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Cell Type-Specific Protein-DNA Interactions in the Human ζ-Globin Upstream Promoter Region: Displacement of Sp1 by the Erythroid Cell-Specific Factor NF-E1

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The protein-DNA interactions of the upstream promoter region of the human embryonic ζ-globin gene in nuclear extracts of erythroid K562 cells and nonerythroid HeLa cells were analyzed by DNase I footprinting, gel mobility shift assay, methylation interference, and oligonucleotide competition experiments. There are mainly two clusters of nuclear factor-binding sites in the ζ promoter. The proximal cluster spans the DNA sequence from −110 to −60 and consists of binding sites for CP2, Sp1, and NF-E1. NF-E1 binding is K562 specific, whereas CP2 binding is common to both types of cells. Overlapping the NF-E1- and CP2-binding sites is a hidden Sp1-binding site or CAC box, as demonstrated by binding studies of affinity-purified Sp1. In the distal promoter region at −250 to −220, another NF-E1-binding site overlaps a CAC box or Sp1-binding site. Extract-mixing experiments demonstrated that the higher affinity of NF-E1 binding excluded the binding of Sp1 in the K562 extract. NF-E1 factors could also displace prebound Sp1 molecules. Between the two clusters of multiple-factor-binding sites are sequences recognized by other factors, including ζ-globin factors 1 and 2, that are present in both HeLa and K562 extracts. We discuss the cell type-specific, competitive binding of multiple nuclear factors in terms of functional implications in transcriptional regulation of the ζ-globin gene.

For most eucaryotic protein-coding genes, a basal level of transcription can be directed by a core promoter sequence centered at the TATA box. This core promoter is able to form a preinitiation complex with at least three general transcription factors and RNA polymerase II (7, 36, 48). Two other classes of *cis*-acting DNA elements are also involved in modulating the TATA box-driven transcription, the upstream promoter elements and enhancers. Both elements exert their effects through specific DNA-binding proteins.

Whereas the function of enhancers is independent of their orientation and distance from the transcriptional initiation sites (1a, 38), the upstream promoter elements act at short distances from the initiation sites. One group of the upstream promoter elements, e.g., the CCAAT box (2, 3, 9, 25, 37), is found upstream of many different genes. Another group consists of gene-specific promoter elements that in most cases confer tissue or cell specificity to the transcription processes (32).

The eucaryotic α- and β-globin gene families, or gene clusters (12, 42), provide an interesting system with which to investigate the roles of protein-DNA and protein-protein interactions in coordinate and differential gene regulation (26a, 45, 45a). Thus far, much of the information has been derived from studies of the β-globin gene family.

Transient expression studies of chicken embryonic ε- and adult β-globin genes in primitive and definitive erythroid cells have suggested that the tissue-specific and developmental stage-specific control of the chicken β-like globin gene regulation is mediated by both the promoters and by a tissue-specific enhancer (10, 24). The enhancer contains multiple functional sequence motifs and interacts with multiple nuclear factors (18, 19), one of which is an erythrocyte-specific nuclear factor, Eryf1. Specific DNA-binding of Eryf1 to the chicken globin enhancer appears to be essential for the enhancer activity (11, 19). Interestingly, a core sequence (5′-$^{T}_{A}$GATA$^{A}_{G}$-3′) of the Eryf1-binding site is found in all chicken α- and β-globin promoters (11, 19).

An Eryf1-like factor also exists in human erythroid cells and has been named NF-E1 (49) or GF-1 (34). Binding sites of NF-E1 have been mapped upstream of the human γ- and β-globin genes and in the β-globin 3′ enhancer region (14, 33, 34, 49). The presence of NF-E1-binding sites in *cis* within the upstream promoter regions mediates the inducibility of the human β-globin gene in transfected mouse erythroid MEL cells (14) and also enhances transient expression of transfected human γ-globin gene in human K562 cells (34). K562 is an erythroleukemic cell line (30) in which all of the human globin genes except β are actively transcribed. These data suggest that the binding of a common erythroid lineage-specific factor to promoter and enhancer sequences is one of the fundamental regulatory mechanisms of transcription of all eucaryotic globin genes. A cDNA encoding the mouse NF-E1 factor has been cloned (46).

Globin expression studies in hybrid cells and in heterokaryons have demonstrated the presence of diffusible, *trans*-acting regulatory factors in erythroid cells for the mammalian α-like globin genes (1, 4, 15). However, very few studies presenting detailed analyses of the functional elements within the promoter regions of mammalian α-like globin genes have been reported. One study has established the importance of CCAAT and TATA boxes for transcription of the human adult α-globin gene in nonerythroid COS 7 cells (37). Transcriptional analysis of transfected clones that have been integrated into K562 cells and nonerythroid cells has suggested (39, 41) that sequences from −555 to +38 of the

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human embryonic ζ-globin gene are sufficient for its tissue-specific and, perhaps, developmental stage-specific transcription. Regarding nuclear factor-promoter DNA interaction, only studies of mouse adult α globin (3, 27) and human θ1 globin (27a) have been reported.

In this communication, we describe studies of interactions between the upstream promoter region of the human embryonic ζ-globin gene and nuclear factors present in erythroid K562 cells and nonerythroid HeLa cells. Interesting patterns of cell type-specific interactions of multiple nuclear factors with the globin promoter have been identified, which appear to be the results of competitive binding of different proteins to the DNA helices.

MATERIALS AND METHODS

Cell lines. Both HeLa and K562 cells were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum, 50 U of penicillin per ml, and 50 μg of streptomycin per ml (GIBCO Laboratories).

Nuclear extracts and factor Sp1. Nuclear extracts of HeLa and K562 cells were prepared by the procedures of Dignam et al. (16). DNA affinity column-purified factor Sp1 of HeLa cells (13, 26) was a kind gift from Al Courey and Robert Tjian.

DNA probes. Restriction fragments were isolated from a plasmid containing the human ζ-globin promoter region of *Eco*RI (−567) to *Pvu*II (+38).

To prepare probes for gel mobility shift assays, two restriction fragments, *Bst*NI (−249)-*Bal*I (−201) and *Dde*I (−131)-*Bst*NI (−50), were 3′ end labeled with ^{32}P with Klenow enzyme and purified from polyacrylamide gels (31). For DNase I footprint assays, three restriction fragments, *Ava*II (−359)-*Pst*I (−85), *Ava*II (−359)-*Sau*3A (−108), and *Hin*fI (−207)-*Ava*II (−18), were labeled at one of the 3′ ends with ^{32}P and purified by polyacrylamide gel electrophoresis.

Several double-stranded oligonucleotides were used either as probes in gel mobility shift assays or as competitors in DNase I footprinting and gel mobility shift assays. These oligonucleotides included (i) an NF-E1 oligonucleotide containing the sequence from −122 to −90 of the human ζ-globin promoter and a *Bam*HI restriction site at each of the two ends (5′-GATCCTTTGTCACTGGATCTGATAAGAAACA CCACCCG-3′/3′-GAAACAGTGACCTAGACTATTCTTT GTGGTGGGCCTAG-5′); (ii) an Sp1 oligonucleotide containing the core binding consensus of nuclear factor Sp1 (5′-GATCGGGGCGGGGC-3′/3′-CCCCGCCCCGCTAG-5′), kindly provided by Al Courey and Robert Tjian; and (iii) a COUP-RIPE mutant oligonucleotide containing a low-affinity binding site for the transcription factor COUP (6) (5′-TATGGTCTCAAAGGTCAAACTTCT-3′/3′-ATACC AGAGTTTCCAGTTTGAAGA-5′). Of these three oligonucleotide probes, only the Sp1 oligonucleotide was concatemerized through a ligation step before use.

DNase I footprinting. A DNase I footprint assay (21) was used to study nuclear factor-DNA interactions as described in detail elsewhere (27a). Usually, 100 to 125 μg of nuclear extract was first preincubated at room temperature for 10 min with 1.5 μg of poly(dI-dC) (Pharmacia, Inc.) in 50 μl of 50 mM KCl–25 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES; pH 7.9 at 4°C)–0.05 mM EDTA–0.5 mM dithiothreitol–10% glycerol–1 mM phenylmethylsulfonyl fluoride–0.2 μg of leupeptin per ml. After the preincubation step, 1 ng of end-labeled restriction fragments (approximately 10^4 cpm) was added. After another 1 to 2 h of incubation, the reaction mixture was adjusted to 5 mM $MgCl_2$, and an appropriate amount of DNase I enzyme (grade DPFF; Worthington Diagnostics) was added. After digestion for 1 min at room temperature, the DNA sample was purified from the reaction mixture by proteinase K digestion, phenol-chloroform extraction, and ethanol precipitation. The digestion products were then analyzed by electrophoresis on denaturing 6 or 8% polyacrylamide–8 M urea gels and autoradiography on X-ray films (31). DNase I footprint analysis with affinity-purified Sp1 was carried out as described by Courey and Tjian (13).

For competition experiments, 5- to 200-fold excess amounts of appropriate oligonucleotides, as indicated, were included in the preincubation step.

Gel mobility shift. A gel mobility shift assay (44) was used to resolve different protein-DNA complexes on gels. Generally, 10 μg of nuclear extract was preincubated with 5 μg of poly(dI-dC) and 1 μg of salmon sperm DNA on ice in a total volume of 20 μl. Other conditions, including salt concentrations, were the same as those used in the DNase I footprint assay. After preincubation, a ^{32}P-end-labeled DNA probe was added, and the binding reaction was allowed to proceed for 30 min on ice. The reaction mixture was then analyzed by electrophoresis on 5% native polyacrylamide gels at 4°C, followed by autoradiography.

For competition experiments, excess amounts of cold oligonucleotides were included in the preincubation step.

Methylation interference analysis. NF-E1 oligonucleotide was partially methylated with dimethyl sulfate (35). The probe was then incubated in K562 extract and subjected to gel mobility shift analysis as described above. The protein-DNA complexes and the free probe were eluted from the gel onto an NA-45 membrane (Schleicher & Schuell), washed off with 1 M NaCl, and purified by ethanol precipitation. The DNA samples were then cleaved with piperidine and analyzed on DNA sequencing gels.

RESULTS

A CCAAT box-binding factor (CBF) interacts with the proximal promoter region of ζ globin. Nuclear factor-DNA interactions in vitro were first assayed by DNase I footprinting. A DNA fragment covering the region of −207 to −18 upstream of the human ζ-globin gene was labeled at the 3′ end and used as the probe in DNase I digestion reactions. The patterns of digestion of this fragment by DNase I in the presence of the HeLa or K562 nuclear extract are shown in Fig. 1.

In comparison with the controls (Fig. 1, lane 1) nuclear extract prepared from either HeLa (lane 2) or K562 (lane 3) cells protected a common DNA region from DNase I digestion. This protected region (CBF in Fig. 1) contained the CCAAT box promoter element of the ζ-globin gene. Thus, it is most likely the binding site for a CBF (9). This binding was manifested by two footprints extending from −87 to −61 on the sense strand and from −78 to −60 on the antisense strand (Fig. 1 and 2). The CBF that bounds to this region was probably CP2 (see Discussion).

A K562 cell-specific factor, NF-E1, binds to sequences upstream of the CBF-binding site. Immediately upstream of the CP2 footprint was a DNA region protected by the nuclear extract from K562 but not HeLa cells (Fig. 1, lane 3). The K562-specific footprint, (NF-E1 in Fig. 1) extended from −112 to −96 on the sense strand and from −108 to −100 on the antisense strand and was bounded by enhanced DNase I cleavage sites (Fig. 1 and 2). This footprint region contained the tetranucleotide sequence 5′-GATA-3′, which

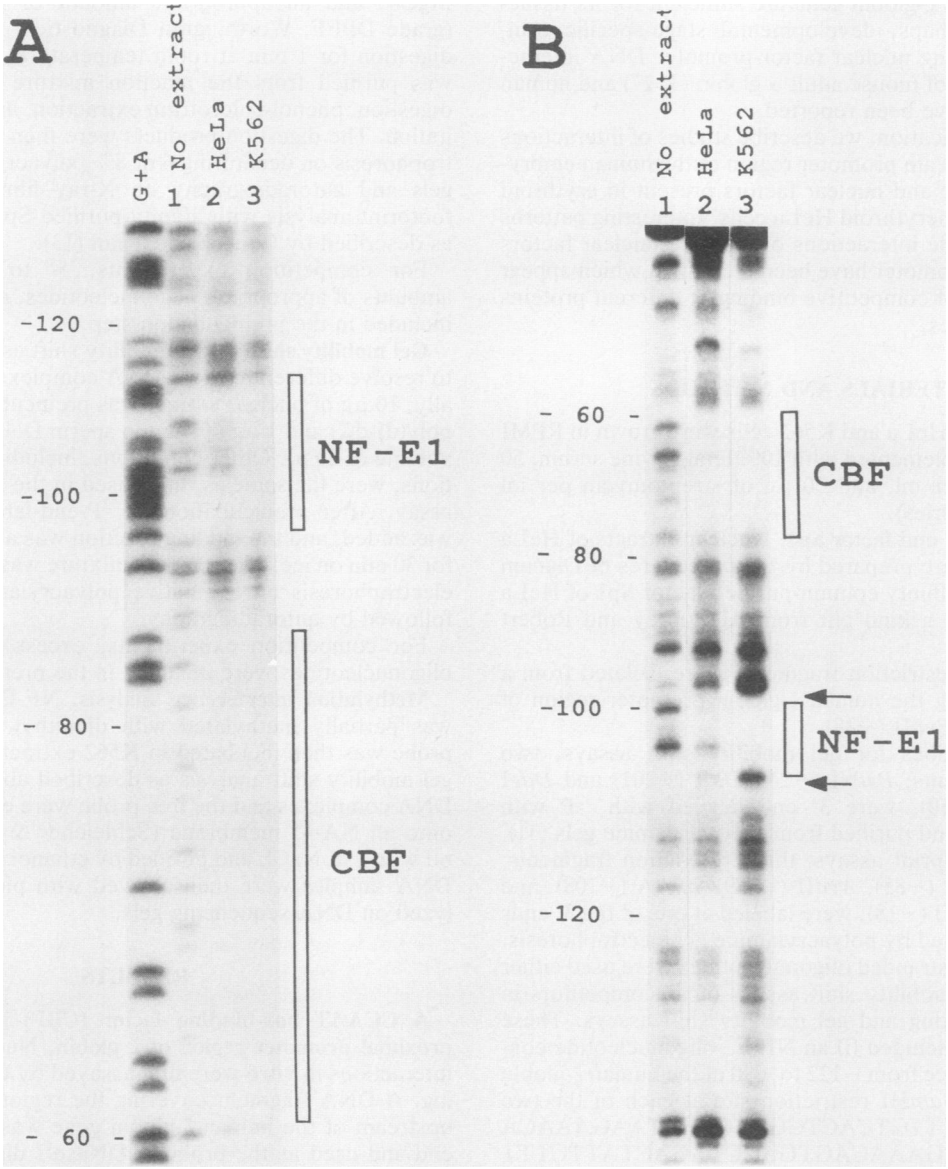

FIG. 1. Footprint analysis of the proximal promoter region of the ζ globin gene. A 190-bp *Hin*fI-*Ava*II fragment containing the ζ-globin upstream promoter sequence from −207 to −18 was labeled at either the *Ava*II (A) or *Hin*fI (B) end. It was then analyzed by DNase I footprinting in an HeLa or K562 nuclear extract as described in Materials and Methods. Results for the sense (A) and antisense (B) strands are shown. The control digestion reactions (lanes 1) were carried out in a similar way except that 30 μg of bovine serum albumin (Sigma Chemical Co.) was used for incubation instead of the nuclear extracts. Symbols: □, DNA regions protected from DNase I digestion in the nuclear extracts; ←, sites of enhanced DNase I cleavages. Numbers to the left of the panels indicate nucleotide positions relative to the cap site. G+A sequencing markers were prepared from probes described previously (35). A detailed map of the footprints is shown in Fig. 2.

is the core of the consensus binding sequence of the erythroid cell-specific factor NF-E1 (14, 19, 34, 40, 46).

The binding specificity of NF-E1 to the ζ proximal promoter region was further evidenced by the following experiments. First, a double-stranded synthetic oligonucleotide containing sequence from −122 to −90 was used as the probe in a gel mobility shift assay. Two protein-DNA complexes were formed in the K562 but not the HeLa extract (Fig. 3A). Second, the specific methylation interference pattern of the major protein-DNA complex (Fig. 3A, band 1) indicated that the G residue at position −106 and, to a lesser extent, the G residue at −111 were essential for the NF-E1-binding reaction (Fig. 3B, lane 2). This interference pattern was similar to those observed for other erythroid cell-specific gene promoters (14, 34). The minor protein-DNA complex of Fig. 3A (band 2) had a methylation interference pattern similar to that of the major complex (compare lanes 3 and 2 in Fig. 3B). Therefore, it was probably the result of the binding of NF-E1 that had been altered by covalent modification or proteolysis. Finally, addition of this NF-E1 oligonucleotide to the K562 extract efficiently segregated active NF-E1 molecules and thus eliminated the specific NF-E1 footprint on an end-labeled restriction fragment (Fig. 3C).

Sp1-binding sites overlap both CBF- and NF-E1-binding sites. Although the DNase I digestion assay in the K562 extract showed only two footprints, NF-E1 and CBF (Fig. 1), there was at least one other nuclear factor, Sp1, that was

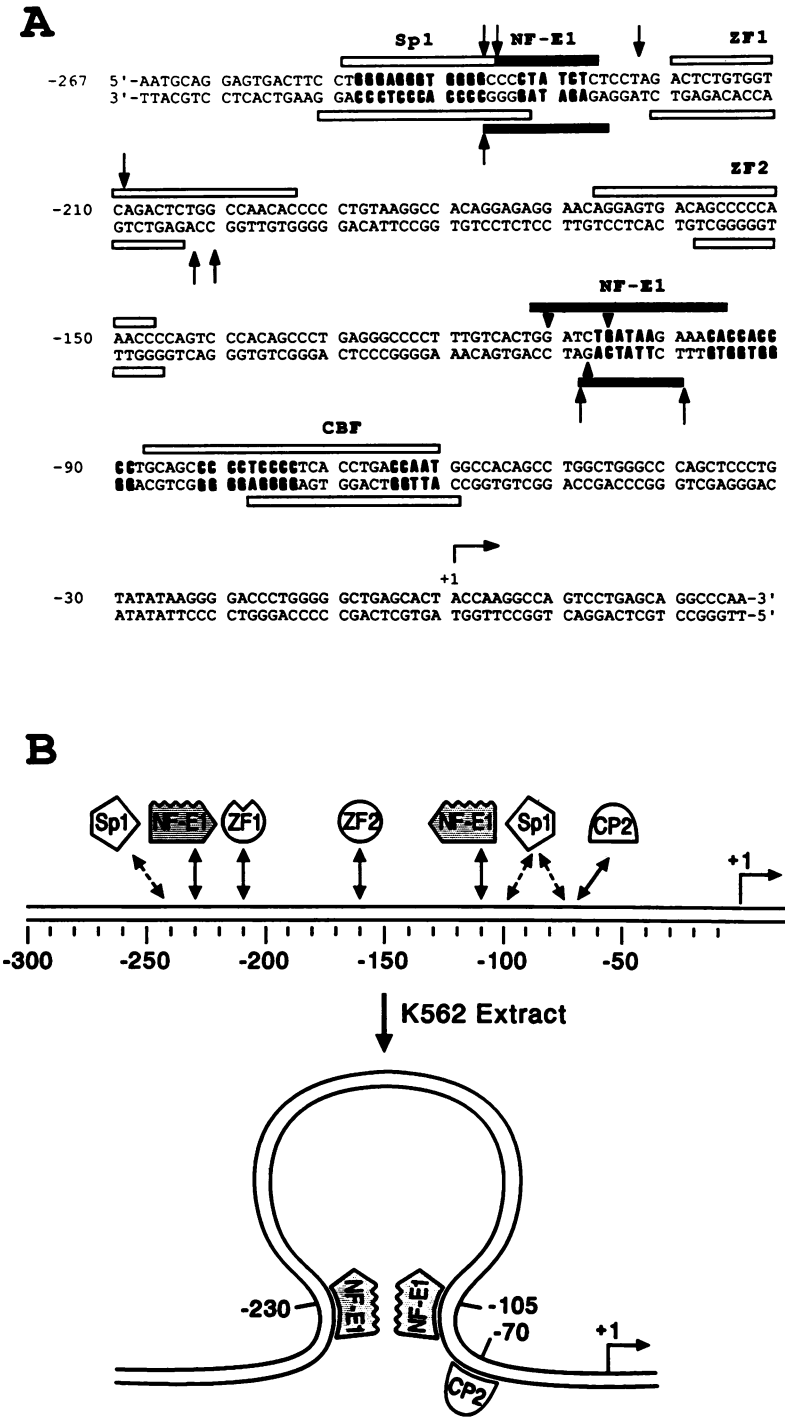

FIG. 2. (A) Summary of DNase I footprints of the human ζ-globin upstream promoter region. Numbers indicate nucleotide positions relative to the cap site (+1) of the ζ-globin gene. Symbols: ▭, footprints generated in both HeLa and K562 extracts; ▬, footprints seen only in the K562 extract; ↓, ↑, enhanced DNase I cleavages in the extracts; ▼, G residue in the proximal promoter region that is protected from methylation by the K562 extract (Fig. 3B and data not shown). The core binding sequences of nuclear factors NF-E1, Sp1, and CP2 are indicated in boldface. Note that there are two potential Sp1-binding sites upstream of the CCAAT box (Fig. 4C), which are also indicated in boldface. (B) Schematic representation of nuclear factor-DNA interactions in the ζ-globin upstream promoter region. The binding sites of Sp1 and NF-E1 at the distal and proximal promoter regions are of opposite orientations, as indicated by the asymmetry of the shapes of the factor molecules. Looping of the DNA helix via an NF-E1 dimer could bring the distal and proximal promoter regions into juxaposition (see Discussion).

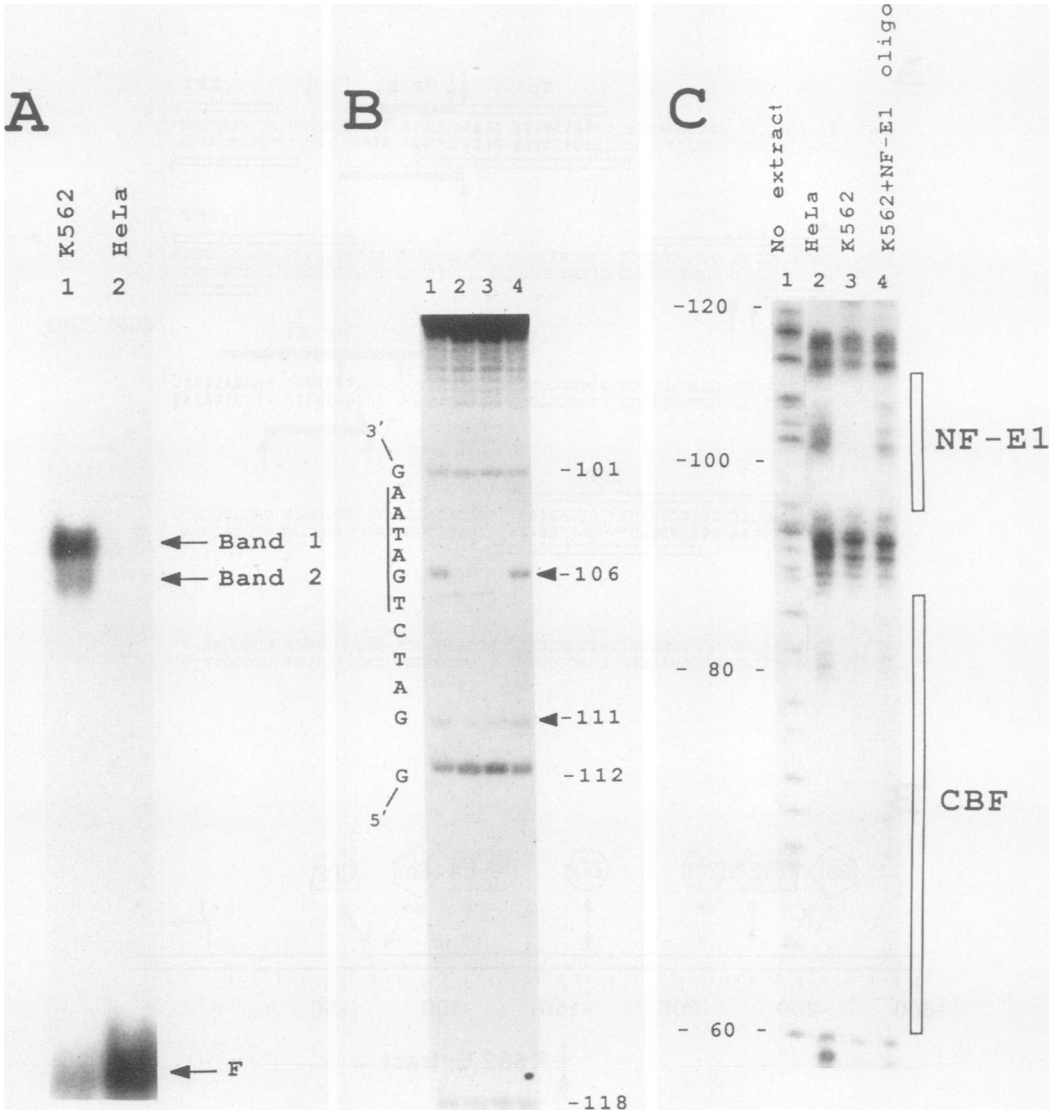

FIG. 3. NF-E1-binding activities in a K562 nuclear extract. (A) Gel mobility shift assay. A synthetic NF-E1 oligonucleotide spanning the DNA sequence from −122 to −90 was labeled with ^{32}P at its 5′ end by kinase reaction (31) and subjected to gel mobility shift assays as described in Materials and Methods. Lanes: 1, after incubation in a K562 extract; 2, after incubation in a HeLa extract. Positions of the two retarded protein-DNA bands (1 and 2) in the K562 extract and of the free probe (F) are indicated. (B) Methylation interference assay. The two protein-DNA complex bands and the free probe from panel A were analyzed by methylation interference assay. Lanes: 1 and 4, free probe; 2, band 1; 3, band 2. Sequences of the central part of the NF-E1 oligonucleotide are shown on the left. The hexanucleotide sequence homologous to the eucaryotic NF-E1 factor-binding consensus (see text) is indicated by a vertical line. Positions of the four G residues are indicated on the right. Two of these G residues, at −106 and −111, were protected from methylation by K562 extract. (C) Competition footprinting experiment. The DNase I footprint of the sense strand was analyzed as for Fig. 1A with or without preincubation of the K562 extract with the NF-E1 oligonucleotide. Lanes: 1, no extract; 2, DNase I footprint in the HeLa extract; 3, DNase I footprint in the K562 extract; 4, DNase I footprint in the K562 extract plus a 50-fold molar excess of NF-E1 oligonucleotide competitor. Note that the NF-E1 footprint, but not the CBF footprint, was effectively abolished by the oligonucleotide competitor.

able to bind to the proximal promoter region of ζ-globin gene.

A DNA fragment containing sequences from −131 to −50 was end labeled and used as the probe in gel mobility shift assays (Fig. 4). Under these conditions, only a small percentage of the DNA molecules bind to nuclear factors in the extracts, thus allowing the separation and detection of different protein-DNA complexes in the gel. Incubation of the fragment in the HeLa extract gave rise to one major band (S1) and five minor bands (C, S2, S3, S4, and S5) (Fig. 4A, lane 1). When a synthetic oligonucleotide containing the binding site of nuclear factor Sp1 (13) was used as the competitor, all bands except band C disappeared (Fig. 4A, lane 2). This result suggested that bands S1 through S5 were all protein-DNA complexes formed between the restriction fragment and Sp1 molecules of the HeLa extract. This possibility is further supported by the fact that at least three of these bands, S1, S2, and S4, comigrated on gels with those protein-DNA complexes formed between the DNA restriction fragment and affinity-purified Sp1 (compare lanes 1 and 6 of Fig. 4B).

When the restriction fragment was incubated in K562

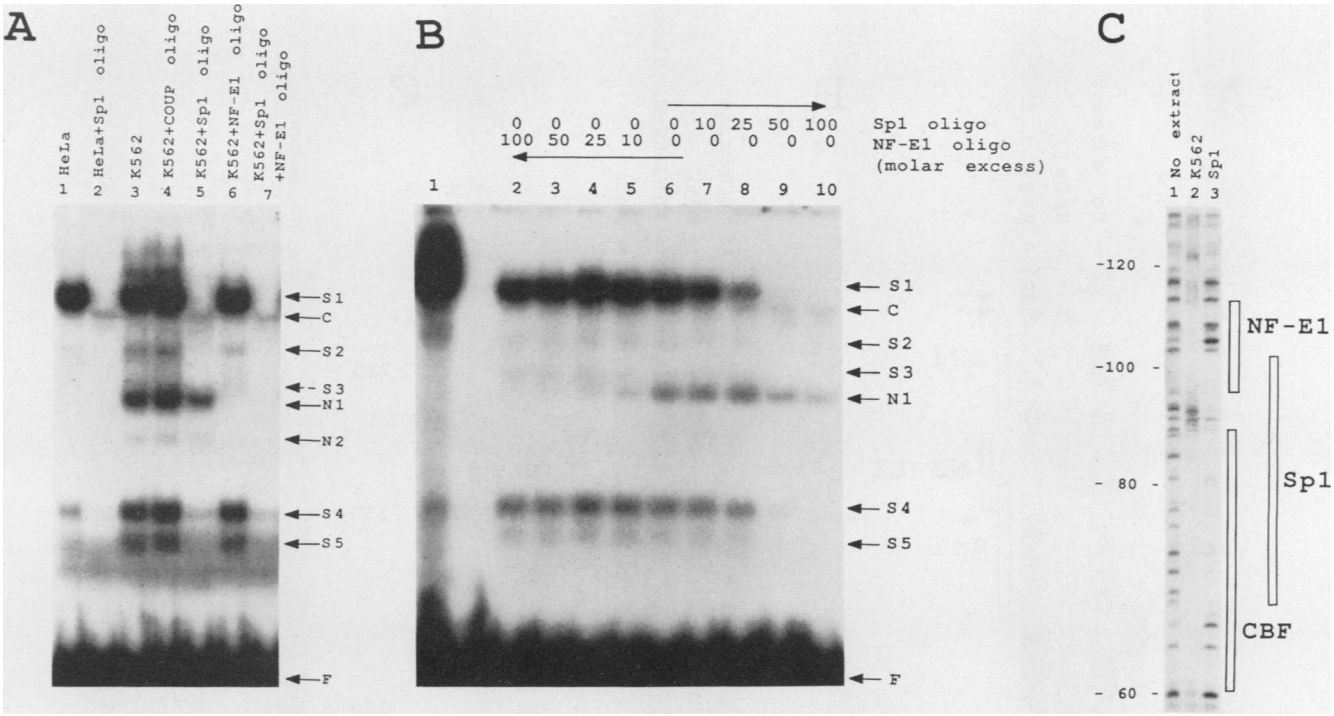

FIG. 4. (A) Gel mobility shift assays of the proximal promoter region with different competitors. A *Dde*I-*Bst*NI restriction fragment spanning the ζ proximal promoter sequence of −131 to −50 was labeled with ^{32}P and subjected to gel mobility shift analyses with or without the presence of 100-fold molar excess amounts of various oligonucleotide competitors. Lanes: 1, HeLa extract, no competitor; 2, HeLa extract plus Sp1 oligonucleotide competitor; 3, K562 extract, no competitor; 4, K562 extract plus COUP oligonucleotide competitor; 5, K562 extract plus Sp1 oligonucleotide competitor; 6, K562 extract plus NF-E1 oligonucleotide competitor; 7, K562 extract plus both Sp1 and NF-E1 oligonucleotide competitors. The various retarded protein-DNA complex bands were identified as follows: S1 to S5, putative Sp1-DNA complexes; N1 and N2, putative NF-E1-DNA complexes; C, the putative CBF-DNA complex. Band C is not easily visible in lanes 1, 3, 4, and 6 because of the high intensity of band S1. Also, band S3 is not easily seen on this gel but is more visible in panel B. (B) Gel mobility shift assays with varying concentrations of NF-E1 and Sp1 oligonucleotide competitors. The sample in lane 6 is the retardation pattern in the K562 extract of the probe used in panel A. On this autoradiograph, however, band N2 is not as visible as in panel A because of the shorter exposure time. Binding reactions in lanes 2 to 5 are the same as in lane 6 but in the presence of 100-, 50-, 25-, and 10-fold, respectively, excess amounts of the NF-E1 oligonucleotide. Lanes 7 to 10 are from binding reactions containing 10-, 25-, 50-, and 100-fold, respectively, excess amounts of the Sp1 oligonucleotide. Lane 1 shows the pattern of protein-DNA complexes formed between affinity-purified Sp1 and the DNA probe. (C) DNase I footprint of affinity-purified Sp1 on the proximal promoter region. The DNase I footprint of the sense strand was analyzed as for Fig. 1A. Lanes: 1, no extract; 2, footprint in the K562 extract; 3, footprint of affinity-purified Sp1. Note that the Sp1 footprint pattern is distinct and overlaps that in the K562 extract.

extract, in addition to the six bands seen with the HeLa extract, two other bands, N1 and N2, were observed (Fig. 4A, lane 3). Although an oligonucleotide containing the binding site for the transcription factor COUP could not act as a competitor (lane 4), incubation with an excess of oligonucleotides containing Sp1-binding sites effectively eliminated most of bands S1, S2, S3, S4, and S5 but not bands C, N1, and N2 (lane 5). On the other hand, bands N1 and N2 were eliminated by competition with the NF-E1 oligonucleotide probe containing an NF-E1-binding site (lane 6). Combined use of Sp1 and NF-E1 oligonucleotides as competitors eliminated all bands except band C (lane 7).

A more systematic oligonucleotide competition experiment of protein-DNA complexes formed in the K562 extract is shown in Fig. 4B. Again, the Sp1 oligonucleotide specifically inhibited formation of complexes S1, S2, S3, S4, and S5 but not bands N1, N2, and C (compare lanes 6 to 10 of Fig. 4B), whereas the NF-E1 oligonucleotide specifically eliminated only bands N1 and N2 (compare lanes 2 to 6 of Fig. 4B). The sample loaded in lane 1 of Fig. 4B contained protein-DNA complexes formed between affinity-purified Sp1 and the DNA probe. It had three bands comigrating with S1, S2, and S4, respectively, formed in the K562 extract (Fig. 4B, lane 6). This result further confirmed that S1, S2, and S4 were Sp1-DNA complexes. We suspect that S3 and S5 were formed between the DNA probe and some degradation products or modified forms of Sp1 in the nuclear extracts.

Similarly abundant and specific Sp1-DNA complexes could form in either the HeLa or K562 nuclear extract. However, NF-E1-DNA complexes could form only in the K562 extract, and their quantities were probably much lower than those of the Sp1-DNA complexes, as judged from the relative band intensities of the autoradiographs (Fig. 4A and B). Two candidates for Sp1-binding sites in the proximal promoter region of ζ globin are DNA sequences from −82 to −74 (5′-CCCCTCCCC-3′/3′-GGGGAGGGG-5′) and from −97 to −88 (5′-CACCACCCC-3′/3-GTGGTGGGG-5′). Except for a base mismatch of the second sequence, both sequences have been demonstrated to be substrates of an Sp1-binding reaction in vitro (29). We have demonstrated by DNase I footprinting that affinity-purified Sp1 is able to protect the DNA region of −101 to −68 (Fig. 4C). This region covers the two putative Sp1-binding sites mentioned

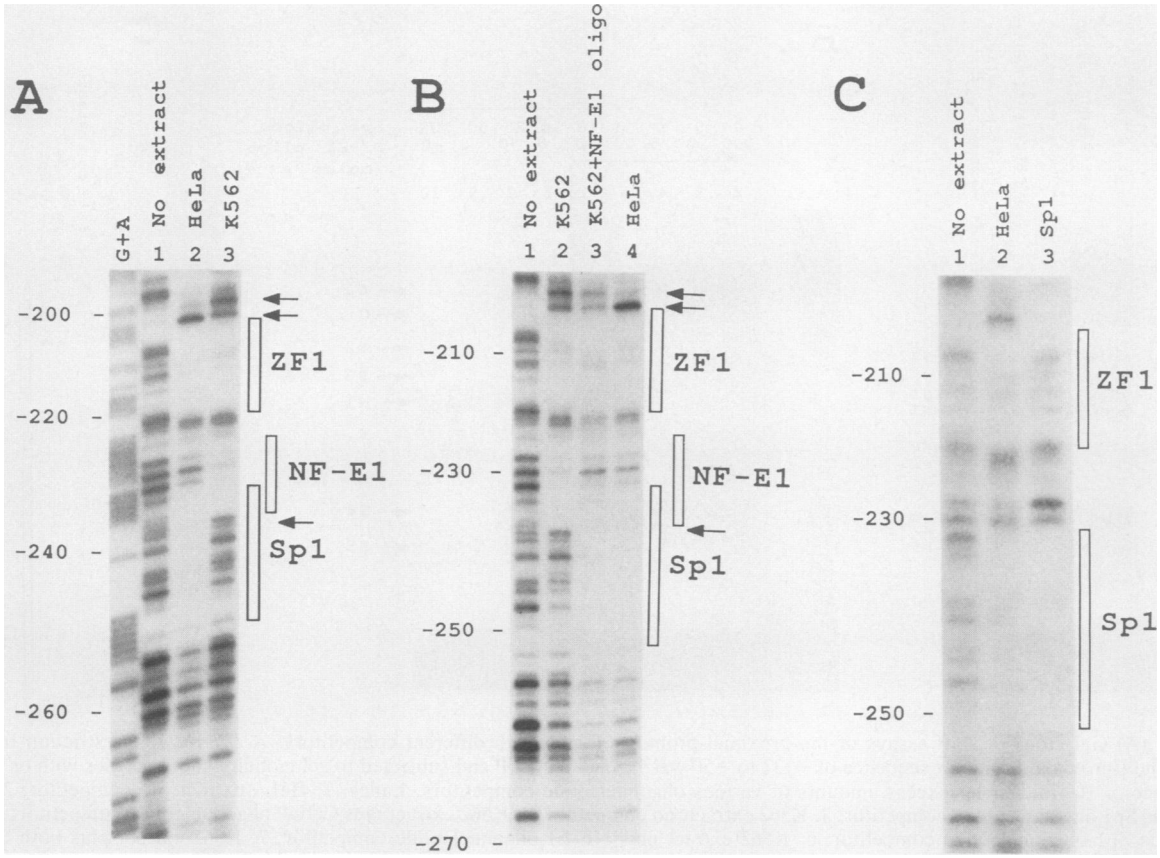

FIG. 5. Footprint analysis of the distal promoter region of the ζ-globin gene. (A) Footprint of the antisense strand. Restriction fragment *Ava*II-*Pst*I, which spans the region of −359 to −85, was end labeled with ^{32}P at the *Ava*II end and used as a probe for DNase I analysis in different nuclear extracts. Lanes: 1, no extract; 2, HeLa extract; 3, K562 extract. The footprints of Sp1, NF-E1, and ZF1 are indicated on the right. Also indicated (←) are sites of enhanced DNase I cleavages. A detailed map of the footprints is shown in Fig. 2. (B) Competition footprint analysis. The DNase I footprint analysis of panel A was repeated in the presence of a 100-fold excess of the NF-E1 oligonucleotide competitor. Lanes: 1, no extract; 2, K562 extract, no competitor; 3, K562 extract plus competitor; 4, HeLa extract, no competitor. (C) Footprinting analysis of the distal promoter region of ζ globin with affinity-purified Sp1. The DNA probe used was the same as that used for panel A. Lanes: 1, no extract; 2, footprint in the HeLa extract; 3, footprint of purified Sp1. Note that the same footprint was generated at the putative Sp1-binding site both on the crude HeLa extract and by Sp1 purified from HeLa cells. The extra DNase I cleavage at −232 in lane 2 is due to either the binding of ZF1 or incomplete protection by Sp1 in the crude extract.

above and apparently overlaps both the CBF and NF-E1 footprints (compare lanes 2 and 3 of Fig. 4C).

The distal promoter region of the ζ-globin gene also contains a cluster of nuclear factor-binding sites: NF-E1, Sp1, and ζ-globin factor 1 (ZF1). When restriction fragments spanning the DNA sequence from −395 to −108 were used as probes for the DNase I footprint assay, three factor-binding sites were found to be clustered in the region of −251 to −194 (Fig. 5).

In the HeLa extract, two footprints were observed (Fig. 5A, lane 2). One was located at −251 to −233 on the antisense strand (Fig. 2 and 5A). It contained the sequences 5′-CCCACCC-3′ and 5′-CCCTCCC-3′, both of which are in vitro binding sites for affinity-purified Sp1 (29). Relative to the crude HeLa extract, the affinity-purified Sp1 generated the same footprint as shown in Fig. 5C. The other footprint in the HeLa extract, which was labeled ZF1, mapped to −221 to −204 on the antisense strand (Fig. 2 and 5A). This region is probably a binding site for activation transcription factor (ATF) (28), since it contains the sequence 5′-GTGG TCA-3′, which has a one-base mismatch to the consensus binding site for ATF (28). We have not characterized this binding activity in further detail.

The K562 nuclear extract gave an interesting footprint pattern in contrast to that of the HeLa extract (compare lanes 2 and 3 of Fig. 5A). Whereas the ZF1 footprint was still present, the Sp1 footprint observed in the HeLa extract was not detectable in the K562 extract. Instead, a new footprint overlapping the HeLa Sp1 footprint region appeared. This K562 extract-specific footprint spanned the DNA region of −237 to −226 on the antisense strand and contained the core binding consensus for NF-E1 (Fig. 2). It was therefore labeled NF-E1 (Fig. 5A) to indicate that this footprint also resulted from binding of NF-E1 to the DNA helix.

Mutually exclusive interaction of NF-E1 and Sp1 at the ζ distal promoter region. Binding of NF-E1 of the K562 extract to the region of −237 to −226 was further tested by oligonucleotide competition experiments. When the NF-E1 oligonucleotide competitor was included in the preincubation step of DNase I digestion reactions, the NF-E1 footprint at the ζ distal promoter region disappeared, whereas the ZF1 footprint remained (compare lanes 2 and 3 of Fig. 5B). Furthermore, disappearance of the NF-E1 footprint was accompanied by the appearance of a footprint identical to the Sp1 footprint seen in the HeLa extract (compare lanes 3 and 4 of Fig. 5B). This result showed that NF-E1 binding in

the K562 extract was more dominant than Sp1 binding, and it excluded further binding of Sp1 to DNA sequence from −251 to −233.

The dominance of binding of NF-E1 could be the result of a higher concentration of NF-E1 than of Sp1 in the K562 extract. Alternatively, NF-E1 may have a higher affinity for its binding site than does Sp1. In this regard, the gel mobility shift assay of protein-DNA complexes formed between the K562 extract and the proximal ζ promoter sequences (Fig. 4) suggested that the concentration of NF-E1 was much lower than that of Sp1, which was of similar abundance in HeLa and K562 extracts. A competition mobility shift experiment using a DNA probe containing the overlapping NF-E1- and Sp1-binding sites of the distal ζ promoter region is shown in Fig. 6.

This DNA probe also formed abundant Sp1-DNA complexes in the K562 extract (Fig. 6, lane 4). These complexes, which included S1, S2, S3, and S4, could all be eliminated by Sp1 oligonucleotides (lanes 5 to 7) but not by NF-E1 oligonucleotides (lanes 1 to 3). The putative NF-E1–DNA complex (band N), however, could be effectively eliminated only by NF-E1 oligonucleotides (lanes 1 to 3). Similar to the results in Fig. 4, the concentration of Sp1 molecules in the K562 extract was much higher than that of NF-E1 (Fig. 6). All of these data indicate that the dominant binding of NF-E1 over Sp1 to the distal ζ promoter region is due to a higher DNA-binding affinity of NF-E1.

Displacement of Sp1 binding by NF-E1 binding. To further study the dominance of NF-E1 binding over that of Sp1, the following experiments were performed (Fig. 7). First, a titration experiment of the footprints in the K562 extract was carried out by using the same DNA probe as was used for Fig. 5A. Faint ZF1 and NF-E1 footprints appeared with 25 μg (Fig. 7A, lane 3) but not 10 μg (lane 2) of extract. They both became more evident with 50 μg of extract (lane 4). With 100 μg of extract, most of the DNA molecules were protected from DNase I digestion at the ZF1- and NF-E1-binding sites (lane 5). Throughout the range of K562 extract amounts used (lanes 2 to 5), no clear Sp1 footprint could be seen.

A similar experiment was performed with the HeLa extract (Fig. 7B). In this case, no NF-E1 binding could be detected throughout the titration range. However, the appearance of an Sp1 footprint in the HeLa extract (Fig. 7B) paralleled that of the K562-specific NF-E1 footprint (Fig. 7A). That is, the HeLa-specific Sp1 footprint was also absent in 10 μg of extract, faint in 25 μg of extract, and most clear in 100 μg of extract (Fig. 7B). To further compare the binding ability of Sp1 in the K562 extract with that in the HeLa extract, the experiment of Fig. 7A was repeated with the inclusion of a 50-fold molar excess of NF-E1 oligonucleotide as the competitor in each reaction. The resulting Sp1 footprint patterns at different concentrations of the K562 extract were similar, if not identical, to those of the HeLa extract (compare Fig. 7B and C). These results demonstrate that the K562 and HeLa extracts have similarly abundant and active Sp1 molecules and that the lack of binding of Sp1 in the K562 extract to the ζ distal promoter region was indeed due to the presence of the NF-E1 molecules.

On the basis of the data of Fig. 7, we chose 50 μg of the K562 extract for an extract-mixing experiment (Fig. 8A). In this experiment, DNase I digestion was performed in 50 μg of K562 extract (Fig. 8A, lane 2), 50 μg of K562 extract plus 50 μg of HeLa extract (lane 3) or 50 μg of K562 extract plus 100 μg of HeLa extract (lane 4). A 50-μg amount of K562 extract alone gave ZF1 and NF-E1 footprints (Fig. 8A),

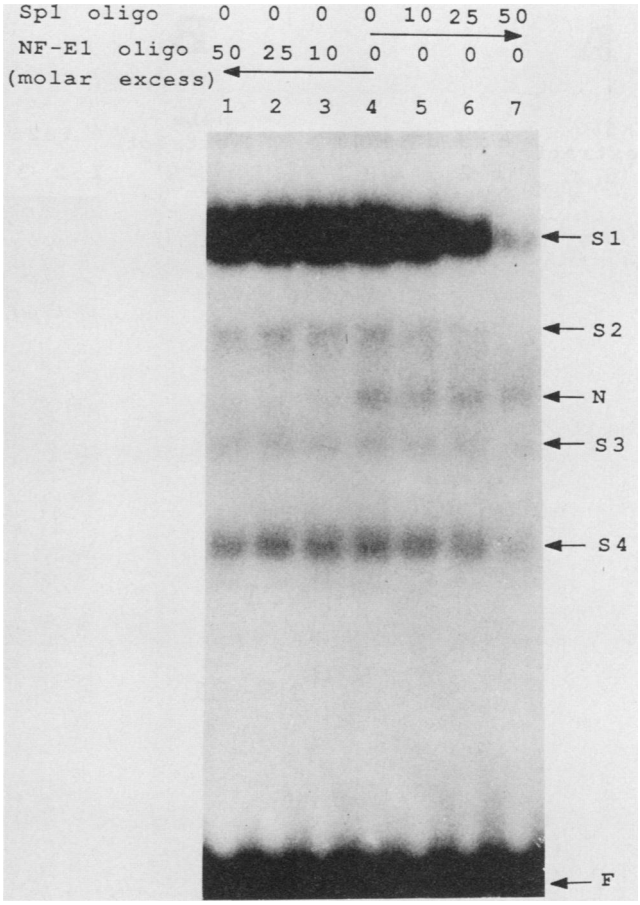

FIG. 6. Gel mobility shift assay and competition experiments. Restriction fragment *Bst*NI-*Bal*I, which spans the region of −249 to −201, was end labeled with ^{32}P and used as the probe for a gel mobility shift assay in the K562 extract. Different concentrations of Sp1 and NF-E1 oligonucleotides were used as competitors. Lanes: 1 to 3, mobility shift assays in the K562 extract containing 50-, 25-, and 10-fold, respectively, excess amounts of NF-E1 oligonucleotide; 4, K562 extract, no competitor; 5 to 7, mobility shift assay in the K562 extract containing 10-, 25-, and 50-fold, respectively, excess amounts of the Sp1 oligonucleotide competitor. The putative Sp1-DNA complexes are labeled S1, S2, S3, and S4. N, The major NF-E1–DNA complex; F, free probe. The ZF1-DNA complex is not seen, probably because of a low concentration of ATF or conditions not favoring formation of the complex.

similar to the results shown in Fig. 7. Addition of neither 50 nor 100 μg of HeLa extract could abolish the NF-E1 footprint or generate the Sp1 footprint. In the reverse experiment, DNase I digestion was performed in a constant amount (100 μg) of the HeLa extract plus various amounts of the K562 extract (Fig. 8B). In this case, the HeLa extract alone (Fig. 8B, lane 2) gave clear ZF1 and Sp1 footprints, as described earlier. The copresence of increasing amounts of K562 extract (lanes 3 to 6) had no apparent effect on the ZF1 footprint. However, the Sp1 footprint gradually disappeared while a NF-E1 footprint was generated (compare lanes 2 to 6).

A third factor-displacement experiment was performed in which the HeLa and K562 extracts were incubated together or sequentially with the DNA probe before it was digested by DNase I (Fig. 8C). Almost identical footprint patterns were obtained when the probe was incubated with premixed

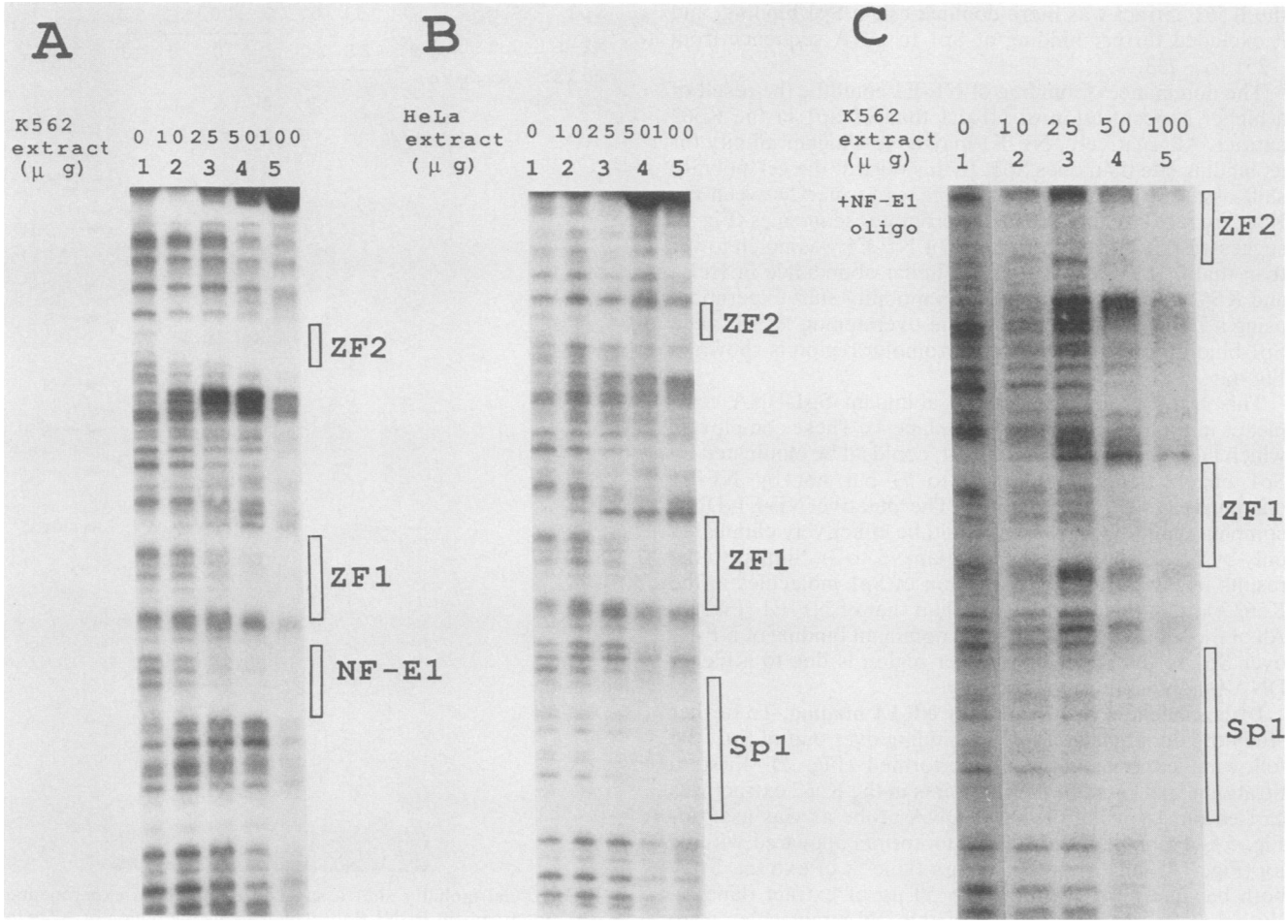

FIG. 7. Titration of DNase I footprints of the distal promoter region with different amounts of extract. A 1-ng sample of the probe used for Fig. 5A was used for DNase I footprinting assays in the presence of various amounts of the (A) K562 extract (A), the HeLa extract (B), and the K562 extract plus NF-E1 oligonucleotide competitor (C). Lanes: 1, no extract; 2 to 5, 10, 25, 50, 100 μg, respectively, of extract. The amount of competitor used in panel C was 50 molar in excess of the probe. ZF2 indicates a footprint seen in both HeLa and K562 extracts that was not further characterized. Its map is shown in Fig. 2.

K562 and HeLa extracts (Fig. 8C, lane 2), with the HeLa extract first then with the K562 extract (lane 3), or with the K562 extract first then with the HeLa extract (lane 4). In all three cases, only ZF1 and NF-E1 footprints, not an Sp1 footprint, were present. These results showed not only that NF-E1 binding to the ζ region of −237 to −226 is dominant over Sp1 binding to the region of −251 to −233 when the two factors are premixed but also that NF-E1 is able to displace prebound Sp1.

DISCUSSION

As with many other eucaryotic genes, the transcription of globin gene families may be modulated at several levels of cellular mechanisms. One of these is the chromatin or chromosome structure, which is manifested by DNase I hypersensitivity in vivo and by nucleotide sequence-dependent DNA bendability for nucleosome formation (5, 20, 22, 23, 42a, 47, 50). Another level is DNA modification. Hypermethylation within and surrounding the coding regions is closely associated with transcriptional inactivity of the eucaryotic globin genes (references 43 and 51 and references therein). Finally, interactions between specific DNA sequences and cell type-specific or developmental stage-specific transcription factors are also expected to play an essential role in globin gene regulation (for references, see above).

Our studies of binding of cell type-specific nuclear factors to the human ζ-globin promoter region (summarized in Fig. 2) have revealed several interesting characteristics that may be relevant to the regulatory mechanisms of the ζ-globin gene transcription. As shown in Results, comparative binding studies in nuclear extracts of erythroid K562 cells and nonerythroid HeLa cells identified multiple protein-binding sites in the upstream promoter region of human ζ globin. These sites are distributed mainly in two regions upstream of the ζ-globin initiation sites: from nucleotides −112 to −60 and from nucleotides −251 to −194. Both regions show distinctive patterns of nuclear factor-DNA interactions between K562 and HeLa cells.

The region of −112 to −60. DNase I footprint analysis of the proximal promoter region revealed two factor-binding sites located adjacent to each other in the region of −112 to −60 (Fig. 1 to 4). The upstream site interacts with NF-E1 (Fig. 1 and 3), which is an erythroid cell-specific nuclear factor and therefore is present only in K562, not HeLa, cells. The downstream site interacts with a factor that is present in

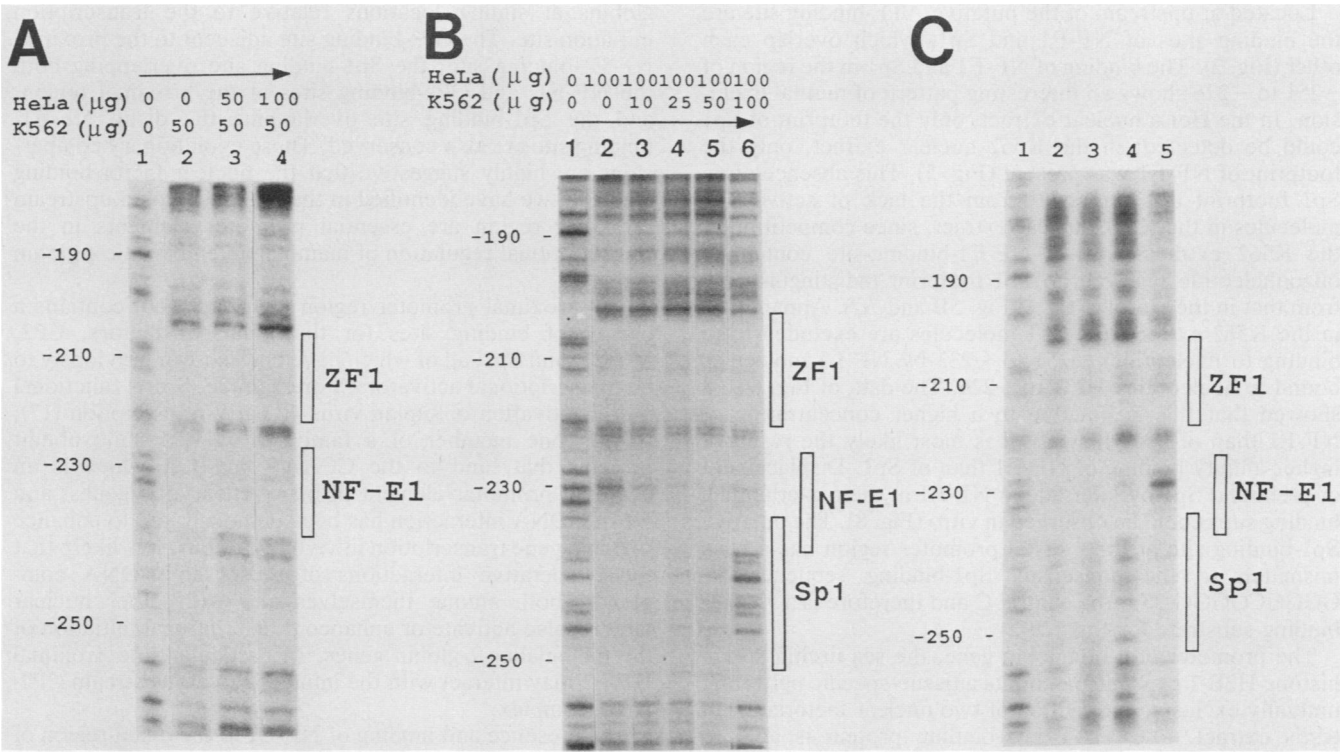

FIG. 8. Dominant binding of NF-E1 over Sp1 at their overlapping binding sites. (A) A 50-μg sample of the K562 extract was premixed with different amounts of the HeLa extract before the two were added together to the DNA probe for DNase I footprinting. Lanes: 1, no extract; 2, K562 extract alone; 3, 50 μg each of K562 and HeLa extracts; 4, K562 extract plus 100 μg of HeLa extract. The DNA probe is that used for Fig. 5A. (B) A 100-μg sample of the HeLa extract was premixed with increasing amounts of the K562 extract before the two were added together to the DNA probe for DNase I footprinting. Lanes: 1, no extract; 2 to 6, K562 extract in amounts of 0, 10, 25, 50, and 100 μg, respectively. (C) Before DNase I digestion, the DNA probe was incubated with the HeLa and K562 extracts together for 1 h (lane 2), with the HeLa extract for 1 h and then with the K562 extract for 1 h (lane 3), with the K562 extract for 1 h and then with the HeLa extract for 1 h (lane 4), or with the HeLa extract alone for 1 h (lane 5). Lane 1, No extract. A 125-μg amount of the HeLa or K562 extract was used in lanes 2 to 5.

both HeLa and K562 extracts (Fig. 1 and 2). Since it contains the CCAAT motif, the factor bound to this downstream site is most likely one of the previously characterized CBFs. We speculate that this CBF is CP2 (see below).

There are two potential Sp1-binding sequences located in between the proximal NF-E1-binding site and the CCAAT box (Fig. 2). Footprint analysis using affinity-purified Sp1 (Fig. 4C) demonstrated that they are indeed bound by Sp1. The footprint generated with purified Sp1 spans the DNA region of −105 to −67 (Fig. 4C, lane 3), which overlaps both the NF-E1 footprint seen in the K562 extract and the CP2 footprint seen in the K562 and HeLa extracts (Fig. 4C, lane 2). Thus, the failure to observe an Sp1 footprint in both extracts (Fig. 1) is most likely due to the dominant binding of CP2 or NF-E1 over Sp1. Since there is no NF-E1-binding activity in the HeLa extract, CP2 binding alone is probably sufficient to exclude the binding of Sp1 to the DNA region of −112 to −60.

Several mammalian CBFs have been isolated and characterized. The DNA-binding specificities of these protein factors have been studied by gel mobility shift assays, DNase I footprinting analysis, and methylation interference assay (2, 3, 9, 25). It was shown that different CBFs bind to different CCAAT box-containing promoter sequences with distinct patterns of affinities. For example, NF-1 binds to a CCAAT box-containing origin with an affinity 3 orders of magnitude higher than the affinity with which it binds to the human α-globin CCAAT element. However, the latter element is one of the preferred binding substrates for CP1. Both CP1 and CP2 also bind with high affinities to CCAAT elements of the *H-2K*^{b} promoter (9). Although preferred CP1 and CP2 binding sites have very similar consensus sequences surrounding the CCAAT box, these two factors show distinct protein-DNA contacts outside the CCAAT box. High-affinity CP1 binding shows preference for a CA dinucleotide immediately 3′ to the CCAAT box, whereas high-affinity CP2 binding shows a strong preference for sequences extending to at least 10 base pairs (bp) 5′, but not 3′, to the CCAAT box (9). NF-1, on the other hand, preferentially binds to sequences containing two inverted CCAAT boxes separated by 7 bp (9, 25). Comparison of DNA sequence surrounding the human ζ-globin CCAAT element and its footprint pattern in nuclear extracts (Fig. 1 and 2) with those of the different CBFs described above (9, 25) suggests that it is a preferred binding site for CP2.

The region of −251 to −194. The complex footprint patterns of the distal promoter region of the ζ-globin gene (Fig. 5) indicate that there are at least three different nuclear factors interacting with the DNA helix from −251 to −194. Two of these factors, Sp1 and ZF1, exist in both K562 and HeLa cells, and the third, NF-E1, is K562 cell specific. ZF1 binds to the DNA region of −221 to −194, and this binding induces enhanced DNase I cleavages flanking the footprints (Fig. 2 and 5). The ZF1-binding site contains the sequence 5′-GTGGTCA-3′, with a one-base mismatch to the consensus binding sequence for transcription factor ATF (28).

Located at upstream of the putative ATF-binding site are the binding sites of NF-E1 and Sp1, which overlap each other (Fig. 2). The binding of NF-E1 and Sp1 in the region of −251 to −226 shows an interesting pattern of mutual exclusion. In the HeLa nuclear extract, only the footprint of Sp1 could be detected; in the K562 nuclear extract, only the footprint of NF-E1 was present (Fig. 5). This absence of an Sp1 footprint did not result from the lack of active Sp1 molecules in the K562 nuclear extract, since competition of the K562 extract with an NF-E1-binding-site containing oligonucleotide restored an Sp1 footprint indistinguishable from that in the HeLa extract (Fig. 5B and 7C). Apparently, in the K562 extract, the Sp1 molecules are excluded from binding to nucleotides −251 to −233 by NF-E1 molecules bound to nucleotides −237 to −226. The data of Fig. 6 to 8 showed that this is not due to a higher concentration of NF-E1 than of Sp1. Instead, it is most likely the result of higher-affinity binding of NF-E1 than of Sp1. Displacement of prebound Sp1 by later-added NF-E1 at their overlapping binding sites could be observed in vitro (Fig. 8). The putative Sp1-binding site at the ζ distal promoter region has a base mismatch to the consensus Sp1-binding sequence 5′-GGGGCGGGG-3′ at the central C and therefore is a weaker binding substrate for Sp1 (29).

The promoter of a nonglobin gene, the sea urchin sperm histone H2B-1 gene, also exhibits a tissue-specific pattern of mutually exclusive interaction of two nuclear factors (2). In testis extract, a CCAAT box-binding protein is able to interact with both CCAAT elements in the sperm H2B promoter. However, in embryonic extract, another factor, CCAAT displacement protein, binds with high affinity to a sequence overlapping the proximal CCAAT box. This binding eliminates the interaction of CCAAT box-binding protein to either the distal or the proximal CCAAT box (2). The phenomenon of mutual exclusion of different nuclear factors at overlapping binding sites has also been implicated in studies of other globin promoters. Several nuclear factors have overlapping binding sites in the human β-globin promoter and its 3′ enhancer region (14, 49). The interaction between the mouse adult α-globin gene promoter and specific nuclear factors of mouse erythroid MEL cells and nonerythroid cells has also been studied (3, 27). In that case, the binding sites of two CBFs α-CP1 and α-CP2, which are the mouse homologs of human CP1 and CP2 (9), overlap at the mouse α CCAAT box (3). The binding of α-CP2 is dominant in MEL nuclear extracts. In nuclear extracts derived from nonerythroid cells, however, the binding of α-CP1 is dominant. Interestingly, the binding of α-CP1 to the mouse α CCAAT box in nonerythroid nuclear extracts also excludes the binding of α-IRP, a mouse Sp1-like transcription factor (3).

Functional implications. Although the biological roles of nuclear factor-DNA interaction at the ζ-globin promoter region await further detailed expression studies, the analysis presented here has interesting implications with respect to the functions of several promoter sequences.

The nucleotide sequences of the upstream promoter regions of several mammalian ζ-globin genes have been analyzed and compared (8). Similarity in the sequences extending to 600 bp upstream was noted among the functional ζ-globin genes of human, mouse, and goat and a ζ-globin pseudogene of rabbit. Among these sequences are several short stretches of high homology, and these include sequences identified in this study to be the binding sites for specific nuclear factors. In particular, the two human NF-E1-binding sites are conserved in the mouse and goat ζ globins at similar locations relative to the transcription initiation site. The CP2-binding site adjacent to the proximal NF-E1-binding site, the Sp1-binding site overlapping both the NF-E1- and CP2-binding sites at the proximal region, and the Sp1-binding site overlapping the distal NF-E1-binding site are also conserved. These evolutionary comparisons are highly suggestive that the nuclear factor-binding sites that we have identified in the human ζ-globin upstream promoter region are essential promoter elements in the transcriptional regulation of mammalian embryonic ζ-globin genes.

The proximal promoter region (−112 to −60) contains a cluster of binding sites for three nuclear factors, CP2, NF-E1, and Sp1, all of which have been shown previously to be transcriptional activators. For example, Sp1 is functional in the activation of simian virus 40 early transcription (17); CP2 is one member of a family of related multisubunit proteins that bind to the CCAAT box (9), which is an essential promoter element of many eucaryotic genes; and NF-E1–DNA interaction has been demonstrated to enhance γ-globin gene transcription in vivo (34). Thus, it is likely that the cooperative interactions of these factor-DNA complexes, both among themselves and with other nuclear factors, also activate or enhance transcriptional initiation of the mammalian ζ-globin genes. In particular, the proximal NF-E1 may interact with the immediately downstream CP2-DNA complex.

The presence and binding of NF-E1 to the distal region of −237 to −226 may also participate in the activation of human ζ-globin gene transcription in erythroid cells. The conservation of the overlapping pattern of this NF-E1-binding site and an Sp1-binding site among mammals (see above) suggests that the mutually exclusive interaction between these two factors plays a fundamental role in transcriptional regulation of the ζ-globin gene. It is not clear, though, why Sp1 is involved, since this factor is a transcriptional activator for other eucaryotic genes and yet, in the ζ-globin gene, the binding of Sp1 to the region of −251 to −233 is displaced by binding of NF-E1 in erythroid K562 extracts. Could Sp1 act as a transcriptional repressor of the ζ-globin gene in nonerythroid cells?

A higher order of interactions among different nuclear factor-DNA complexes may also be involved in the activation of ζ-globin gene transcription in erythroid cells. For example, the pair of NF-E1-binding sites in the ζ proximal and distal promoter regions are directed in opposite orientations (Fig. 2). Recently, it has been shown that the murine NF-E1 factor has two zinc fingers (46) and that both the murine and human NF-E1 factors are able to bind to two NF-E1-binding sites at the same time (34, 46). Furthermore, NF-E1 could form a dimer (46). Thus, via a single NF-E1 molecule or a dimer of NF-E1, the proximal and distal NF-E1-binding sites of the mammalian ζ-globin promoter could be brought into close proximity, as shown in the looping model of Fig. 2B. Whether this conformation participates in ζ-globin gene activation also awaits further detailed chromatin structure analysis and functional studies of a mutagenized ζ-globin promoter.

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