov-

ered that vanadate strongly increases the DNA binding activity of NF-E2 in crude nuclear extracts. Figure 1 shows an EMSA examining the binding of proteins in nuclear extracts from human K562 (panel B) and mouse MEL (panel C) erythroleukemia cells to an oligonucleotide from HS2, which spans the tandem NF-E2 sites (panel A). Three major protein–DNA complexes were observed (lane 2). The formation of the lower complex was inhibited by preincubating the extract with a polyclonal antibody against the p45 component of NF-E2 (lane 3), but not a preimmune antibody (lane 4). The middle and upper complexes were not affected by either antibody. The anti-p45 antibody was produced against purified recombinant p45 and has been shown by Western blot analysis to react specifically with p45 (Andrews *et al.*, 1993). The antibody does not react with the ubiquitous transcription factor AP-1, which also binds to NF-E2 sites (Ney *et al.*, 1990). Based on the antibody inhibition results, and comparison with previous work (Solomon *et al.*, 1993; Andrews *et al.*, 1993; Ney *et al.*, 1993), we conclude that the lower and middle complexes represent NF-E2 and AP-1, respectively.

As the DNA binding assays were performed with a stoichiometric excess of DNA probe, the predominant protein–DNA complexes observed represent occupancy of only one of the two sites on the oligonucleotide. However, the weak upper complex (asterisk, Figure 1A) appears to represent AP-1 bound to both NF-E2 sites. This complex is not affected by the p45 antibody and is not seen with an oligonucleotide that contains a single NF-E2 site (see Figure 3).

A very different pattern of bands was observed when 10 mM vanadate was included in the binding assay (lanes 7 and 5 of panels B and C, respectively). The amount of NF-E2 complex increased considerably, while the AP-1 complex

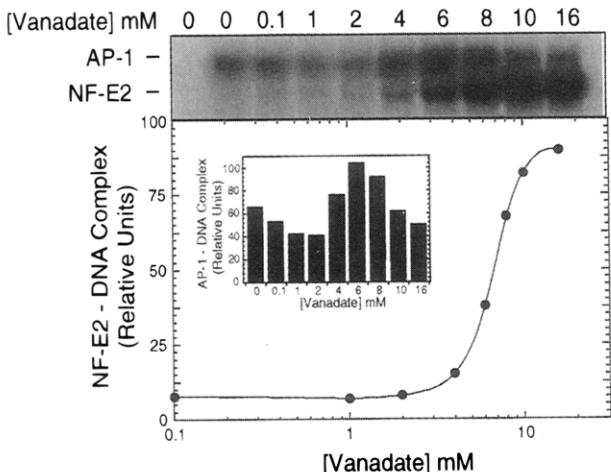


FIGURE 2: Concentration dependence for activation of NF-E2 DNA binding by vanadate. Nuclear extracts from K562 cells were incubated with 0–16 mM vanadate. DNA binding was assayed by EMSA with 20 fmol of a  $^{32}$ P-labeled oligonucleotide, containing tandem NF-E2 sites. Protein–DNA complexes were resolved on a 6.5% nondenaturing polyacrylamide gel. The positions of complexes formed by NF-E2 and AP-1 binding are indicated. The gel was quantitated with a PhosphorImager to determine the amount of NF-E2 and AP-1 complex formed. The amount of each complex is expressed in relative units. The inset graph shows AP-1–DNA complex formation as a function of vanadate concentration.

decreased to a lesser extent. The formation of both complexes was prevented by a 50-fold molar excess of the unlabeled tandem NF-E2 oligonucleotide, but not an unrelated oligonucleotide, containing a binding site for the USF transcription factor (Figure 1B, lanes 10 and 11; Figure 1C, lanes 8 and 9). The anti-p45 antibody was used to determine if the strong band, with an identical mobility as the NF-E2–DNA complex, represents NF-E2 bound to the oligonucleotide. The anti-p45 antibody prevented formation of the lower complex (Figure 1B and 1C, lanes 8 and 6, respectively), while the preimmune antibody had no effect (Figure 1B and 1C, lanes 9 and 7, respectively), similar to the results in the absence of vanadate. The formation of the weak upper complex with the K562 extract was partially inhibited by the p45 antibody (Figure 1B, lane 8). This complex may result from occupancy of both sites by NF-E2. Thus, inclusion of 10 mM vanadate in the binding assay strongly increases the formation of the NF-E2–DNA complex. The stimulatory effect of vanadate is consistently observed with undialyzed and dialyzed extracts, which are assayed immediately after preparation, or subsequent to freezing and storage at  $-70^{\circ}\text{C}$ .

The concentration dependence for vanadate to stimulate NF-E2 DNA binding was determined. DNA binding was assayed by EMSA with K562 nuclear extract and the oligonucleotide containing tandem NF-E2 binding sites (see Figure 1A). As shown in Figure 2, concentrations of vanadate between 2 and 16 mM progressively increased the amount of the NF-E2–DNA complex. In contrast, the AP-1–DNA complex was not reproducibly affected by vanadate and varied somewhat between assays.

In the initial experiments, a sodium orthovanadate solution in water (150 mM) was used for additions to DNA binding reactions. The pH of the concentrated vanadate solution was 11.4, consistent with the known behavior of orthovanadate (Willsky, 1990). When vanadate was added to the DNA binding reaction, at a final concentration of 10 mM, the pH

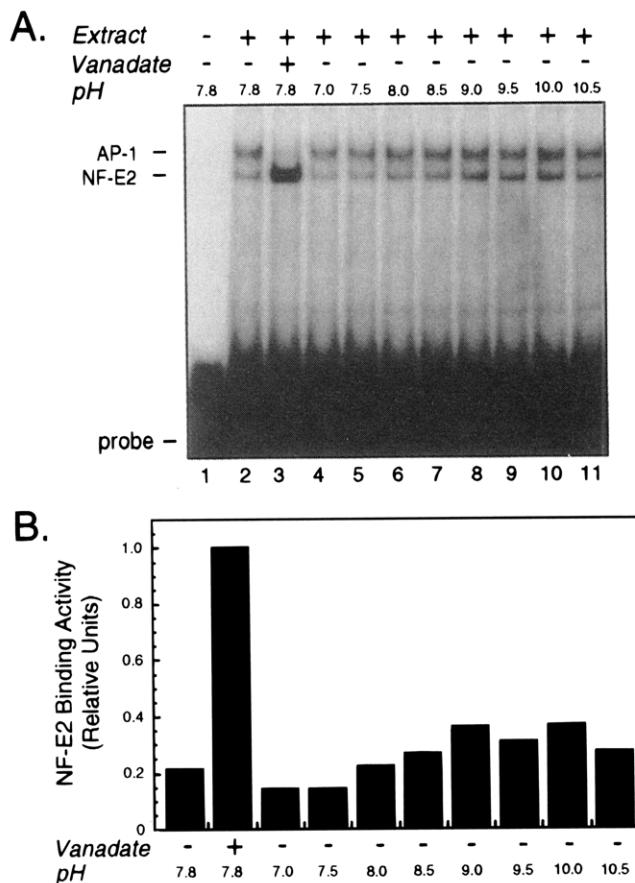


FIGURE 3: pH dependence for NF-E2 DNA binding. Nuclear extracts from MEL cells were incubated in buffers of varying pH, in the absence or presence of 10 mM vanadate. (A) DNA binding was assayed by EMSA with 20 fmol of a  $^{32}$ P-labeled oligonucleotide, containing a single NF-E2 binding site (5'-ACCTGTGCT-GACTCACTGGAG). Protein–DNA complexes were resolved on a 6.5% nondenaturing polyacrylamide gel. The positions of complexes formed by NF-E2 and AP-1 binding are indicated. (B) The gel was quantitated with a PhosphorImager to determine the amount of NF-E2–DNA complex formed. The amount of complex is expressed in relative units.

increased to approximately 9.7. This raised the issue as to whether the stimulation of NF-E2 binding is due to the pH change, or the presence of vanadate. Analysis of the pH dependence of NF-E2 binding, in the absence of vanadate, revealed only small effects on binding between pH 7.0 and 10.5 (Figure 3). DNA binding was assayed by EMSA with an oligonucleotide containing a single NF-E2 site. The amount of binding, in the presence of vanadate, was considerably higher than the binding under optimal pH conditions. Thus, the vanadate-induced increase in pH is not responsible for the enhancement of NF-E2 DNA binding.

The stimulatory effect of vanadate on NF-E2 binding appears to require an elevated pH. A vanadate stock solution (150 mM), prepared in 10 mM HEPES and then adjusted to pH 7.8, does not stimulate NF-E2 binding. In addition, binding assays were performed with 40 mM HEPES buffer (pH 7.0), which maintains the pH of the assay at 8.0, when the vanadate/water solution is added to a final concentration of 10 mM vanadate. Vanadate did not stimulate NF-E2 binding under these conditions (data not shown). Based upon the known chemistry of vanadate (Willsky, 1990) (see Discussion), it is likely that the active species is formed at high pH.

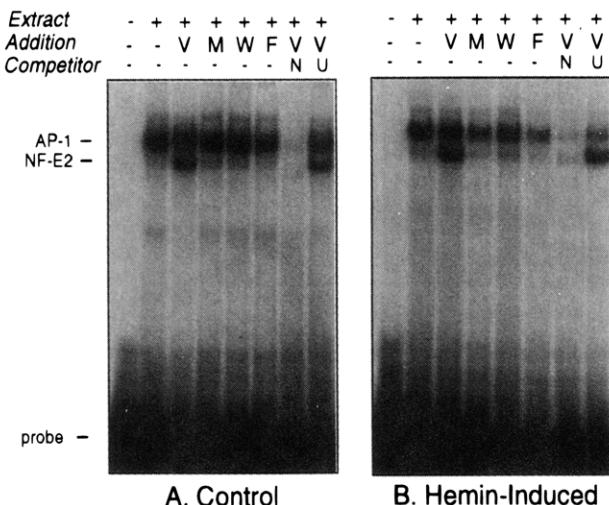


FIGURE 4: Oxyanion specificity for the activation of NF-E2 DNA binding. Nuclear extracts from untreated (A) or hemin-induced (B) K562 cells were incubated with buffer, 10 mM sodium vanadate (V), 10 mM sodium molybdate (M), 10 mM sodium tungstate (W), or 20 mM sodium fluoride (F). DNA binding was assayed by EMSA with 20 fmol of a  $^{32}$ P-labeled oligonucleotide, containing tandem NF-E2 sites. A 50-fold molar excess of unlabeled NF-E2 (N) or USF (U) oligonucleotide was included in the reaction to assess the specificity of binding. Protein-DNA complexes were resolved on a 6.5% nondenaturing polyacrylamide gel. The positions of unbound probe and complexes formed by NF-E2 and AP-1 binding are indicated.

Vanadate and the other oxyanions molybdate and tungstate are known to influence the activity of a variety of proteins (Willsky, 1990). For example, they stabilize certain protein-protein interactions, such as the association of the glucocorticoid receptor and pp60v-src with hsp90 (Hutchison *et al.*, 1992). The oxyanions are also inhibitors of protein phosphatases (Gresser & Tracy, 1990). Vanadate is commonly used as an inhibitor of tyrosine phosphatases. The relative efficacy of vanadate, molybdate, and tungstate to inhibit tyrosine versus serine/threonine phosphatases differs (Zhao *et al.*, 1993). We initially hypothesized that there may be a phosphorylation requirement for NF-E2 DNA binding. Thus, the stimulatory effect of vanadate on NF-E2 DNA binding might result from inhibition of a protein phosphatase.

To assess the oxyanion specificity of the vanadate effect on NF-E2 DNA binding, we asked if molybdate, tungstate, and an inhibitor of serine/threonine phosphatases, sodium fluoride, also increase NF-E2 DNA binding. Nuclear extracts were prepared from control and hemin-treated K562 cells. The K562 extracts used in Figures 1 and 2 were also from hemin-treated cells. It has been reported that extracts from hemin-treated cells contain slightly higher levels of NF-E2 DNA binding activity (Solomon *et al.*, 1993). Hemin is known to induce K562 cells to develop a more mature phenotype, characterized by higher levels of hemoglobin synthesis (Dean *et al.*, 1981).

Extracts from untreated (Figure 4A) and hemin-treated K562 cells (Figure 4B) were incubated with the oxyanions or fluoride. DNA binding activity was assayed by EMSA, with the tandem NF-E2 binding site oligonucleotide. In the absence of vanadate, the NF-E2 binding activity was low in control and hemin-treated extracts. Vanadate, but not the other oxyanions or fluoride, strongly increased the formation of the NF-E2-DNA complex with both extracts.

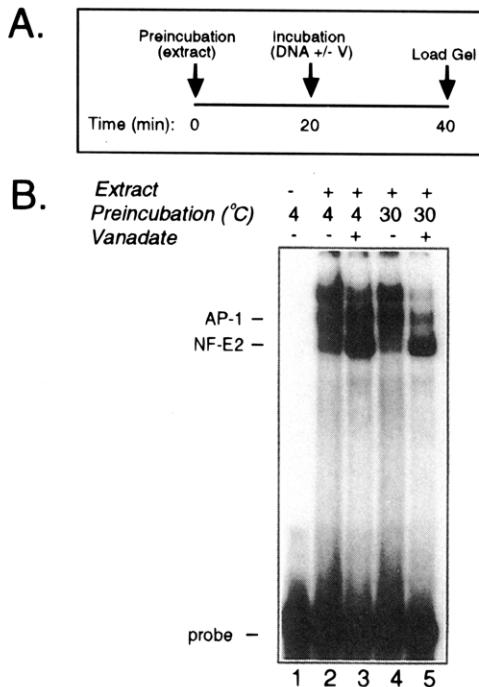


FIGURE 5: Preincubation of nuclear extract at elevated temperature does not preclude the activation of NF-E2 DNA binding by vanadate. Nuclear extract from K562 cells was preincubated for 20 min at 4 or 30 °C. A  $^{32}$ P-labeled oligonucleotide, containing tandem NF-E2 sites (20 fmol), was then added and incubated for 20 min at 25 °C, in the absence or presence of vanadate. Protein-DNA complexes were resolved on a 6.5% nondenaturing polyacrylamide gel. The positions of unbound probe and complexes formed by NF-E2 and AP-1 are indicated.

*Evidence That Stimulation of NF-E2 DNA Binding by Vanadate Is Not Mediated by Inhibition of a Phosphatase.* We further explored the possibility that the stimulatory effect of vanadate on NF-E2 DNA binding might involve inhibition of a protein phosphatase. A reasonable model would be that a phosphatase acts on one of the components of NF-E2 when the extracts are incubated at 25 °C, during the DNA binding reaction. If phosphorylation is required for generation of the active form of NF-E2, or for the DNA binding reaction itself, vanadate could potentially increase DNA binding by preventing dephosphorylation of the relevant component. To test this possibility, we reasoned that incubation of the nuclear extract at elevated temperature, in the absence of vanadate, should allow the phosphatase to inactivate binding. Thus, if vanadate is added after the preincubation, it should not stimulate binding. On the other hand, if vanadate has a direct positive effect on DNA binding, there may be a requirement for vanadate to be present during the DNA binding reaction. It is unlikely that kinases are active during the incubations at 25 °C, as dialyzed nuclear extracts should not contain significant amounts of ATP.

The K562 nuclear extract was divided into two aliquots and incubated for 20 min at 4 or 30 °C. The extracts were then incubated at 25 °C, in the absence or presence of vanadate. DNA binding activity was assayed by EMSA, with the tandem NF-E2 oligonucleotide (Figure 5). Preincubating the extract at 30 °C had no effect on the ability of vanadate to increase NF-E2 DNA binding. As phosphatases would be expected to be active at the elevated temperature, this result suggests that the stimulatory effect of vanadate on NF-E2 DNA binding is not mediated by inhibition of a

protein phosphatase. It is unlikely that kinases are active under these conditions, as the extracts are dialyzed and should not contain significant levels of free ATP. This is further supported by the experiments of Figures 7–9. Fractionation of the extract on gel filtration and ion exchange columns does not result in loss of the stimulatory effect of vanadate on NF-E2 DNA binding. These columns would separate NF-E2 from any residual ATP.

Finally, we asked if another tyrosine phosphatase inhibitor, phenylarsine oxide (Igarashi *et al.*, 1993), also activates NF-E2 DNA binding. The K562 nuclear extract was treated with concentrations of phenylarsine oxide between 0.1 and 80  $\mu$ M. DNA binding was assayed by EMSA as in Figure 4. Phenylarsine oxide had no effect on the formation of the NF-E2–DNA complex (data not shown). Taken together with the preincubation experiment described above, these results are consistent with a model in which vanadate has a direct stimulatory effect on one of the components of the NF-E2 complex.

**Vanadate Stimulates NF-E2 DNA Binding by Recruiting a Non-DNA Binding Pool of NF-E2.** Quantitative DNA binding experiments were performed to determine whether vanadate stimulates the DNA binding activity of NF-E2 by increasing the affinity of DNA binding or by recruiting molecules of NF-E2 that were previously incompetent for DNA binding. EMSA assays were carried out with variable concentrations of labeled oligonucleotide, containing a single NF-E2 binding site, and a constant amount of nuclear extract. This allows one to estimate the affinity of a protein for its DNA binding site ( $K_D$ ) and the number of molecules that are competent to bind to DNA ( $B_M$ ) (Bresnick & Felsenfeld, 1993).

Quantitative EMSA assays were performed with MEL nuclear extract, in the absence or presence of 10 mM vanadate. The gels were analyzed with a PhosphorImager to determine the amount of NF-E2 complex formed. The amount of complex formed (picomolar per microgram of protein) was plotted as a function of the DNA concentration in the assay, yielding a hyperbolic binding isotherm (Figure 6). Nonlinear regression analysis was used to estimate the  $K_D$  and  $B_M$  parameters for NF-E2 binding to the oligonucleotide. The amount of NF-E2–DNA complex formed in the presence of vanadate was much higher than in the absence of vanadate, at all DNA concentrations. The  $K_D$  values of  $3.7 \pm 1.4$  nM and  $2.6 \pm 1.1$  nM (mean  $\pm$  standard error,  $n = 3$ ) for control and vanadate-treated samples, respectively, were not significantly different. In contrast, there was a 9.6-fold lower concentration of active NF-E2 molecules in control versus vanadate-treated extracts ( $B_M$  of  $2.7 \pm 0.6$  and  $26 \pm 5.7$ , respectively (mean  $\pm$  standard error,  $n = 3$ ). These results demonstrate that the stimulatory effect of vanadate on NF-E2 DNA binding is not due to a small increase in the affinity of NF-E2 for the oligonucleotide, but rather involves recruitment of a non-DNA binding pool of NF-E2.

**Gel Filtration Analysis of NF-E2.** One can envision that vanadate modulates NF-E2 DNA binding by facilitating the formation of the active species. It is known that the erythroid-specific component p45 must heterodimerize with the ubiquitous component p18 to form the active heterodimer (Andrews *et al.*, 1994; Igarashi *et al.*, 1994). Two possible mechanisms were considered by which vanadate could facilitate the formation of an active NF-E2 complex. First, if only a small fraction of the active complex is assembled

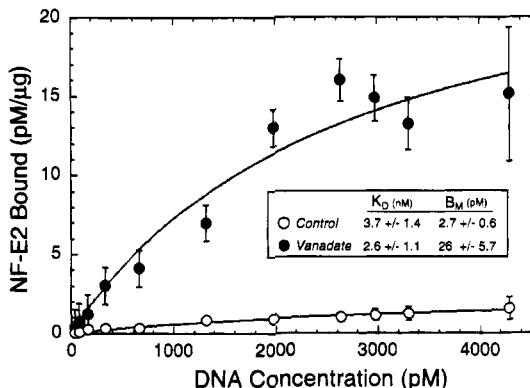


FIGURE 6: Evidence for a non-DNA binding pool of NF-E2. Nuclear extract from MEL cells was incubated with various concentrations of a  $^{32}$ P-labeled oligonucleotide, containing a single NF-E2 site for 20 min at 25 °C. Protein–DNA complexes were resolved on a 6.5% nondenaturing polyacrylamide gel. The dried gel was analyzed with a PhosphorImager to determine the amount of unbound and bound probe. The amount of bound probe was plotted against the total DNA concentration. Nonlinear regression analysis was used to determine the apparent  $K_D$  and  $B_M$  for NF-E2 binding to the oligonucleotide. The  $K_D$  and  $B_M$  values (mean  $\pm$  standard error,  $n = 3$ ) for NF-E2 in control and vanadate-treated extracts are indicated in the inset.

in the nuclear extract, vanadate might increase the assembly or stability of this species. This action could involve stabilizing the p45–p18 heterodimer or, potentially, the association of an unknown component with the heterodimer. Second, the NF-E2 complex may exist in two conformations with an identical subunit composition. By shifting the equilibrium between the inactive and active conformational states, vanadate would stimulate NF-E2 DNA binding.

To begin to address these possibilities, we examined the physical properties of NF-E2 by gel filtration chromatography. If the active species is indeed unstable, and the complex dissociates, the 18-kDa and 45-kDa components should be separable on a gel filtration column. The consequence of resolving the individual components would be loss of inducibility and low DNA binding activity. On the other hand, if NF-E2 exists in active and inactive conformations, with an identical subunit composition, the fractionation would not separate an essential component. Thus, the binding activity would remain inducible. The assumption is that the gel filtration column would be incapable of resolving two conformers with an identical subunit composition.

MEL nuclear extract was fractionated on a Superdex 200 column, in the absence (Figure 7A) or presence (Figure 7B) of 10 mM vanadate. Aliquots of fractions were assayed for NF-E2 activity by EMSA. Figure 7A shows a representative elution profile of NF-E2 DNA binding activity, assayed in the absence or presence of vanadate. Low levels of NF-E2 activity were detected in the absence of vanadate, and vanadate strongly increased the binding activity. Thus, the DNA binding activity of NF-E2 remains inducible after fractionation on the gel filtration column. Figure 7B shows a representative elution profile of NF-E2 DNA binding activity from the Superdex 200 column, which was equilibrated in vanadate. NF-E2 activity was assayed in the presence of vanadate.

By comparing the elution profile of NF-E2 with standard proteins (Figure 7C), it was determined that NF-E2 has a Stokes' radius of  $45 \pm 3.2$  Å (mean  $\pm$  standard error,  $n = 3$ ). The NF-E2 recovered from the column, in the presence

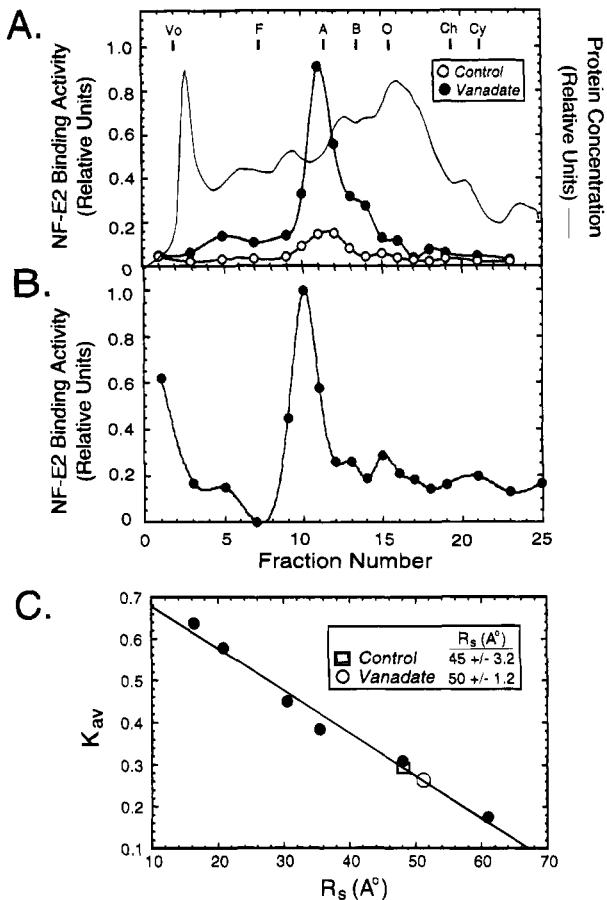


FIGURE 7: Gel filtration analysis of NF-E2 in the absence and presence of vanadate. MEL nuclear extract ( $200 \mu\text{L}$ ) was fractionated by chromatography on a Superdex 200 HR 10/30 column, in the absence (A) or presence of  $10 \text{ mM}$  vanadate (B). Aliquots of fractions ( $5 \mu\text{L}$ ) were assayed for NF-E2 DNA binding activity by EMSA with  $20 \text{ fmol}$  of a  $^{32}\text{P}$ -labeled oligonucleotide, containing a single NF-E2 binding site. In panel A, fractions were assayed in the absence or presence of  $10 \text{ mM}$  vanadate. In panel B, fractions were assayed in the presence of  $10 \text{ mM}$  vanadate. Protein–DNA complexes were resolved on a 6.5% nondenaturing polyacrylamide gel. The dried gel was analyzed with a PhosphorImager to quantitate the amount of NF-E2 complex formed. The relative binding values, in the absence (○) or presence (●) of vanadate, were plotted as a function of fraction number (panels A and B). In panel B, the apparent NF-E2 binding activity in fraction 1 is an artifact, resulting from the positive control in the adjacent lane. The relative protein concentration (absorbance at  $A_{280}$ ) is shown by the solid line (A). The elution positions of standard proteins, of known Stokes' radius ( $R_s$ ), used to calibrate the column are shown at the top:  $V_o$ , void volume; F, ferritin; A, aldolase; B, bovine serum albumin; O, ovalbumin; Ch, chymotrypsinogen; Cy, cytochrome c. (C) The  $K_{av}$  values for the standard proteins indicated above were plotted against the known  $R_s$  values. Linear regression was used to generate the standard curve. The  $K_{av}$  value for NF-E2 was calculated from the elution profile of panel A, and the corresponding  $R_s$  values were determined from the standard curve. The  $K_{av}$  and  $R_s$  values are indicated in the inset.

of vanadate (Figure 7B), has a slightly larger Stokes' radius of  $50 \pm 1.2 \text{ \AA}$  ( $n = 3$ ). These results are inconsistent with a model in which vanadate prevents dissociation of the active NF-E2 heteromer into individual components.

**Resolution of Two Distinct Forms of NF-E2 by Ion Exchange Chromatography.** To further explore the possibility that vanadate activates DNA binding by stabilizing an active conformation of NF-E2, ion exchange chromatography was utilized to fractionate NF-E2. As discussed above, if the active NF-E2 heteromer is unstable and dissociates in

the nuclear extract, chromatographic separation of the individual components should result in loss of DNA binding activity.

Nuclear extracts from MEL cells were fractionated on a Resource-S cation exchange column. Aliquots of fractions were assayed for NF-E2 DNA binding activity, in the absence or presence of vanadate. An oligonucleotide containing a single binding site for NF-E2 was used for the EMSA assays. Figure 8A shows a representative column profile and a summary of NF-E2 binding data from four experiments. NF-E2 activity bound tightly to the column and eluted between fractions 31 and 40. This single chromatographic step separated NF-E2 from at least 78% of the total nuclear protein.

The binding activity of the material applied to the column (0) was strongly induced by vanadate, similar to the NF-E2 activity that eluted in fraction 40. In contrast, the binding activity that eluted between fractions 31 and 37 was only weakly induced by vanadate. Both the strongly- and weakly-inducible DNA binding activities reacted with anti-p45 (Figure 8C, lanes 6 and 8), but not preimmune antibodies (Figure 8C, lanes 5 and 7). Taken together with the result that strongly- and weakly-inducible activities form protein–DNA complexes with identical mobilities on nondenaturing gels, NF-E2 appears to segregate into two distinct populations. As the majority of the binding activity remains strongly inducible after fractionation of the extract on the column, it is likely that the heteromeric NF-E2 complex is quite stable under these conditions.

A small amount of an additional protein–DNA complex, with a slightly slower mobility than the NF-E2 complex, is observed with the crude extract, and the fractions that did not bind to the column (fraction 4). The anti-p45 antibody does not inhibit the formation of this complex (Figure 8C, lane 4). Taken together with the observation that NF-E2 binds tightly to the column, it is likely that a protein distinct from NF-E2 forms the weak complex.

The nuclear extract was also fractionated on a Q-Sepharose anion exchange column. A representative column profile is shown in panels A and B of Figure 9. The NF-E2 activity bound tightly to the column and eluted between fractions 36 and 54. This single chromatographic step separated NF-E2 from 80% of the total nuclear protein.

The binding activity of the material applied to the column (0) was strongly induced by vanadate, similar to the NF-E2 activity that eluted between fractions 36 and 42. In contrast, the binding activity that eluted between fractions 45 and 54 was only weakly induced by vanadate. Formation of the strongly- and weakly-inducible complexes was prevented by the anti-p45 antibody (Figure 9C, lanes 4 and 6), but not the preimmune antibody (Figure 9C, lanes 3 and 5).

An additional protein–DNA complex, with a slightly slower mobility than the NF-E2 complex, was observed with the crude extract, as described above. The formation of this complex was not prevented by the anti-p45 antibody (Figure 9C, lanes 2 and 6). In contrast to the Resource-S column, this activity bound tightly to the Q-Sepharose column and eluted toward the end of the gradient.

As was observed with the Resource-S column, the majority of the NF-E2 activity remained strongly inducible after fractionation of the extract on the column. The NF-E2 segregated into two populations, which were either strongly or weakly induced by vanadate, similar to the Resource-S

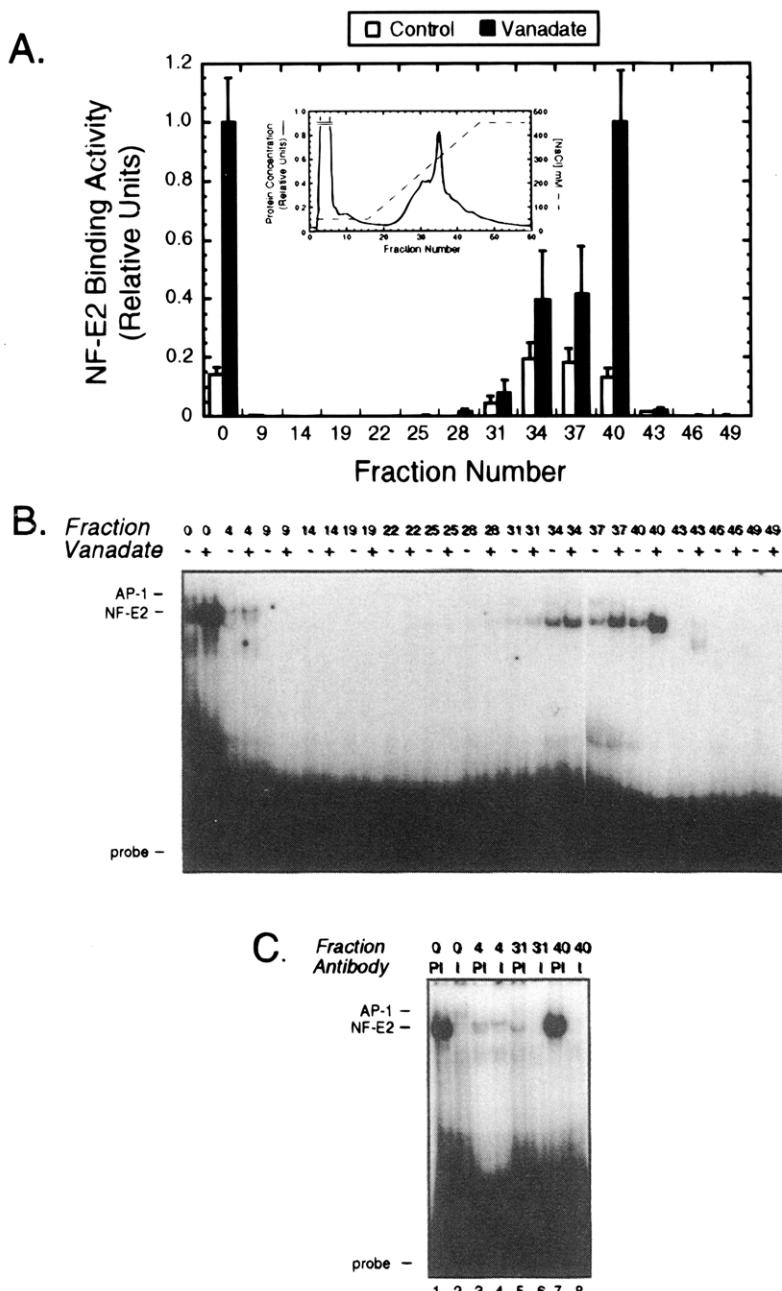


FIGURE 8: Resolution of distinct forms of NF-E2 on a Resource-S column. MEL nuclear extract ( $800 \mu\text{L}$ ) was fractionated on a Resource-S column, and proteins were eluted with a  $50\text{--}450 \text{ mM NaCl}$  gradient (dashed line of inset). Aliquots of fractions ( $3 \mu\text{L}$ ) were assayed for NF-E2 DNA binding activity, in the absence or presence of vanadate, by EMSA with  $20 \text{ fmol}$  of  $^{32}\text{P}$ -labeled oligonucleotide, containing a single binding site for NF-E2. The dried gel was analyzed with a PhosphorImager to quantitate the amount of NF-E2 complex formed. (A) The relative binding values, in the absence (open bars) or presence (solid bars) of vanadate, were plotted against the fraction number (mean  $\pm$  standard error,  $n = 4$ ). Each fractionation used an independent preparation of nuclear extract. The inset shows a plot of the relative protein concentration of the fractions eluting from the column and the NaCl gradient used to elute the bound proteins. (B) EMSA analysis of NF-E2 binding activity in column fractions, in the absence or presence of vanadate. The positions of unbound probe and complexes formed by NF-E2 and AP-1 binding are indicated. (C) Specificity of protein-DNA complexes. Aliquots of fractions were preincubated with anti-p45 or preimmune antibodies and then assayed for NF-E2 DNA binding activity.

column. In contrast to the cation exchange column (Resource-S), in which the strongly-inducible activity eluted at a higher NaCl concentration than the weakly-inducible activity, the strongly-inducible activity eluted at a lower NaCl concentration from the anion exchange column (Q-Sepharose). These results are consistent with distinct charge properties for the strongly- and weakly-inducible forms of NF-E2.

Two experiments were performed to determine if the strongly-inducible NF-E2 activity was stable to further fractionation. First, the strongly-inducible material from the Resource-S column was rechromatographed on the Re-

source-S column. The eluted NF-E2 activity remained strongly inducible (data not shown). Second, the strongly-inducible material from the Resource-S column was rechromatographed on the Q-Sepharose column. The NF-E2 activity eluted also remained strongly inducible (data not shown).

## DISCUSSION

*Stimulation of NF-E2 DNA Binding by Vanadate.* The erythroid-specific transcription factor NF-E2 binds to promoters and enhancers of multiple erythroid-specific genes

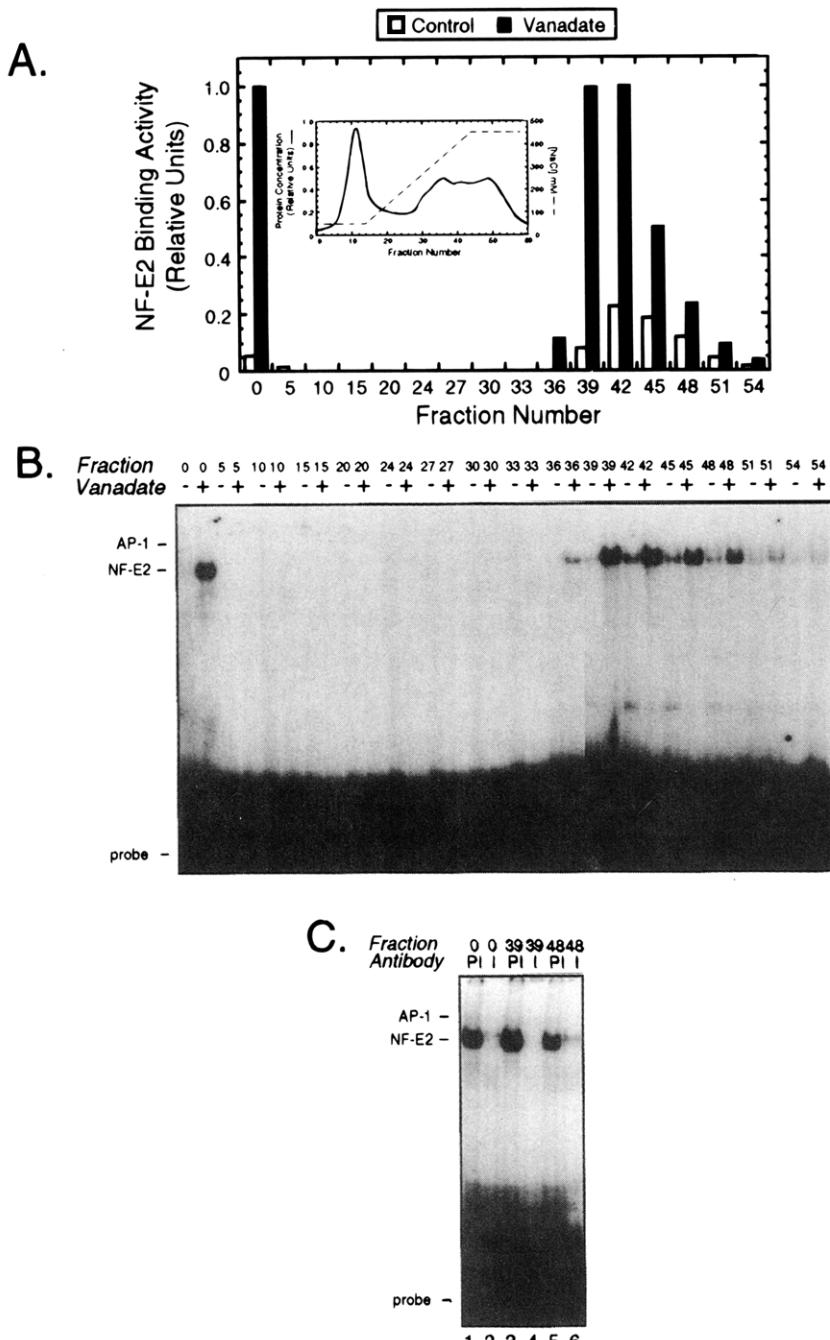


FIGURE 9: Resolution of distinct forms of NF-E2 on a Q-Sepharose column. MEL nuclear extract ( $800 \mu\text{L}$ ) was fractionated on a Q-Sepharose column. Proteins were eluted with a  $50-450 \text{ mM NaCl}$  gradient (dashed line of inset). Aliquots of fractions ( $3 \mu\text{L}$ ) were assayed for NF-E2 DNA binding activity, in the absence or presence of vanadate, by EMSA with  $20 \text{ fmol}$  of  $^{32}\text{P}$ -labeled oligonucleotide, containing a single binding site for NF-E2. The dried gel was analyzed with a PhosphorImager to quantitate the amount of NF-E2 complex formed. (A) The relative binding values, in the absence (open bars) or presence (solid bars) of vanadate, were plotted against the fraction number. The inset shows a plot of the relative protein concentration of the fractions eluting from the column and the NaCl gradient used to elute the bound proteins. (B) EMSA analysis of NF-E2 binding activity in column fractions, in the absence or presence of vanadate. The positions of unbound probe and complexes formed by NF-E2 and AP-1 binding are indicated. (C) Specificity of protein-DNA complexes. Aliquots of fractions were preincubated with anti-p45 or preimmune antibodies and then assayed for NF-E2 DNA binding activity.

(Andrews *et al.*, 1993). NF-E2 sites are also found within the  $\beta$ -globin LCR (Tuan *et al.*, 1989; Ney *et al.*, 1990; Talbot *et al.*, 1990), which controls the transcriptional activity and replication timing of the entire  $\beta$ -globin domain (Forrester *et al.*, 1990). Two observations support a role for NF-E2 in activating globin genes in MEL cells (Kotkow & Orkin, 1995). First, expression of a dominant-negative mutant of p18 inhibits globin gene expression. Second, introduction of a tethered NF-E2/p18 molecule into CB3 cells, a variant of MEL cells which do not express p45, increases globin

gene expression. Surprisingly, deletion of the p45 gene in mice by homologous recombination had no obvious effect on globin gene expression, suggesting that redundant activating factors may exist. These factors may include the LCR-F1 (Caterina *et al.*, 1994b), Nrf-1 (Chan *et al.*, 1993), and Nrf-2 (Moi *et al.*, 1994) proteins, which interact with a similar DNA binding site as NF-E2. A major phenotype of the NF-E2 $^{-/-}$  mice was the lack of circulating platelets, demonstrating a crucial role for NF-E2 in megakaryocyte maturation and platelet production (Shivdasani *et al.*, 1995).

The NF-E2 DNA binding activity in nuclear extracts from erythroid cell lines has been observed to be very low by ourselves and others (for example, Solomon *et al.*, 1993; Ney *et al.*, 1993). In this paper, a strong stimulatory effect of vanadate on NF-E2 DNA binding is described. Vanadate influenced the activity of NF-E2 in every system tested—uninduced and induced human K562 and mouse MEL cells. Even after resolving the nuclear extract sequentially on two high-resolution ion exchange columns, the majority of NF-E2 DNA binding activity remains inducible by vanadate.

**Mechanism of the Vanadate Effect.** Vanadate is commonly used as an inhibitor of tyrosine phosphatases (Gresser & Tracey, 1990). Several observations argue against a role for phosphatases in mediating the vanadate effect. First, the active vanadate concentrations (see Figure 2) are higher than the micromolar concentrations typically used to inhibit tyrosine phosphatases. Second, another tyrosine phosphatase inhibitor, phenylarsine oxide, has no effect on NF-E2 binding. Third, preincubation of the extract at elevated temperature does not prevent the stimulatory effect of vanadate. The preincubation would be expected to abrogate the vanadate effect, if a phosphatase converts NF-E2 to a non-DNA binding form (see Figure 5).

Vanadium (V) exists as a heterogeneous mixture of mono-, di-, and tetrameric forms in aqueous solution (Willsky, 1990; Butler, 1990). The relative abundance of the various forms depends upon the vanadate concentration, the pH, and the redox state of the solution. The predominant form at alkaline pH is V<sub>2</sub>, whereas the major form between pH 6 and 8 is V<sub>4</sub>. Under acidic conditions, the major form is decavanadate (V<sub>10</sub>), which is characterized by an orange-yellow color.

In the experiments described herein, a sodium orthovanadate solution in water (150 mM) was used for the additions to DNA binding reactions. As noted under Results, this increased the pH of the reaction. Analysis of the pH dependence of NF-E2 binding, in the absence of vanadate, revealed only small effects on binding (Figure 3). The vanadate-induced increase in pH, therefore, is not responsible for the enhancement of NF-E2 DNA binding. However, the stimulatory effect of vanadate on NF-E2 binding appears to require an elevated pH, as a vanadate stock solution (150 mM), prepared with a pH 7.8 HEPES buffer, does not stimulate NF-E2 binding. This buffered vanadate solution had an orange-yellow color, suggesting that V<sub>10</sub> was present. It is unlikely that V<sub>10</sub> is the active form, as it is predominant at low pH. Based upon the avidity of vanadium (V) forms for a variety of ligands (Gressler & Tracey, 1990), and the results described above, it is reasonable to suggest that vanadate interacts directly with one or more components of the NF-E2 complex to generate the active DNA binding state.

The gel filtration studies (see Figure 7) argue against a role for vanadate in preventing dissociation of the active NF-E2 heteromer. The Stokes' radius of NF-E2, in the absence and presence of vanadate, is quite similar (45 and 50 Å, respectively). Thus, the NF-E2 heteromer does not appear to be unstable under these conditions. The Stokes' radius values are larger than would be expected for a *globular* heterodimer with 45-kDa and 18-kDa subunits. However, they are similar to another leucine zipper-containing transcription factor, USF, which is an *elongated* 68-kDa homodimer, with a Stokes' radius of 44 Å (Bresnick & Felsenfeld, 1994b).

A trivial explanation for the vanadate effect is that NF-E2 may be susceptible to proteolysis, and vanadate stabilizes NF-E2 against proteolysis. Two results are inconsistent with this possibility. First, the extract can be preincubated at elevated temperature, in which proteases would be active. Once proteolysis has occurred, vanadate should not stimulate binding. However, vanadate is equally effective at stimulating binding when added before or after a preincubation (Figure 5). Second, if vanadate increases binding by inhibiting NF-E2 proteolysis, the vanadate effect should be lost upon purification of NF-E2. The stimulatory effect of vanadate persists even after NF-E2 has been fractionated sequentially over two high resolution ion exchange columns. The fractionation experiments on ion exchange columns also argue against the trivial possibility that DNA fragments, potentially generated during preparation of the extract, are bound to NF-E2 and displaced by vanadate.

**Evidence for Distinct DNA Binding Forms of NF-E2.** The quantitative DNA binding experiment of Figure 6 demonstrates that the stimulation of DNA binding by vanadate is due to an increase in the number of NF-E2 molecules that are competent for DNA binding, rather than a small change in the affinity of binding. It is not clear whether the molecules that are recruited to bind with high affinity completely lack DNA binding activity or if the binding affinity is too low to measure by EMSA assay. Nevertheless, there appears to be two forms of NF-E2 in the nuclear extract with distinct DNA binding properties.

The experiments of Figures 8 and 9 provide further evidence for distinct DNA binding forms of NF-E2. The NF-E2 DNA binding activity that eluted from Resource-S and Q-Sepharose columns was not uniformly responsive to vanadate. With the Resource-S column, the activity that eluted first was only slightly affected by vanadate. In contrast, the activity that eluted at a higher ionic strength was strongly stimulated by vanadate. The opposite chromatographic behavior was observed with the Q-Sepharose column. The activity that eluted first was strongly stimulated by vanadate, whereas the activity eluting at a higher ionic strength was weakly stimulated by vanadate. The constitutive binding activity, in the absence of vanadate, represents only a small fraction of the total binding, in the presence of vanadate. However, the constitutive binding was indeed high-affinity binding (see Figure 6). These results suggest that two forms of NF-E2 exist in the nuclear extract, which are characterized by distinct charge properties and DNA binding activities. The constitutive binding activity may have a greater overall negative charge, as this material binds tightest to the anion exchange column. The activity that is strongly stimulated by vanadate appears to be more positively charged, as this material binds tightest to the cation exchange column.

One might expect the two forms of NF-E2 to be either inducible or entirely nonresponsive to vanadate. What could be the basis for the partial inducibility? If the chromatographic profiles for the two forms partially overlap, this would result in weak inducibility. For example, if a fraction contains a small amount of the inducible form, mixed with a predominance of the noninducible form, this would yield a weakly-inducible activity.

Based on the gel filtration studies (Figure 7), the distinct forms of NF-E2 may have a similar subunit composition. They potentially could differ in a covalent modification (such

as phosphorylation) and/or conformation. We propose that vanadate directly interacts with NF-E2 to (1) stabilize an active conformation or (2) vanadate mimics a bound phosphate molecule, which is required for the active state. Vanadate has been reported to have both activities in biological systems. Vanadate converts a portion of p53 molecules into a form that is recognized by conformation-specific monoclonal antibodies (Landesman *et al.*, 1994). In addition, vanadate mimics inorganic phosphate in binding to F-actin and stabilizing the actin filament (Combeau & Carlier, 1988).

If there is a phosphorylation requirement for NF-E2 DNA binding, the constitutive binding form may reflect the phosphorylated form, which would be consistent with a greater negative charge. The inducible form may be dephosphorylated, consistent with greater positive charge.

It is not known if NF-E2 is endogenously phosphorylated. It was recently reported that NF-E2 DNA binding activity increases 3.3-fold after treatment of MEL cells for 72 h with the differentiation inducing agent HMBA (Garingo *et al.*, 1995). The HMBA-induced increase in NF-E2 activity did not occur with mutant cells that lack the cAMP-dependent kinase. Evidence was presented that p45 and p18 can be phosphorylated by the cAMP-dependent kinase *in vitro*. However, phosphorylation did not affect NF-E2 DNA binding activity. It was concluded that the elevation of NF-E2 DNA binding activity, during the 72 h incubation with HMBA, requires the cAMP-dependent protein kinase; albeit an indirect mechanism appears to be responsible.

The quantitative DNA binding experiments (Figure 6) estimate the concentration of active NF-E2 molecules in the vanadate-treated nuclear extract to be 26 pM. Assuming a heterodimer of 45-kDa and 18-kDa subunits, this would correspond to approximately 1.6 ng of NF-E2 protein per milliliter of MEL nuclear extract. Our optimized DNA binding assay can detect very small amounts of NF-E2 in a nuclear extract (approximately 160 fg of NF-E2 in 0.1  $\mu$ L of a nuclear extract). Based upon the very low abundance of NF-E2, it may not be possible to directly analyze the protein composition of NF-E2 complexes. The generation of an erythroid cell system that overexpresses NF-E2 subunits will be an important step toward testing the model described above, in which two forms of NF-E2 differ in conformation, but not subunit composition. It should be possible to label p45 with an epitope tag and stably express the marked p45 in K562 or MEL cells. The endogenous complexes could be immunoadsorbed, after metabolic labeling with radiolabeled orthophosphate or methionine and cysteine, to examine the polypeptide composition and the phosphorylation state of the two forms.

The ultimate reconstitution of the active NF-E2 heteromer from recombinant protein will allow one to determine if p45 and p18 are sufficient to generate the active state and for the vanadate stimulation. Attempts to overexpress p45 and p18 in bacteria have not been very successful. To date, only fragments of recombinant p45 have been expressed in bacteria, and p18 appears to have a toxic effect on the bacteria (Andrews *et al.*, 1993).

**Implications for Regulation of Erythroid-Specific Genes.** The assembly of nucleoprotein complexes that regulate gene transcription requires the binding of multiple protein components to clusters of cis-acting elements (Tjian & Maniatis, 1994). Once bound to DNA, it is likely that a series of

protein-protein interactions must occur between the individual components to generate the active element. The results described herein demonstrate that the tissue-specific transcription factor, NF-E2, which binds to the erythroid-specific LCR (Andrews *et al.*, 1993), can exist in two states with distinct DNA binding properties. The presence of a non-DNA binding pool of NF-E2 suggests that the generation of an active NF-E2 heteromeric complex may be a regulated step in the cell. This contrasts with a model in which the active NF-E2 species is assembled immediately upon translation, and simply must bind to DNA to function. Studies with hormone-responsive cells will allow one to ask if the relative distribution of the two NF-E2 forms is modulated by physiologic stimuli that control erythroid cell growth or development.

## ACKNOWLEDGMENT

We are grateful to Dr. Nancy Andrews for generously providing antisera to p45. We thank Drs. Moshe Sadovsky, Jeffrey Hayes, and Sam John for a critical review of the manuscript and Dr. Brent Hamstra for helpful discussions on vanadate chemistry.

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BI951873M