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# A DNA-Bending Protein Interacts with an Essential Upstream Regulatory Element of the Human Embryonic β-Like Globin Gene

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**The mammalian β-like globin gene family has served as an important model system for analysis of tissue- and developmental stage-specific gene regulation. Although the activities of a number of regulatory proteins have been implicated in the erythroid cell-specific transcription of globin genes, the mechanisms that restrict their expression to discrete stages of development are less well understood. We have previously identified a novel regulatory element (PRE II) upstream from the human embryonic β-like globin gene (ε) that synergizes with other sequences to confer tissue- and stage-specific expression on a minimal ε-globin gene promoter in cultured embryonic erythroid cells. Binding of an erythroid nuclear protein (PRE II-binding factor [PREIIBF]) to the PRE II control element is required for promoter activation. Here we report on some of the biochemical properties of PREIIBF, including the characterization of its specificity and affinity for DNA. The embryonic and adult forms of PREIIBF recognize their cognate sequences with identical specificities, supporting our earlier conclusion that they are very similar proteins. PREIIBF binds DNA as a single polypeptide with an $M_r$ of ~80,000 to 85,000 and introduces a bend into the target DNA molecule. These results suggest a mechanism by which PREIIBF may contribute to the regulation of the embryonic β-like globin gene within the context of a complex locus.**

Hemoglobin switching is a complex biological process requiring tight regulation of two large chromosomal loci, α and β, such that individual globin genes are sequentially and exclusively expressed in erythroid cells at the correct developmental stage (reviewed in reference 54). Within the human β-globin locus on chromosome 11, two developmental switches are observed; the embryonic-to-fetal switch occurs during weeks 5 to 6 of gestation, while the fetal-to-adult switch occurs around the time of birth. An accumulating body of evidence suggests that these developmental transitions require interactions between regulatory sequences proximal to each globin gene and sequences within the locus control region (24) located many kilobases upstream (reviewed in references 13, 16, and 19). The molecular details of these interactions are not well understood, but they are thought to be mediated, at least in part, by combinations of lineage-specific, ubiquitous, and stage-specific regulatory proteins. According to this view, protein-protein interactions would facilitate and stabilize DNA looping, thereby bringing specific subdomains of the locus control region into proximity with globin gene promoters and upstream regulatory sequences.

Strong evidence for the presence of tissue- and stage-specific *trans*-acting regulators of globin genes in erythroid cells first came from experiments in heterokaryons, or short-term, multinucleated hybrid cells. These studies demonstrated that the expression of globin genes could be rapidly reprogrammed following fusion of erythroid with nonerythroid cells (5, 6). The erythroid cell contributes to the heterokaryon a number of proteins that activate previously silent globin genes; these include (4, 34) the lineage-specific transcription factor GATA-1 (62), basic helix-loop-helix proteins (39), and presumably several additional regulators (e.g., NF-E2 [2], RBTN2 [66], and EKLF [38, 41, 42]) known to be required for normal erythroid cell development and differentiation.

The apparent plasticity of mouse and human embryonic β-like globin gene expression in erythroid heterokaryons (5, 6) prompted us to examine the mechanism of its regulation in greater detail. Analysis of a series of deletion mutations within the first 849 bp of the human embryonic β-like (ε) globin 5′-flanking region (61) in several of the same erythroid and nonerythroid (HeLa) cell lines used in the earlier heterokaryon studies indicated the presence of multiple positive and negative regulatory elements (59), including a previously identified silencer (9, 25, 47, 65). Deletion of one of the positive regulatory elements (ε-PRE II; simplified in this report to PRE II) preferentially abolished activation of a minimal human ε-globin gene promoter in cultured erythroid cells (K562 and GM979) that express endogenous ε-globin genes but had little or no effect in adult erythroid (MEL) or nonerythroid (HeLa) cells (59). While PRE II had no activity on its own (monomeric or multimerized form), it synergized with other elements located further upstream or downstream to activate the minimal human ε-globin gene promoter (59). A nuclear factor highly enriched in erythroid cells binds specifically within a novel, conserved sequence in PRE II (59, 60). Protein binding to PRE II is required for transcriptional activation of a minimal promoter (60), identifying the PRE II-binding factor (PREIIBF) as a potential regulator of the embryonic β-like globin gene. PREIIBF was shown to have different forms in embryonic and adult erythroid cells (59, 60) which likely result from posttranscriptional modification.

To examine the properties and function of PREIIBF, we have carried out an extensive purification from a human embryonic erythroid cell line (K562). We show here that PRE-

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TABLE 1. Oligonucleotides used in this study

| Oligonucleotide | Sequence[a] |
|---|---|
| For generation of mutations in PRE II | |
| Top strand (wild type, primer 1) | 5′-AAAAGATCTCCCAGTGAGAAGTATAAGCA-3′ |
| Bottom strand (mutated, primer 2) | 5′-CGGGATCCT**AAAGCTGTTAAAACAGAATC**TCACCC-3′[b] |
| For circular permutation assay | |
| Top strand | 5′-AATTCGAGATTCTGTTTTAACAGCTTTG-3′ |
| Bottom strand | 5′-AATTCAAAGCTGTTAAAACAGAATCTCG-3′ |
| For phasing analysis | |
| 0-Phase | |
| Top strand | 5′-CTAGAGCAAAAACGGGCAAAAACGGGCAAAAACGG-3′ |
| Bottom strand | 5′-TCGACCGTTTTTGCCCGTTTTTGCCCGTTTTTGCT-3′ |
| 2-Phase | |
| Top | 5′-CTAGAGCAAAAACGGGCAAAAACGGGCAAAAACGGTG-3′ |
| Bottom | 5′-TCGACACCGTTTTTGCCCGTTTTTGCCCGTTTTTGCT-3′ |
| 3-Phase | |
| Top | 5′-CTAGAGCAAAAACGGGCAAAAACGGGCAAAAACGGTACG-3′ |
| Bottom | 5′-TCGACGTACCGTTTTTGCCCGTTTTTGCCCGTTTTTGCT-3′ |
| 6-Phase | |
| Top | 5′-CTAGAGCAAAAACGGGCAAAAACGGGCAAAAACGGTACTGG-3′ |
| Bottom | 5′-TCGACCAGTACCGTTTTTGCCCGTTTTTGCCCGTTTTTGCT-3′ |
| 8-Phase | |
| Top | 5′-CTAGAGCAAAAACGGGCAAAAACGGGCAAAAACGGTACTGGTG-3′ |
| Bottom | 5′-TCGACACCAGTACCGTTTTTGCCCGTTTTTGCCCGTTTTTGCT-3′ |
| 10-Phase | |
| Top | 5′-CTAGAGCAAAAACGGGCAAAAACGGGCAAAAACGGTACTGGTACG-3′ |
| Bottom | 5′-TCGACGTACCAGTACCGTTTTTGCCCGTTTTTGCCCGTTTTTGCT-3′ |
| PREII.SalI, top strand | 5′-TCGACGAGATTCTGTTTTAACAGCTTTC-3′[c] |
| PREII.XhoI, bottom strand | 5′-TCGAGAAAGCTGTTAAAACAGAATCTCG-3′[c] |
| For preparation of DNA affinity column | |
| MAD32, top strand | 5′-AATTCGAGATTCTGTTTTAACAGCTTTG-3′ |
| MAD33, bottom strand | 5′-A**A**TTCAAAGCTGTTAAAACAGAATCTCG-3′[d] |

[a] Underlined sequences indicate restriction sites (see Materials and Methods).
[b] PRE II sequence is in boldface.
[c] Note that *Sal*I and *Xho*I ends are compatible for ligation.
[d] Nucleotide in boldface represents the alkylamine-tethered dA residue used to couple the DNA to the activated carboxyl support (33).

IIBF is an extremely stable protein of ~80 to 85 kDa and binds DNA as a single polypeptide. Embryonic and adult forms of PREIIBF bind PRE II with identical specificities, consistent with our earlier conclusion (60) that they are very similar proteins. Both forms of PREIIBF are phosphorylated, but this modification alone does not account for the observed mobility differences (59, 60) between the two proteins. By Scatchard analysis, PREIIBF binds to PRE II with a relatively high thermodynamic dissociation constant ($K_d$) of ~14.6 nM, and this binding introduces a bend into the DNA helix. Together with the observation that PRE II interacts synergistically with other regulatory elements, PREIIBF-induced bending suggests a looping mechanism by which this protein might function in activation of the human embryonic β-like globin gene.

## MATERIALS AND METHODS

**Mutagenesis of PRE II and plasmid constructions.** Point mutations were introduced into the PRE II sequence through a chemical synthesis in which phosphoramidites were mixed at a ratio of 19:1 (wild type to non-wild type) at each nucleotide position. Non-wild-type nucleotides were chosen such that a transversion mutation would result at each position: G to T, A to C, T to G, and C to A. Oligonucleotide sequences are shown in Table 1. Primer 1 contained wild-type sequence corresponding to positions −770 to −751; primer 2 contained mutations introduced during chemical synthesis of the sequence corresponding to positions −444 to −418. Oligonucleotides were synthesized on an Applied Biosystems 380B DNA synthesizer and contained *Bgl*II and *Bam*HI restriction sites (underlined in Table 1) to facilitate subcloning of PCR products into a pBSK vector (Stratagene). Wild-type top-strand primer and a mixture of mutated bottom-strand primers were used for PCR amplification of the sequence from −770 to −418 upstream of the human ε-globin gene promoter (61). Following PCR amplification, the products were digested with *Bam*HI and *Bgl*II and ligated into the *Bam*HI site of the pBluescript (pBSK$^+$) phagemid vector (Stratagene). Sequence analysis of miniprep DNA from 61 colonies indicated that there were 27 (44%) wild-type colonies, 19 (31%) colonies with single point mutations, and 11 (18%) colonies with double mutations; the remainder contained multiple mutations.

For electrophoretic mobility shift assays (EMSAs), plasmids containing selected mutations were digested by treatment with *Xba*I (position −533) and *Bam*HI (−418) to generate a probe of 115 bp which was then purified by polyacrylamide gel electrophoresis (PAGE), end labeled with α-$^{32}$P-deoxynucleoside triphosphates (see below), and then purified again by PAGE.

For the circular permutation assays, a double-stranded oligonucleotide containing PRE II was cloned into the *Sma*I site of pCY4 (46). The oligonucleotide sequences are shown in Table 1 and contained *Eco*RI half sites that were filled in by treatment with the Klenow fragment of DNA polymerase prior to ligation into pCY4. To generate circularly permuted versions of PRE II for binding assays, the pCY4-PRE II construct was digested in separate reactions with the appropriate restriction enzymes (see Results), and the PRE II-containing fragment was gel purified.

Phasing plasmids (provided by James Falvo, Harvard University) were prepared by inserting a set of oligonucleotides (Table 1) between the *Xba*I and *Sal*I sites of pBEND2 (31). A double-stranded oligonucleotide containing PRE II (Table 1) was then cloned into the *Sal*I site of each of the phasing vectors modified as described above.

**Cell culture.** Several ε-globin gene-expressing K562 cell sublines were tested for PREIIBF activity and were found to give comparable yields (15). The RA6 subline of K562 cells (49) was used for all subsequent experiments. Cells were grown in 3-liter spinner flasks (Bellco) to a density of $0.8 \times 10^6$ to $1.2 \times 10^6$ cells per ml in RPMI 1640 medium (JRH Bioscience) supplemented with 10% bovine calf serum (HyClone). GM979 cells (7) and MEL cells (semiadherent line 585S [10]) were grown in the same manner as the K562 cells except that Dulbecco's modified Eagle's medium (Gibco-BRL) was used instead of RPMI 1640.

**Preparation of nuclear extracts.** Nuclear extracts were prepared as described by Dignam et al. (12) except that the high-salt (0.42 M KCl) nuclear extract from K562 cells (to be used in subsequent purification steps; see below) was frozen at −70°C without dialyzing against buffer D. MEL and GM979 nuclear extracts were prepared as described by Dignam et al. (12) and were dialyzed against buffer D. All buffers contained 0.5 mM dithiothreitol (DTT; Sigma), 0.5 mM

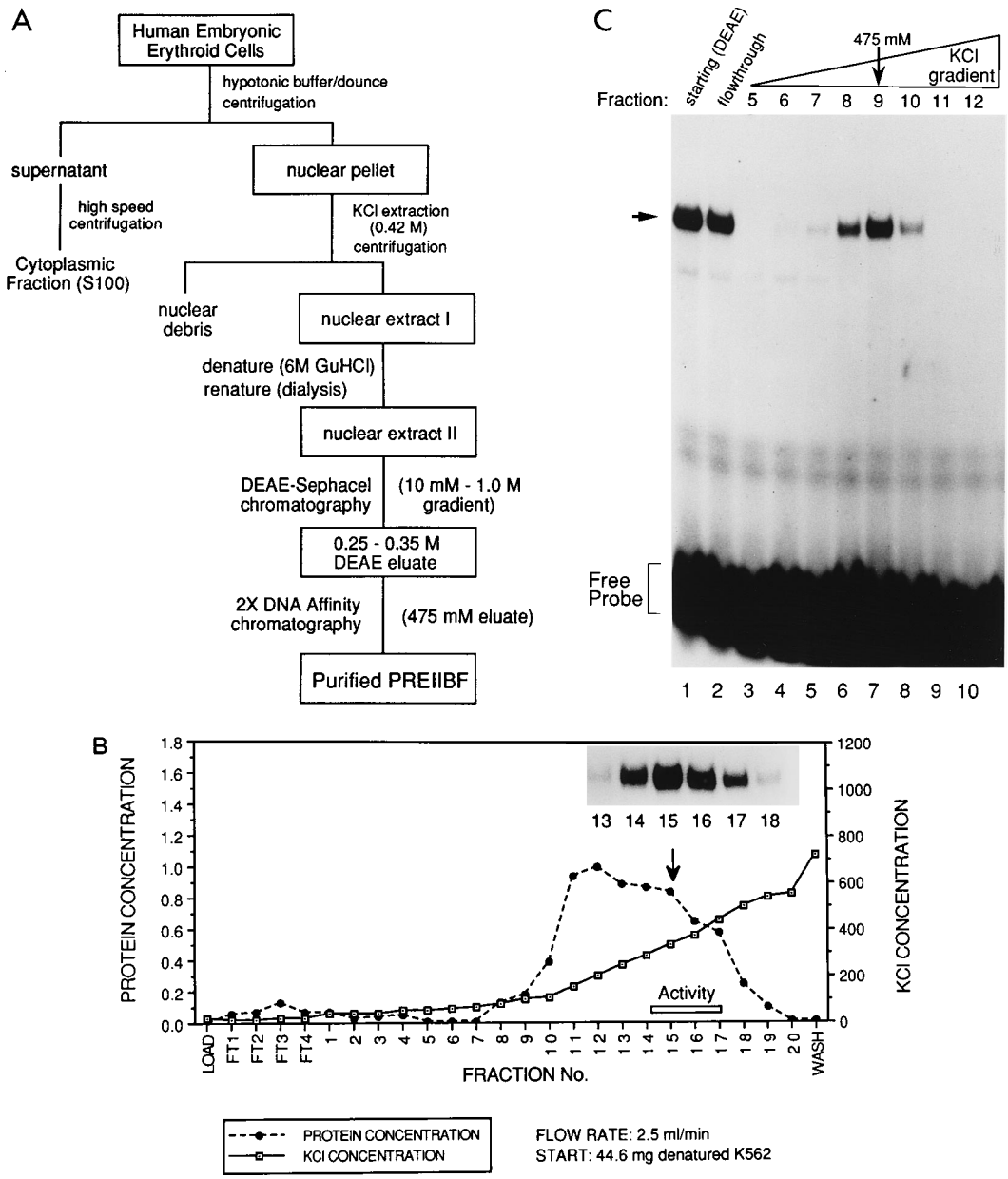


FIG. 1. Purification of PREIIBF from human embryonic erythroid (K562) cells. (A) Purification scheme. The steps in purification of PREIIBF from K562 cells are indicated in simplified form as a flowchart. For details, see Materials and Methods. Nuclear extracts from the murine erythroid lines GM979 and MEL showed behavior similar to that of extracts from the human K562 cells, through DEAE-Sephacel chromatography. (B) DEAE-Sephacel chromatography of PREIIBF. Protein (milligrams per milliliter) and KCl (millimolar) concentrations were plotted against fraction number (5 ml of each) from a column through which NE II (denatured-renatured nuclear extract) was chromatographed. PREIIBF activity eluted predominantly in fractions 14 to 17 (see inset for autoradiograph from EMSA). FT1 to FT4, flowthrough fractions. (C) Site-specific DNA affinity chromatography of PREIIBF. DEAE-Sephacel-purified PREIIBF (lane 1) was fractionated on a DNA affinity column as described in Materials and Methods. Approximately 10% of input PREIIBF bound to the column (see Results), and the flowthrough fraction (lane 2) was generally rechromatographed to recover additional PREIIBF. Specific binding activity eluted at around 475 mM KCl.

phenylmethylsulfonyl fluoride (PMSF; Sigma), and 1 μg each of leupeptin, aprotinin, and pepstatin A (all from Sigma) per ml. We obtained ~30 to 50 mg of nuclear extract per liter of K562 cells.

**Purification of PREIIBF.** PREIIBF was purified from K562 nuclear extracts by ion-exchange and site-specific DNA affinity chromatography following denaturation and renaturation from guanidine hydrochloride (GuHCl) (Fig. 1A). The amount of PREIIBF activity was determined by a quantitative electrophoretic mobility shift assay (EMSA) in which 1 U of binding activity was defined as the amount of protein required to shift 10% of the input probe. Briefly, crude nuclear extract (NE I), containing more than 80% of the cellular binding activity, was denatured by addition of GuHCl to a final concentration of 6 M. The denatured nuclear extract was then renatured by dialysis against buffer $HG_{10}K_{10}$ (20 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [HEPES; pH 7.9], 10% glycerol, 10 mM KCl, 0.5 mM DTT, 0.5 mM PMSF) and centrifuged in a Sorvall SA600 rotor for 10 min at 10,000 rpm to remove undissolved protein. This step resulted in a five- to sixfold purification and removed most of a nonspecific DNA binding activity (complex C in reference 59) found at variable levels in different preparations of crude K562 cell nuclear extract.

The supernatant (NE II) was applied to a column of DEAE-Sephacel (Pharmacia) in buffer $HG_{10}K_{10}$. The column was developed with a 10 mM to 1 M linear gradient of KCl in $HG_{10}K$ (20 mM HEPES [pH 7.9], 10% glycerol, 0.5 mM DTT, 0.5 mM PMSF). Aliquots (0.5 ml) of each fraction (5 ml) were dialyzed into $HG_5K_{50}$ (20 mM HEPES [pH 7.9], 5% glycerol, 50 mM KCl, 0.5 mM DTT, 0.5 mM PMSF) and assayed for PREIIBF activity by EMSA (see below). PREI IBF activity eluted between 200 and 300 mM KCl (Fig. 1B). This step resulted in a total purification of approximately 30-fold and served to remove contaminating

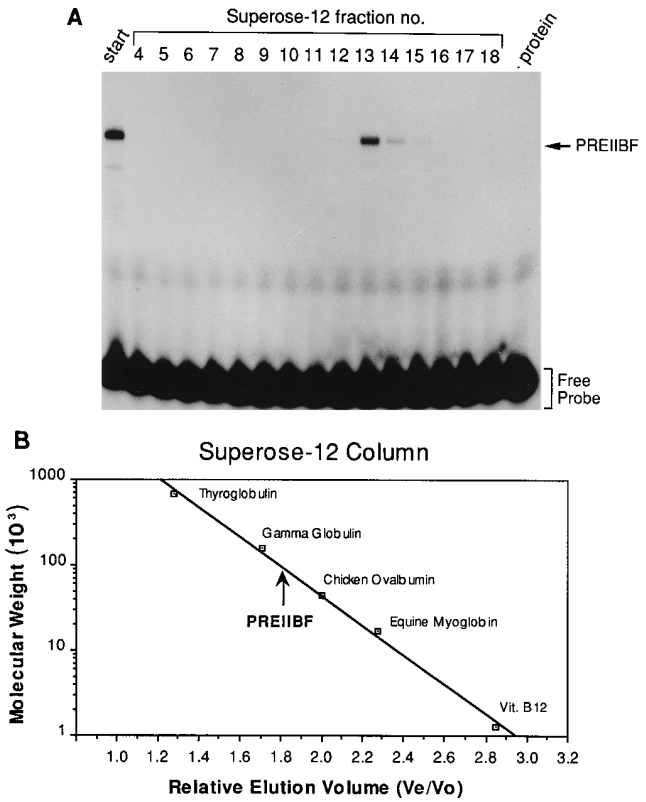


FIG. 2. Gel filtration chromatography of PREIIBF from K562 cells on Superose-12. (A) DNA binding analysis. DEAE-Sephacel-purified PREIIBF (0.25 mg in 0.5 ml) was chromatographed on a Superose-12 gel filtration FPLC column (1 by 30 cm; Pharmacia) as described in Materials and Methods. Protein standards (Bio-Rad) were run under identical conditions. Column fractions were tested for DNA binding activity by EMSA. (B) Determination of $M_r$. Molecular weights for Superose-12 gel filtration standards were plotted on a logarithmic scale against relative elution volume (Ve/Vo, where Ve = elution volume and Vo = void volume). The resulting standard curve was used to determine the $M_r$ for PREIIBF as ~85,000. Protein standards were thyroglobulin (670,000), gamma globulin (158,000), chicken ovalbumin (44,000), equine myoglobin (17,000), and vitamin (Vit.) $B_{12}$ (13,000).

nucleic acids, nucleases, and other proteins. Fractions containing PREIIBF activity were pooled and dialyzed into $HG_5K_{50}$.

For the final steps of the purification, a high-capacity DNA affinity column (33) was used. This column offered an important advantage over conventional oligonucleotide affinity resins (27): its high binding capacity (~100 times greater than for the standard protocol) has allowed the purification of DNA-binding proteins that have eluded purification by standard DNA affinity methods (36, 45). The DNA affinity column was prepared according to the protocol of Larson and Verdine (33), using ligated multimers of a PRE II oligonucleotide (Table 1) containing *Eco*RI half sites. An alkylamine-tethered dA residue was used to couple the DNA to the activated carboxyl support (33). The column contained ~375 nmol of PRE II binding site, with a bed volume of 1 ml.

Calf thymus DNA (Sigma) was added to DEAE-purified PREIIBF to a final concentration of 40 μg/ml, and the sample was loaded onto the PRE II affinity column, which was washed with 5 volumes of $HG_5K_{50}$ buffer and developed with a linear gradient of 50 mM to 1 M KCl in $HG_5K$ buffer. PREIIBF eluted from the DNA affinity column in the 375 to 475 mM KCl fractions (Fig. 1C), resulting in an additional purification of 25- to 30-fold (total purification, 750- to 900-fold). For subsequent rounds of affinity chromatography, the active fractions were diluted in $HG_5$ buffer (20 mM HEPES, 5% glycerol, 0.5 mM DTT, 0.5 mM PMSF) to a KCl concentration of 100 mM. A second round of DNA affinity chromatography resulted in an overall purification of at least 20,000-fold, though these fractions still contained several polypeptides and therefore did not represent homogeneous preparations of PREIIBF (not shown). Partially purified PREIIBF was used in subsequent studies that were not feasible with use of crude nuclear extracts. To date, the largest preparation of K562 cells that we have taken from crude nuclear extract through at least one round of DNA affinity chromatography was 25 liters, with a final yield of ~0.59 mg of protein (5 to 6 pmol of DNA binding activity).

**EMSAs.** Restriction fragments containing PRE II sequences (mutated, circular permutation, and phase-sensing probes) were gel purified and radiolabeled with [α-$^{32}$P]dCTP and/or [α-$^{32}$P]dATP (3,000 Ci/mmol; New England Nuclear) to comparable specific activities, using the Klenow fragment of DNA polymerase. All other EMSAs were performed with the MAD32 and MAD33 oligonucleotide probes described in Table 1. Binding reaction mixtures contained 20,000 cpm of probe (0.1 to 0.5 ng), 10 mM Tris-HCl (pH 7.5), 50 mM KCl, 10% glycerol, 0.5 mM DTT, and 3 μg of poly(dI-dC) (K562, MEL, or GM979 cell NE I) or 0.25 μg of calf thymus DNA (K562 NE II, DEAE- or DNA affinity-purified PREIIBF). For the partially purified preparations of K562 PREIIBF, calf thymus DNA was substituted for poly(dI-dC), with equivalent results. The reactions were analyzed by EMSA using a nondenaturing 4 or 6% polyacrylamide gel at 4°C in 1× Tris-borate-EDTA (90 mM Tris-borate, 2 mM EDTA) containing 5% glycerol. Quantitative analyses of the binding reactions were performed by excising the band containing the protein-DNA complex from the dried gel and counting in a liquid scintillation counter in 2 ml of Econofluor (DuPont-NEN). For calculation of total input DNA, several dilutions of unbound probe were also run on the same gel, excised, and counted.

**Size fractionation of PREIIBF.** DEAE-Sephacel-purified PREIIBF (0.25 mg in 0.5 ml) was chromatographed on a Superose-12 gel filtration fast protein liquid chromatography (FPLC) column (1 by 30 cm; Pharmacia) in $HG_5K_{50}$ buffer at a flow rate of 0.5 ml/min. Gel filtration standards (Bio-Rad) were run under identical conditions.

**Recovery of active PREIIBF from denaturing gels.** DEAE Sephacel-purified PREIIBF (2.5 mg) was concentrated by addition of ammonium sulfate to 80% saturation. Precipitated protein was centrifuged, resuspended in 1× sodium dodecyl sulfate (SDS)-PAGE sample buffer, and then subjected to electrophoresis through a preparative SDS–10% polyacrylamide gel. The gel was sliced horizontally into nine 0.5-cm fractions. Protein was eluted from each fraction, denatured by treatment with 6 M GuHCl essentially as described (45), and renatured by dialysis against $HG_{20}K_{50}$ buffer to remove GuHCl. A portion (20 μl) of the 50-μl eluate was used for the binding assay (EMSA).

**Southwestern (DNA-protein) blot analysis of PREIIBF.** The protocol used for Southwestern blotting was an adaptation of oligonucleotide library screening procedures (52, 64) (details available upon request). The filter was hybridized with $10^6$ cpm of multimerized PRE II probes (MAD32 and MAD33; see above) per ml.

**$K_d$ determination.** DNA affinity-purified PREIIBF (0.58 μg) was incubated with increasing concentrations (0.73 to 73 nM) of radiolabeled PRE II probes (MAD32 and MAD33; Table 1) and analyzed by quantitative EMSA. The $K_d$ was calculated by Scatchard analysis as described in reference 45.

**Circular permutation and phasing analyses.** The circular permutation analysis (see Results) was performed by using a 4% polyacrylamide gel (29.2:0.8 ratio of acrylamide to bisacrylamide). The bend angle α was calculated from the equation $\mu M/\mu E = \cos(k\,\alpha/2)$ (29), where $\mu M/\mu E$ is the ratio of the most slowly migrating complex to the fastest-migrating complex (binding site in the middle or at the end of the DNA probe, respectively) and $k$ is a coefficient calculated to account for electrophoresis conditions (30). Phasing analyses were performed by using 4% gels (29.2:0.8 acrylamide/bisacrylamide) and the phasing probes shown in Table 1. Bend angle was determined by linear interpolation using bent DNA standards (57) (a generous gift from Barbara Nikolajczyk, Brandeis University) as described previously (40). For both circular permutation and phasing analyses, the mobilities of the protein-DNA complexes were corrected for the mobilities of the free probes. DEAE-Sephacel fractions of PREIIBF from K562 cells were used for all of the bending studies. It was not possible to obtain unambiguous results in the circular permutation and phasing analyses by using crude preparations of nuclear extract, because additional (though nonspecific) bands appeared on the gel.

## RESULTS

**PREIIBF binds DNA as a single polypeptide of ~85 kDa.** To facilitate more detailed analysis of the function of PREIIBF, we devised the purification scheme shown in Fig. 1A (see Materials and Methods for details). After two rounds of DNA affinity chromatography (33), PREIIBF was enriched >20,000-fold. For the experiments reported below, we found that it was not necessary to use protein that had been carried through the second round of DNA affinity chromatography (Fig. 1C), and in some cases the DEAE-Sephacel fraction of PREIIBF (Fig. 1B) was adequate.

We had previously estimated the molecular size of PREIIBF as 80 to 85 kDa from Sepharose CL-6B chromatography (60). As an aid in identifying the polypeptide(s) of interest in purified preparations of PREIIBF, we carried out a higher-resolution determination of $M_r$. DEAE Sephacel-purified protein was subjected to gel filtration chromatography through a Su-

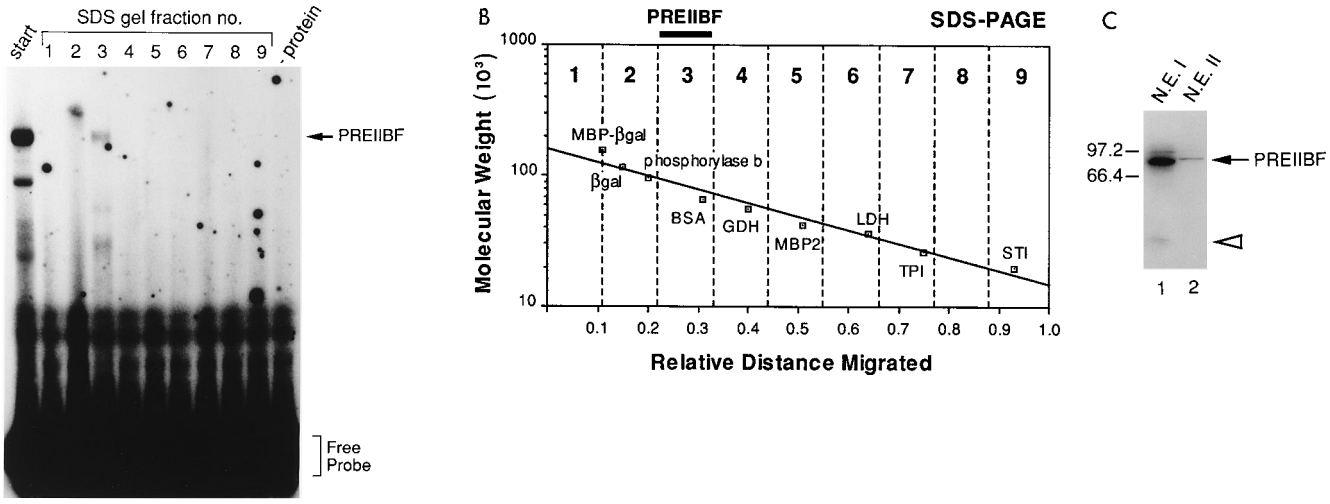


FIG. 3. PREIIBF binds DNA as a single polypeptide. (A) Renaturation of PREIIBF fractionated by SDS-PAGE. PREIIBF was renatured from nine horizontal sections of an SDS-polyacrylamide gel as described in Materials and Methods. Renatured PREIIBF was incubated with radiolabeled PRE II probe and assayed for DNA binding by EMSA. The mobility of the renatured protein-DNA complex (slice 3 of the gel) was identical to that of the native starting material (lane start), indicating that PREIIBF binds DNA as a single polypeptide (either as a monomer or, possibly, a homodimer). (B) Standard curve for preparative SDS-gel. Molecular weights for protein standards (New England Biolabs) were plotted on a semilog scale versus relative distance migrated. Dotted vertical lines represent boundaries of individual gel slices from which protein was eluted. PREIIBF activity was found in gel slice 3. The molecular weight markers were maltose-binding protein–β-galactosidase fusion protein (MBP-βgal; 158,200), β-galactosidase (βgal; 116,300), phosphorylase *b* (97,200), bovine serum albumin (BSA; 66,400), glutamic dehydrogenase (GDH; 55,600), maltose-binding protein 2 (MBP2; 42,700), lactate dehydrogenase M (LDH; 36,500), triosephosphate isomerase (TPI; 26,600), soybean trypsin inhibitor (STI; 20,100), and chicken lysozyme (14,300; used to normalize migration distances). (C) Southwestern blot analysis of PREIIBF. Protein from crude nuclear extract (NE I, 80 μg) or from renatured extract (NE II, 8 μg; see Fig. 1A) was fractionated by SDS-PAGE. Protein was electroblotted onto a nitrocellulose filter, denatured by incubation with 6 M GuHCl, and then slowly renatured by stepwise dilution. The filter was then hybridized with a multimerized, radiolabeled PRE II DNA probe as described in Materials and Methods. The probe detected a predominant band of ~85 to 90 kDa. We do not know the origin of the less abundant, lower-molecular-mass species (open arrowhead, ~28 kDa), but it may represent a degradation product of PREIIBF. Migration positions of two protein standards are indicated in kilodaltons at the left.

perose-12 FPLC column. As shown in Fig. 2, PREIIBF eluted in the ~85-kDa fraction, consistent with our earlier estimate (60).

To determine whether PRE II binding activity can be attributed to a single polypeptide, proteins from active fractions of a DEAE-Sephacel column were fractionated by SDS-PAGE. Protein was eluted from slices of the gel, incubated in GuHCl, renatured by dialysis, and finally analyzed for specific binding by EMSA (Fig. 3A). PRE II binding activity was detected in the gel slice containing protein of ~60 to 88 kDa (Fig. 3B). The mobility of the renatured protein-PRE II complex was identical to that observed with crude or partially purified extracts, suggesting that PREIIBF binds DNA as a monomer or homodimer. This conclusion was strengthened by UV cross-linking experiments (not shown) and Southwestern blot analysis (Fig. 3C). The predominant band in preparations of NE I and NE II (Fig. 3C), as well as in more highly purified DEAE-Sephacel fractions (not shown), was approximately 85 to 90 kDa.

**DNA binding affinity of PREIIBF.** As is evident from inspection of the data in Fig. 1C, only about 10% of the input PREIIBF was bound with each loading of the DNA affinity column, suggesting that the DNA binding affinity of PREIIBF is relatively low. We therefore used the DNA affinity-purified PREIIBF in a quantitative EMSA (Fig. 4A) to determine the equilibrium binding affinity of PREIIBF for its cognate site. The concentrations of free and bound binding site were determined for each concentration of input DNA and plotted in Fig. 4B. The $K_d$ for the interaction of PREIIBF with PRE II was calculated by Scatchard analysis (45) from the slope as ~14.6 nM. This value was confirmed in a reciprocal experiment in which the concentration of PRE II probe was held constant and the concentration of PREIIBF was varied.

**Embryonic and adult forms of PREIIBF bind DNA with identical specificities.** We have previously shown that the PREIIBFs found in adult and embryonic erythroid cells can be distinguished by the mobilities of their complexes with DNA (PREIIBF found in adult cells forms a complex of slightly faster mobility [59, 60]). To examine the properties of the two forms of PREIIBF in greater detail, we synthesized a set of point mutations across PRE II and used the resulting mutated probes (Fig. 5A) in quantitative EMSA experiments. Protein binding was examined for a total of 15 mutated sequences (Fig. 5B); of these, 14 contained single-point mutations and one contained a double mutation. Probes labeled to comparable specific activities were incubated in binding reaction mixtures in the presence of nuclear extracts from human embryonic erythroid (K562; Fig. 5B and C), mouse embryonic erythroid (GM979; Fig. 5C), and mouse adult erythroid (MEL; Fig. 5C) cells. The results of these experiments are summarized in histogram form in Fig. 5C, and a representative mobility shift assay is shown for GM979 (mouse embryonic erythroid) nuclear extracts in Fig. 5D.

As expected, differences in mobility were observed for the embryonic and adult forms of PREIIBF bound to wild-type probe as well as probes containing mutations that reduced but did not eliminate DNA binding (data not shown). Interestingly, embryonic and adult forms of PREIIBF exhibited identical DNA specificities as assessed by this mutational analysis (Fig. 5C). This observation is consistent with other data (notably methylation interference patterns, partial proteolysis studies, and estimates of protein size [60]) suggesting that the embryonic and adult PREIIBFs are very similar proteins. The different mobilities of the protein-DNA complexes on EMSA therefore likely reflect differences in posttranscriptional pro-

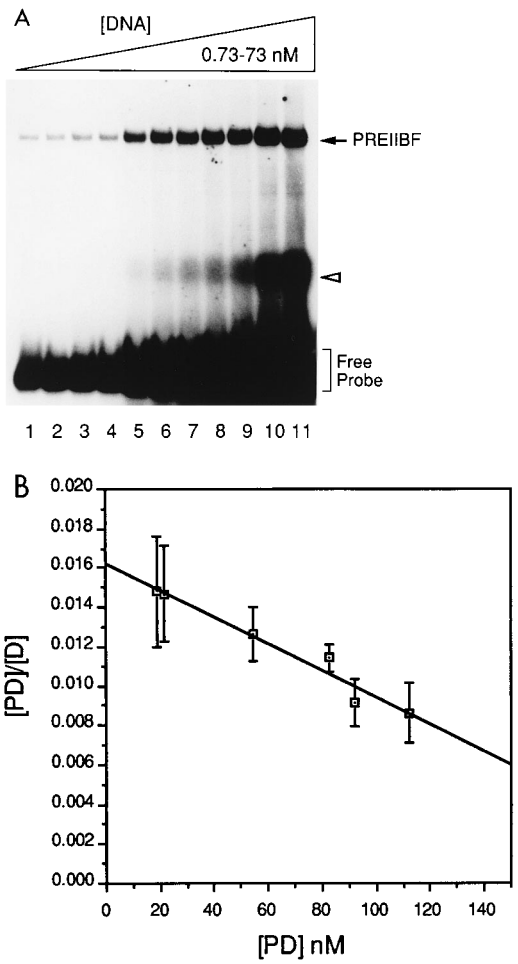


FIG. 4. Determination of DNA binding affinity of PREIIBF. (A) Titration of PRE II probe against a fixed PREIIBF concentration. The equilibrium affinity of PREIIBF for the PRE II site was determined by EMSA. PREIIBF purified through one DNA affinity step (see Fig. 1A) was incubated with various concentrations of the PRE II site probe as described in Materials and Methods. The PRE II probe was titrated at concentrations of 0.73, 1.02, 1.31, 1.46, 4.4, 7.3, 10.2, 13.1, 14.6, 43.8, and 73.0 nM (lanes 1 to 11, respectively), while the PREIIBF concentration was kept constant at 0.2 nM. The concentrations of protein-DNA complexes were determined by quantitative EMSA (see Materials and Methods). The open arrowhead indicates an artifact of unknown origin that we have observed in some preparations of probe; it was unrelated to probe composition or sequence and was observed in the presence or absence of protein. Therefore, it is not due to probe binding by a contaminating protein. (B) Scatchard analysis of binding of PREIIBF to its cognate site. Data from the experiment shown in panel A (lanes 3 to 8) were used to determine the $K_d$, assuming a simple bimolecular interaction between PREIIBF and probe. The amount of free DNA ($[D_{free}]$) was taken as $[D_{total}] - [PD]$, where [PD] is the amount of bound DNA (protein-DNA complex), and the $[PD]/[D_{free}]$ ratio was plotted against [PD] to give a line with slope ($m$) = $-1/K_d$ and $y$ intercept = $K_a[P_{total}]$ (by definition, $K_a = 1/K_d$). This analysis yielded a $K_d$ of 14.6 nM or $1.46 \times 10^{-8}$ M. Each point represents the average of values obtained in two separate experiments.

cessing rather than the presence of distinct proteins in embryonic and adult erythroid cells.

In agreement with our previous methylation interference studies (59), G-to-A transversions at positions −438 and −425 resulted in significant disruption of binding (Fig. 5B and C). A mutation in the third guanosine residue identified by methylation interference (position −432) was not obtained in the group of mutations generated. Among the single-point mutations analyzed, nucleotide transversions in several of the positions that are conserved across species (−436, −434, −431, −429, −425, −423, and −422 in humans, mice, rabbits, and galagos [60]) resulted in a substantial disruption of binding (Fig. 5C). Mutation of position −433 (C in galagos and T in the other three species) enhanced binding (Fig. 5C).

Single-point mutations that, when mutated as a block, abolished protein binding to and transcriptional activation by PRE II were obtained in five of the six nucleotides (60). Each mutation reduced protein binding by 54 to 94% (positions −423, −425, −428, −434, and −438 [Fig. 5B and C]).

**Binding by PREIIBF induces DNA bending.** We have shown previously that at least some of the elements upstream of the human embryonic β-like globin gene (ε) interact synergistically to stimulate transcription from the minimal ε-globin gene promoter, although they are separated by approximately 200 bp (in the case of PRE II and PRE V [59, 60]) in the intact locus. Looping out of the intervening DNA is a plausible mechanism by which PRE II might functionally synergize with other regulatory sites, and it could be achieved by protein-protein interactions between PREIIBF and transcriptional regulators bound at other sites, by protein-induced DNA bending, or by both.

To determine whether binding of PREIIBF at PRE II alters the conformation of the DNA helix, we used a circular permutation assay (11, 68) in which the mobility of the protein-DNA complex was analyzed as a function of the position of the PRE II binding site within the DNA probe. This general strategy is based on the observation that a DNA molecule with a bend near the center will migrate more slowly in nondenaturing gels than a molecule with a comparable bend at the end (for a review, see reference 11). PRE II sequences were subcloned into the circular permutation vector pCY4 (46). Five DNA probes (437 bp) were prepared from this construct and radiolabeled for EMSA, using DEAE-purified PREIIBF. By comparison with probes containing more terminally located PRE II sites (e.g., A and E; Fig. 6), the isomeric probes that contain PRE II near the center (probes C and D; Fig. 6) exhibited significantly slower mobilities when bound by PREIIBF. Therefore, binding by PREIIBF resulted in a position-dependent change in mobility of the DNA probe, suggesting that PREIIBF introduces a bend into the target DNA. A bend angle of 63° was calculated from a graph in which relative probe mobility was plotted against the position of the binding site (see Materials and Methods). The specificity of binding by PREIIBF was confirmed by including an excess of unlabeled specific or nonspecific competitor DNA in separate sets of binding reactions (Fig. 6).

DNA unwinding or nonglobular protein structures can influence the results of circular permutation assays, and some DNA-binding proteins cause unstructured distortions or deformations in DNA (3, 20, 32, 51). Therefore, we assessed whether binding by PREIIBF induces a directed bend by using a phase-sensitive assay (11, 28). PRE II was subcloned into a set of constructs containing adenine tracts phased such that the spacing between a set of three adenine tracts and PRE II was varied, by insertion of different length linkers, over a full turn of the double helix (10 bp) (Fig. 7A). When the phase-sensing probes containing PRE II were incubated with DEAE-Sephacel-purified PREIIBF and analyzed by EMSA, a sinusoidal variation in mobility with respect to linker length was observed (Fig. 7B). Average mobility was calculated for the protein-bound and free PRE II probes as well as for control probes (no PRE II insert) (Fig. 7B). Relative mobility was taken as the ratio of the actual mobility for each probe to the calculated average mobility and was plotted against spacer length after normalizing for the mobility of free probe (Fig. 7C). The amplitude of the curve obtained for the protein-PRE II complexes

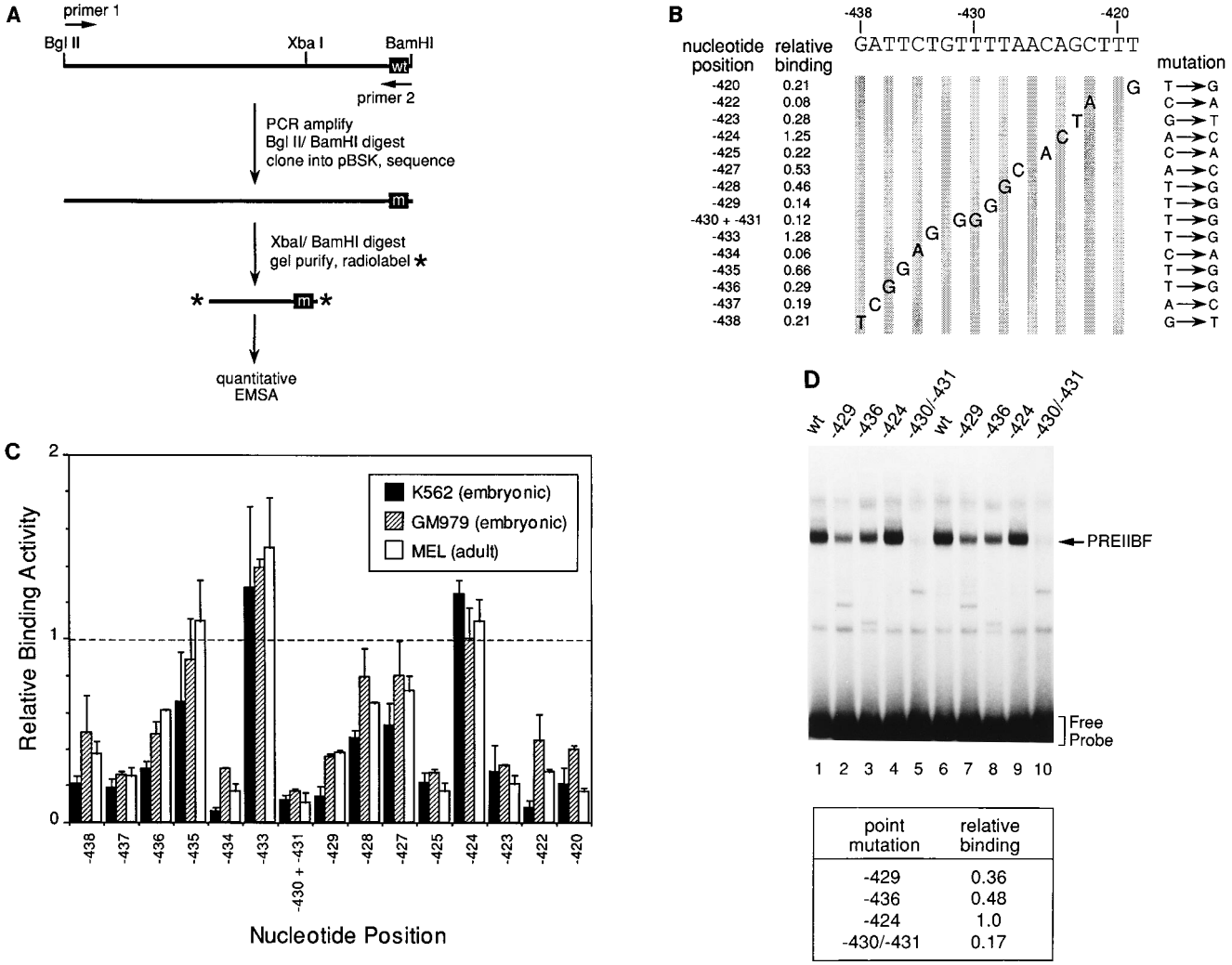


FIG. 5. Mutational analysis of the PRE II binding site. (A) PCR-based strategy for generation of point mutations in PRE II. Primer 1 (wild type [wt]) and primer 2 (containing the PRE II sequence mutated as described in Materials and Methods) were used to amplify the region between −770 and −418 with respect to the human ε-globin gene start site. Amplification products were subcloned into *Bgl*II-*Bam*HI-digested pBSK and sequenced. Point mutations (m) selected for further analysis were excised from the pBSK vectors as *Xba*I-*Bam*HI (115-bp) fragments, gel purified, radiolabeled by end filling, and gel purified once more. The probes were used in quantitative EMSA experiments as outlined in Materials and Methods. (B) Summary of point mutations obtained by the scheme shown in panel A. Nucleotide position and specific transversion mutations are summarized. Relative binding (mutant/wild type) is indicated for PREIIBF from K562 cells (average values taken from the histogram in panel C). Values of <1 correspond to decreased affinity of the mutated sequence for PREIIBF, while values of >1 correspond to increased affinity. (C) Graphical representation of relative binding affinities for mutated PRE II sites. Radiolabeled DNA probes were prepared (see panel A) from the mutated sequences shown in panel B and analyzed by EMSA, using DEAE-purified PREIIBF from human K562 cells or nuclear extracts from murine erythroid (GM979 and MEL) cells. Relative binding affinities were calculated as described in Materials and Methods and plotted for each mutation. Values graphed represent averages of two separate experiments ± standard deviations. (D) Representative mobility shift assay for selected mutated PRE II sites. Binding assays were performed in duplicate, using nuclear extracts from GM979 cells. Both sets of duplicate reactions are shown. The average binding affinities are shown at the bottom and were taken from the data plotted in panel C.

was greater than that for the free PRE II probe, indicating that PREIIBF induces a directed bend when it is bound to PRE II.

Phasing analysis also allowed us to calculate the direction and degree of the PREIIBF-induced bend as described in Materials and Methods. The best-fit cosine curve (Fig. 7C) indicated a bend angle of 54° (in close agreement with the estimate of 63° obtained from the circular permutation analysis), after correction for the slight bend (~1°) contributed by the DNA probe alone. Each poly(A) tract in the phasing vectors confers an intrinsic bend of 18°, resulting in an overall bend of 54°C directed toward the minor groove (11, 28). Because the PREIIBF-induced bend (54 to 63°) reinforced the intrinsic minor groove bend (54°) when it was positioned on the same side of the DNA helix as the poly(A) tracts (mobility minimum at integral number of helical turns), we concluded that PREIIBF bends its target DNA toward the minor groove.

## DISCUSSION

Like other eukaryotic regulatory regions (58), the ε-globin upstream control region contains a complex arrangement of both positive and negative elements (59, 60). Deletion of one of the positive elements, PRE II, reduces activity to the level of the minimal ε-globin gene promoter in embryonic but not adult erythroid cells (59). When linked directly with the minimal promoter, PRE II is inactive in monomeric or multimeric

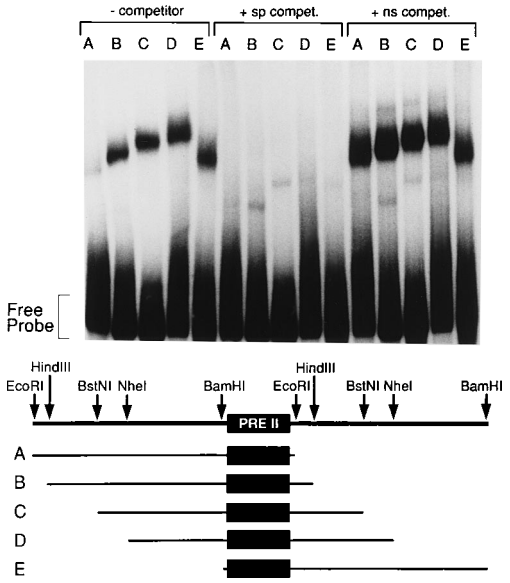


FIG. 6. Alteration of DNA conformation by PREIIBF, determined by a circular permutation assay. A blunt-ended 32-bp oligonucleotide containing a PRE II site was cloned into the *Sma*I site of pCY4 (46), and probes were generated by digestion with the indicated restriction enzymes (e.g., probe A was obtained by digestion with *Eco*RI, probe B was obtained by digestion with *Hin*dIII, etc.). The mobilities of the fastest (*Bam*HI) and slowest (*Nhe*I) complexes were used to estimate a DNA bending angle of 63° as described in Materials and Methods. Shown are results obtained with a 4% polyacrylamide gel. sp compet., specific competitor; ns compet., nonspecific competitor.

form; however, in combination with another element (PRE V) located ~200 bp upstream, PRE II confers tissue- and stage-specific expression in cell culture (59, 60).

PREIIBF is a nuclear protein whose binding to a novel regulatory sequence within PRE II (59) is required for promoter activation in cultured embryonic erythroid cells (60). Therefore, it is a candidate regulator of the human embryonic β-like globin gene (59, 60). In this report, we use fractions of PREIIBF purified from human embryonic erythroid (K562) cell nuclear extracts to characterize some of the properties of this protein.

**DNA binding properties of PREIIBF.** PREIIBF binds DNA as a monomer or homodimer of ~80 to 85 kDa, as estimated from gel filtration and SDS-PAGE. Because no additional bands appeared by EMSA following denaturation and renaturation (either during purification or after SDS-PAGE), it seems most likely that PRE II is bound by a single molecule of PREIIBF.

Estimates of DNA binding affinities for regulatory proteins depend on the experimental conditions and methodologies used and vary over a broad range. Examples include the *lac* repressor of *Escherichia coli* ($10^{-13}$ M [48]), the major histocompatibility complex class I transcription factor H2TF1 ($3 \times 10^{-11}$ M [45]), the *Drosophila* Antennapedia homeodomain peptide ($1.6 \times 10^{-9}$ M [1]), monomers of the yeast α2 protein ($6 \times 10^{-8}$ M [50]), and the high-mobility group protein HMG-1 ($3.7 \times 10^{-7}$ M [44]). PREIIBF binds with a $K_d$ of ~$1.5 \times 10^{-8}$ M; thus, its binding affinity is modest. In vivo, binding of PREIIBF at PRE II may be stabilized by other interactions—for example, with other as yet unidentified protein(s) bound at PRE V. Protein-protein interactions that increase DNA binding affinity have been well documented in other systems (8, 14, 36, 37, 55).

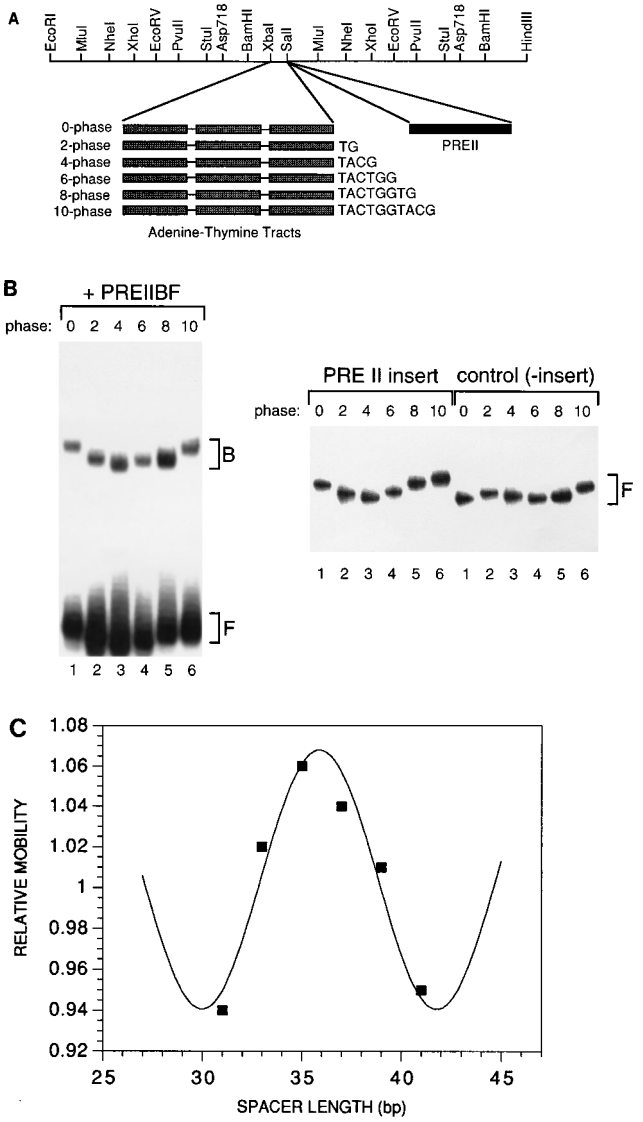


FIG. 7. Phasing analysis of PRE II. (A) Probes used for phasing analysis. The control probes 0-phase through 10-phase were prepared from *Eco*RI-*Hin*dIII digests of vectors containing a set of three in-phase adenine tracts followed by the 0- to 10-bp linker sequence shown (17). The PRE II probes were prepared by inserting a 28-bp PRE II oligonucleotide with *Sal*I-*Xho*I half sites into the *Sal*I site of the phasing vectors. (B) Phase-sensing analysis of PREIIBF bound at PRE II. PREIIBF from K562 cells was incubated with the PRE II phasing probes shown in panel and analyzed by EMSA. The PRE II target itself does not contain a directed bend, as the amplitudes of the sinusoidal curves for the control probes and the PRE II probes in the absence of protein are identical. The slight sinusoidal variation in mobility of both sets of probes in the absence of protein is due to a small inherent bend (~1°) in the adjoining pBEND2 vector sequence (17). Note that the control and PRE II probes are out of phase because the PRE II oligonucleotide (28 bp) contains a nonintegral number of helical turns. (C) Relative mobility plotted against spacer length. The relative mobility of the protein-DNA complex was corrected for the mobility of free probe (see Materials and Methods). Spacer length (in base pairs) was taken as the distance between the centers of the minor groove-bending poly(A) tracts and the PRE II site. Normalized mobilities were plotted against spacer length, using the KaleidaGraph program and a bending angle calculated as described previously (40). The large sinusoidal variation in relative mobility with spacer length for the protein-DNA complexes indicates that PREIIBF induces a directed bend (toward the minor groove; see text) in the DNA when it binds at PRE II. The bend angle obtained by this analysis (54°) is in close agreement with that obtained from the circular permutation assay (63°). The curve has been extended beyond the datum points (spacer lengths of <30 and >40 bp) to emphasize the periodicity of the cosine function.

**Sequence specificity of PREIIBF binding.** A cluster of six point mutations spanning PRE II abolishes binding by PREI IBF and synergistic activation of a minimal promoter by the combination of PRE II and PRE V, suggesting that PREIIBF may play a role in regulation of the ε-globin gene in vivo (60). In the present study, a collection of point mutations in PRE II was generated as a first step in characterizing this protein-DNA interaction in greater detail and to facilitate the analysis of recombinant PREIIBF protein once cDNA clones have been isolated. Point mutations introduced individually into each of five of the six positions previously mutated as a cluster (60) resulted in a significant decrease in protein binding. Other mutations in positions that are evolutionarily conserved within PRE II also reduced binding by PREIIBF. Together with competition binding assays (15, 59, 60), these results indicate that PREIIBF binding is specific for sequences within PRE II.

Because we have not generated all possible base changes at each position, we cannot yet compile a reliable consensus binding sequence. However, we note that the PRE II sequence is pyrimidine rich, and as already indicated, transversion mutations to a purine nucleotide (Fig. 5B) have significant effects on binding (Fig. 5C and D). The mutational analyses in the present study were performed with partially purified fractions of PREIIBF; determination of the optimal PREIIBF sequence by binding site selection will require recombinant protein.

Different forms of PREIIBF are found in embryonic and adult erythroid cells, and several lines of evidence suggest that they are similar if not identical proteins (60). This conclusion was reinforced by the present study, in which we found that the chromatographic properties as well as the binding site specificities of embryonic and adult PREIIBFs are identical. To date, we have been unable to determine the origin of the differences in protein-DNA complex mobility observed for the two forms of PREIIBF. Although both forms are phosphorylated, this modification alone is not sufficient to account for the mobility differences detected by EMSA (26).

**Alteration of DNA conformation and control of gene expression.** Induced DNA bending is a common (but not universal) characteristic of transcription factors and is thought to play a role in gene regulation (18, 21, 30, 40, 43, 53, 56, 63). The ability of the PRE II regulatory element to synergize with sequences located both upstream and downstream (59) suggested the possibility that PREIIBF plays a role in bending or looping of the DNA. We show here that PREIIBF introduces a directed bend of 54 to 63° into the DNA molecule. Notably, this bending is not dependent on the presence of other sequences with which PRE II synergizes.

DNA bending can function by bringing other proteins into closer proximity, thereby stabilizing interactions between transcription factors bound to flanking sequences or between the DNA-bending protein and factors bound at other sites (e.g., reference 23). Such interactions might create stronger (or weaker) binding sites for other proteins (14) or might contribute to regulatory specificity, as observed for different combinations of bZIP proteins (30). It is conceivable that the specificity of binding by PREIIBF derives in part from sequence-dependent deformability of the PRE II binding site. However, we caution that although the apparent bend angle estimated from circular permutation and phasing analyses has in some cases been confirmed by crystallographic studies (e.g., reference 35), in other cases (30) it can be a large overestimate (22).

Recently, Merika and Orkin (37) have demonstrated that GATA-1 and Sp1 synergize from a distance, suggesting one mechanism by which DNA loops between the locus control region and globin promoters/enhancers might be stabilized. The work presented here suggests a plausible second mechanism, involving protein-induced DNA bending, by which multiple *cis*-acting elements that are physically well separated along a stretch of DNA might cooperate in the regulation of downstream genes. Whether PREIIBF acts simply to stabilize interactions at protein-protein interfaces (thereby serving primarily an architectural role [67]) or to juxtapose more promoter-proximal elements such as PRE II and PRE V or PRE II and PRE I (59), or contributes to more long-range interactions with the locus control region, is unknown.

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