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Genetics

Isolation of a differentially regulated splicing isoform of human NF-E2

(basic leucine zipper transcription factor/transcriptional regulation/hemoglobin switching/alternative promoter)

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ABSTRACT **The transcription factor NF-E2 (nuclear factor erythroid 2), interacting via DNA motifs within regulatory regions of several hematopoietic genes, is thought to mediate the enhancer activity of the globin locus control regions. By screening a human fetal liver cDNA library with probes derived from mouse NF-E2, we have isolated a splicing variant of the NF-E2 gene (fNF-E2) that differs in the 5′ untranslated region from the previously reported cDNA (aNF-E2). The fNF-E2 isoform is transcribed from an alternative promoter located in the 3′ end of the first intron and joined by alternative splicing to the second and third exons, which are shared by both RNA isoforms. Although the two forms produce the same protein, they are expressed in different ratios during development. fNF-E2 is more abundant in the fetal liver and less abundant in the adult bone marrow compared to the previously described form. Their distribution apparently follows the differential expression of fetal and adult hemoglobins.**

The expression of the five globin genes in the human β-globin gene cluster is subjected to tight developmental control (1, 2). In the embryonic stage of development, when erythropoiesis is confined to the yolk sac, the ε-globin gene is the only active gene in the cluster. Growth to the fetal stage of development is concurrent with a change in the major site of erythropoiesis from the yolk sac to the fetal liver and a switch from ε- to γ-globin gene expression. Later erythropoiesis switches to the bone marrow and globin production also switches from fetal to adult δ- and β-globin genes. The hemoglobin switching has possible therapeutic implications because its understanding and subsequent manipulation could possibly lead to reactivation of the γ-globin genes in adult life for the treatment of sickle cell anemia and thalassemia (3).

The locus control region upstream of the β-globin cluster plays an important role not only in the high level of expression of globin genes in erythroid cells but also in hemoglobin switching. Currently, one hypothesis proposes that the locus control region sequentially interacts with embryonic, fetal, and adult genes during development (4, 5). These interactions are mediated through a series of DNA–protein and protein–protein interactions. Cis sequences and trans-acting factors have been identified; some are ubiquitous, while others are restricted to the hematopoietic tissues. Among the factors that are specific to the hematopoietic cells are GATA-1 (6), which binds to the WGATAR sequence; EKLF (erythroid Kruppel-like factor) (7), which binds to a CACCC sequence at the β-globin promoter (8); and nuclear factor erythroid 2 (NF-E2) (9–13), which binds to GCTGASTCA sequences. NF-E2 is a basic leucine zipper protein of the AP1 family that is primarily expressed in erythroid and megakaryocytic series, although small amounts of message can be found in other organs such as lung, intestines, and placenta (12). As mice with "knockout" of this gene die early from thrombocytopenia with only mild to moderate anemia (14), it is not yet understood what role NF-E2 plays in erythropoiesis. In this study, we describe an isoform of NF-E2 derived from the usage of an alternative first exon. The effect of these two isoforms with different exons, both of which encode 5′ untranslated regions, is to bring in two different promoters with different putative protein binding motifs. RNA analyses indicate that the new isoform is produced more abundantly in fetal hematopoietic tissue and the previously described form is more abundant in adult hematopoietic tissues. These are designated fNF-E2 and aNF-E2, respectively.

MATERIALS AND METHODS

cDNA Cloning and RNA Analysis. cDNA clones were isolated by screening of a human fetal liver cDNA library (Clontech) with a DNA probe derived from mouse NF-E2 cDNA (15). Genomic clones were isolated from a human placental genomic library in the λ FIX II phage (Stratagene) by screening with the human NF-E2 cDNA. The 5′ ends of fNF-E2 and aNF-E2 cDNAs were amplified by a RACE (rapid amplification of cDNA ends) procedure (5′-AmpliFINDER RACE kit; Clontech) and the PCR products were sequenced up to the cap sites. Sequence analysis was done directly on λ phage DNA, on Bluescript (Stratagene) plasmid subclones, and on PCR products with the dideoxynucleotide chain-termination method (16) using a commercial cycle sequencing kit (fmol DNA sequencing system; Promega).

Northern blots were done on poly(A) mRNA prepared by the method of Chomczynski and Sacchi (17) followed by purification with poly(dT) spin columns (Pharmacia) according to the manufacturer's instructions.

***In Vitro* Transcription and Translation.** *In vitro* transcription and translation reactions were performed with the TNT kit (Promega) using purified plasmid DNA (Qiagen, Chatsworth, CA) and [^{35}S]methionine (Amersham). Translation products were separated on denaturing SDS/polyacrylamide gel and visualized by autoradiography.

Reverse Transcriptase (RT)-PCR Analysis. One microgram of total RNA was reverse transcribed into cDNA with Mohoney murine leukemia virus RT (BRL) according to the manufacturer's conditions. Primer c, which is located in the second NF-E2 exon and shared among the two isoforms, was used for cDNA synthesis. One of five aliquots was subjected to 35 cycles of PCR with isoform-specific primer sets (f and c for

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Abbreviations: EKLF, erythroid Kruppel-like factor; RT, reverse transcriptase; NF-E2, nuclear factor erythroid 2; ORF, open reading frame; SSP, stage selector protein; YY-1, yin and yang 1; CRE, cAMP responsive element.

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fNF-E2 and a and c for aNF-E2). After denaturation at 95°C for 5 min, cycling was initiated by the addition of AmpliTaq thermostable DNA polymerase (Perkin–Elmer) in the manufacturer's suggested buffer (2.5 mM $MgCl_2$) with the following profile: 94°C for 40 sec, 53°C for 40 sec, 72°C for 1 min. Sequences of oligonucleotides used for RT-PCR were as follows: c, 5′-GGCCAGAGTCTGGTCCAG-3′; f, 5′-GAGG GAAAGCAGGGTGA-3′; a, 5′-TCGCAGATTCTCAA AGG-3′.

RESULTS

cDNA Cloning. In the primary screening of the library, we isolated six overlapping cDNA clones that we could assemble into a unique full-length cDNA from the 5′ untranslated region to the poly(A) tail (Fig. 1). The cDNA had a nucleotide sequence almost completely identical to the one previously described for human NF-E2. In all three cDNA clones that extended 5′ enough to include part of the untranslated region, we found 5′ sequences identical among themselves but completely divergent from the published sequences of the untranslated region of human NF-E2. Two initiation codons present in the first exon were immediately followed by termination codons in-frame. Hence, the new sequence did not produce a different or a more extended open reading frame (ORF) from that published before. Since the clones previously described by two groups (12, 13) were both derived from a K562 cDNA library and our clones were isolated from a fetal liver cDNA library, we suspected that fNF-E2 might represent another tissue or developmental isoform of human NF-E2.

Genomic Organization of NF-E2. Using the human NF-E2 cDNA as a probe for the genomic DNA screening, we isolated two overlapping clones from a human placental genomic library. The genomic structure was first defined approximately by PCR amplification (19) with sets of primers located across the putative exon–intron junctions using the organization of the mouse NF-E2 gene as a guide (Fig. 2*A*). We then precisely defined the exon–intron boundaries by sequencing across the putative splice sites (Fig. 2*B*). The whole human NF-E2 gene with the inclusion of the promoter region extends for ≈10 kb of DNA, which embraces both alternative spliced isoforms. The previously described transcript (aNF-E2) was derived from the 10-kb gene, whereas the transcript we have isolated was derived from an alternative exon (1f), which is at the 3′ end of the first exon (1a) and ≈5 kb from it. The sequences of the two isoforms at exons 2 and 3 are identical. Since the NF-E2 translated region starts in the second common exon, it is predicted that the two isoforms will produce identical proteins. Stop codons present in all three reading frames in the 5′ untranslated regions of both isoforms exclude the possibility that two NF-E2 proteins different in their N terminus might be produced by starting translation from a previously unreported upstream initiation codon.

aNF-E2 and fNF-E2 Isoforms Are Transcribed from Two Alternative Promoters. By primer-extension analysis for exon 1f (data not shown) and by direct sequencing of the RACE products for both exons 1a and 1f, we defined the mRNA sequences up to the cap sites and demonstrated that the two exons are different for the entire length and colinear with the genomic sequences up to the cap sites. The absence of a common nucleotide sequence in the 5′ terminus of the mRNA demonstrates that the two splicing isoforms aNF-E2 and fNF-E2 are transcribed from alternative promoters. Another noteworthy difference between the two variants is found in features of the cap site, which is single and well-defined in fNF-E2, whereas it is at least double and ill-defined in aNF-E2 (data not shown).

Promoter Analysis. We sequenced fNF-E2 promoter up to −670 and aNF-E2 promoter up to −367 bp from the cap sites (Fig. 3). As suggested by the multiplicity of transcription initiation sites, aNF-E2 promoter shows a different overall organization from fNF-E2 promoter. The former is more G+C-rich than fNF-E2 (65% vs. 54% in the first 120 nucleotides). fNF-E2 has at −25 and at −70 bp CAAT-like and TATA-like boxes similar to the ones found in globin promoters (Fig. 4). Furthermore, fNF-E2 has several DNA binding sites for transcription factors involved in regulation of erythropoiesis: a GATA-1 site at −153 bp, two EKLF sites at −377 and −510 bp, three SSP (stage selector protein) (20) sites at −133, −143, and −391 bp, and one site for YY-1 (yin and yang 1) (9) at −468 bp. aNF-E2 promoter has an SP1 site at −57 bp and a CRE (cAMP responsive element) site at −167 bp from the first cap site, but it does not have any of the DNA motifs for erythroid transcription factors found in fNF-E2. Also absent were CAAT-like and TATA-like boxes similar to the ones found in the globin genes. Thus, based on the overall nucle-

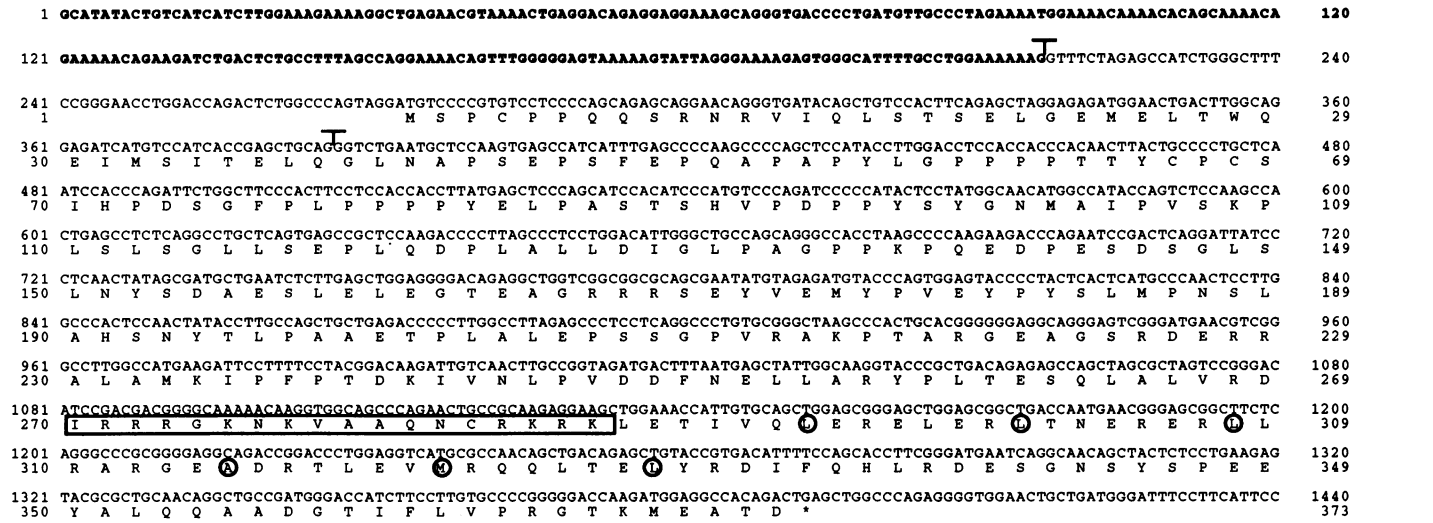

FIG. 1. Complete fNF-E2 cDNA and encoded protein sequence. The alternative exon 1f is in boldface type. Splicing junctions are indicated by a ⊤. The basic DNA binding domain is boxed and the leucine and hydrophobic residues of the zipper domain are circled. Poly(A) addition signal is underlined.

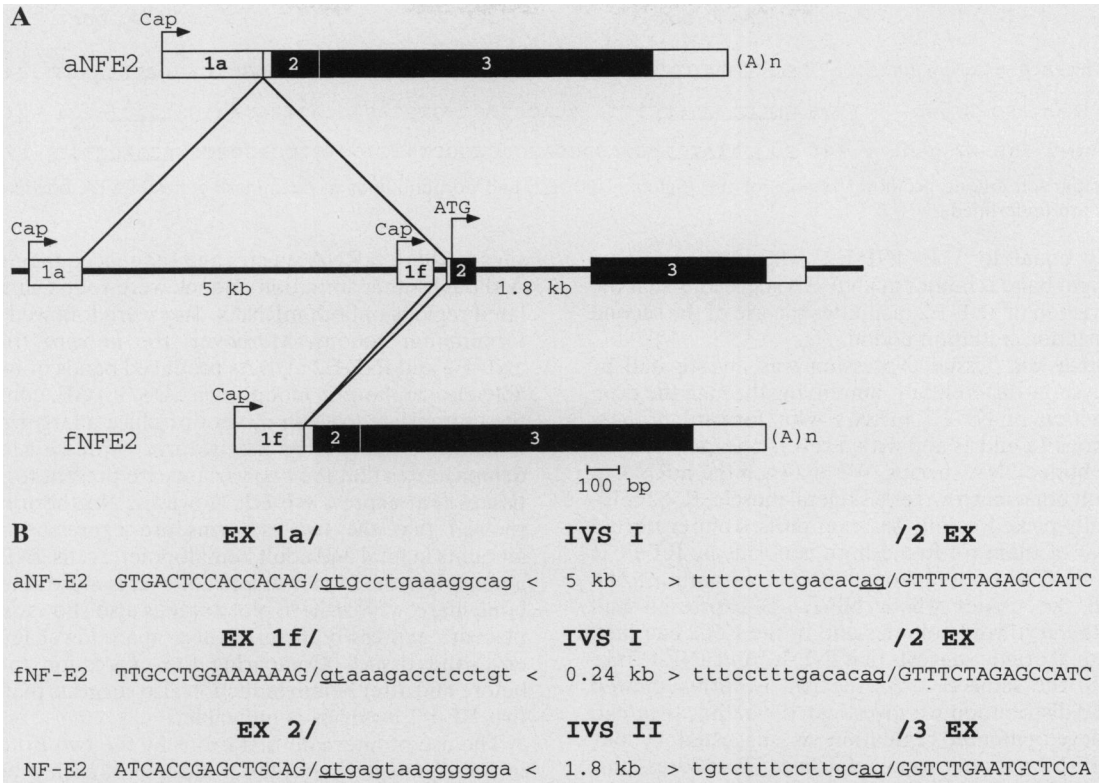

FIG. 2. Diagram of NF-E2 gene organization. (*A*) Origin of the aNF-E2 (*Top*) and fNF-E2 (*Bottom*) mRNA isoforms from an alternative splicing of the gene (*Middle*) is schematically shown. Open and solid rectangles represent untranslated and translated exon regions, respectively. (*B*) Alternative (aNF-E2 and fNF-E2) and shared (NF-E2) exon/intron boundary sequences of the NF-E2 splicing isoforms. Exon and intron DNA sequences are indicated by capital and lowercase letters, respectively. Invariant dinucleotide splicing junctions are underlined.

otide content, on DNA binding motif analysis, and on the presence of multiple cap sites, the aNF-E2 promoter was more like that of a housekeeping gene, whereas fNF-E2 promoter appeared to have more erythroid characteristics.

***In Vitro* Translation and DNA Binding Studies of the Two Isoforms.** We confirm that the two isoforms did not have a different ORF by analyzing the proteins obtained by *in vitro* translation of the different cDNA isoforms. Each shows two identical bands with indistinguishable electrophoretic mobility on SDS/PAGE (Fig. 5). The two bands are the likely result of the alternative use of two in-frame initiation codons as previously suggested (12). The ratio of the two protein bands is

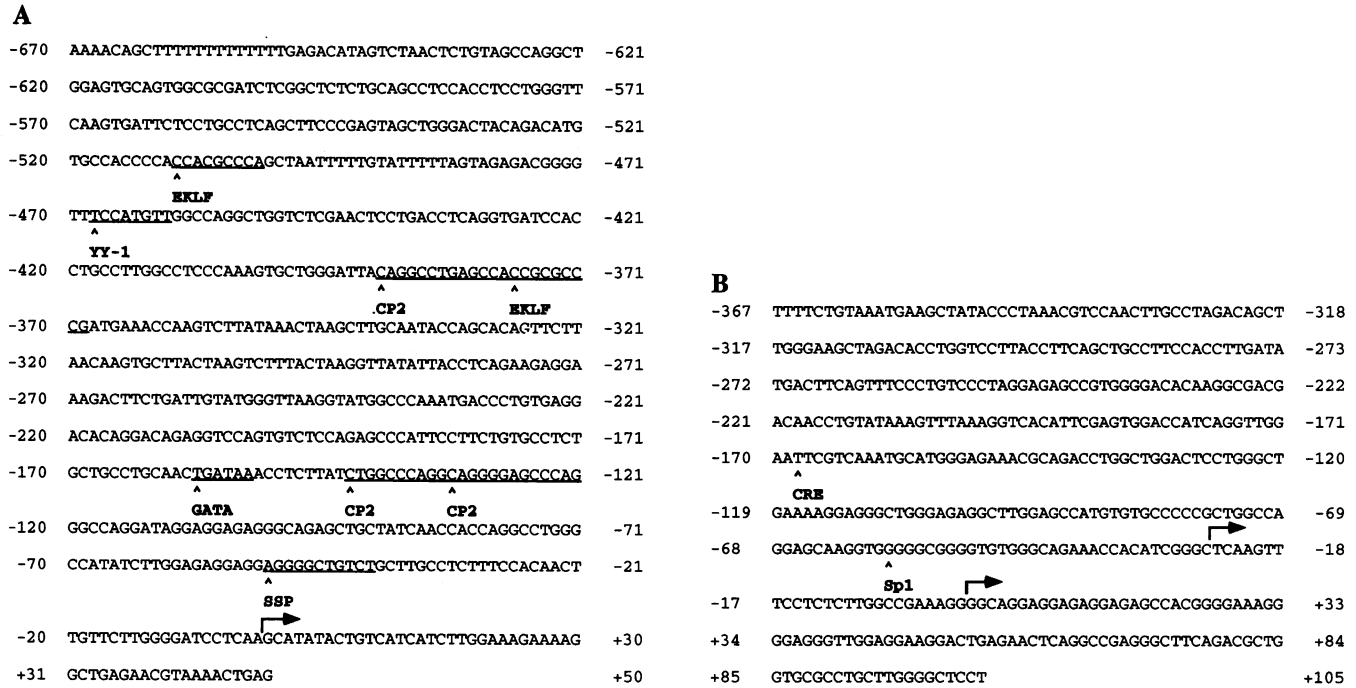

FIG. 3. Nucleotide sequence of the fNF-E2 (*A*) and aNF-E2 (*B*) promoters. Arrows, sites of mRNA transcription initiation. DNA binding motifs for transcription factors that have been implicated in the regulation of globin genes are indicated (GATA-1, SSP, EKLF, YY-1, CRE, SP1).

	CAAT box	TATA box
Human β-globin gene	-78 GGCCAATCTACTCCCAGGAGCAGGGAGGGCAGGAGCCAGGGCTGGGCATAAAAGT	-24
Human PBG-D gen	-78 GGCCTTATCTCTTTACCCACCTGGCTGTGCACAGCACTCCCACTGACAACTGCCT	-24
Human fNF-E2 gene	-70 GGCCATATCTTGGAGAGGAGGAGGGGCTGTCTGCTTGCCTCTTTCCACAACTTGT	-17

FIG. 4. Comparison among promoter regions of the β-globin, fNF-E2, and porphobilinogen deaminase genes. TATA box-like and CAAT box-like motifs are underlined.

approximately equal to 1 in fNF-E2, whereas the higher molecular weight band is fainter in aNF-E2, suggesting that the untranslated region of aNF-E2 facilitates the use of the second in-frame translation initiation codon.

Tissue Expression. Tissue expression was investigated by RT-PCR analysis by differentially amplifying through the exon 1/exon 2 junction of NF-E2 mRNA with forward primers specific for exons 1a and 1f and with a reverse primer on exon 2 common to both cDNA species. We analyzed the mRNA in fetal liver, adult bone marrow, fetal skeletal muscle, K562 cells, and individually picked colony-forming units from erythroid colonies. In all of them we found both isoforms by RT-PCR analysis (Fig. 6). The simultaneous presence of both mRNA species in all the tissues where NF-E2 is expressed and especially in the erythroid colonies and in the K562 cell line, which has a clonal origin, suggests that fNF-E2 and aNF-E2 are coexpressed in the same cell. As the two isoforms showed identical tissue distribution we investigated whether they had a different developmental regulation as suggested by our cloning of fNF-E2 variants from fetal liver. To address this question, we studied the relative abundance of the two isoforms by Northern blot analysis. A Northern blot containing poly(A) RNA derived from fetal liver, bone marrow, fetal and adult lung, placenta, and K562 cells treated or not treated with hemin was sequentially probed with oligonucleotides complementary to fNF-E2, γ-globin, aNF-E2, β-globin gene, and β-actin mRNAs. The mRNA analysis demonstrates that the two isoforms are expressed at different levels in the fetal liver and in the bone marrow (Fig. 7). fNF-E2 mRNA was enriched in the fetal liver, whereas aNF-E2 was more abundant in adult bone marrow cells. Using β-actin to normalize the RNA expression in these two tissues, we estimated that fNF-E2 is ≈3 times more abundant that aNF-E2 in the fetal liver, whereas in the adult bone marrow we see an inverted specular situation with aNF-E2 expressed 2–3 times more than fNF-E2. The hybridization to γ-globin- and β-globin-specific oligonucleotides provides an internal control for the stage of erythroid development and shows that the abundance of fNF-E2 and aNF-E2 mRNA follows that of γ- and β-globin mRNA, respectively.

DISCUSSION

In this paper, we present evidence that the NF-E2 gene has two alternatively spliced forms differing solely in their 5′ untranslated regions in the mature mRNA. We believe that the two mRNA species have remained unrecognized because they have an almost identical length and code for the same polypeptide.

To be sure that the cloned cDNAs were not missing 5′ regions that could generate a longer ORF, we defined the cap sites for both mRNA species and sequenced them completely. Although other initiation codons were found in the untranslated regions of both mRNAs, they were followed by in-frame termination codons. Moreover, the *in vitro* translation of aNF-E2 and fNF-E2 cDNAs produced bands of indistinguishable electrophoretic mobility on SDS/PAGE, confirming that the untranslated region did not produce a larger polypeptide. Qualitative analysis of the isoform expression by RT-PCR demonstrates that the two variants are present together in the tissues that express NF-E2. However, Northern blot analysis showed that the two isoforms are expressed in different amounts in fetal and adult hematopoietic cells. fNF-E2 is more abundant in the fetal liver and aNF-E2 is more abundant in the bone marrow. Northern blot analysis also shows that lung and placenta express both forms at a much lower level than the erythroid tissues. Comparing the expression in K562 cells before and after hemin induction also suggests that none of the two NF-E2 isoforms is inducible.

The use of alternate first exons by the two isoforms results in two different 5′ untranslated regions, each preceded by its own promoter. Promoter sequence analysis suggests differential regulation of the two human NF-E2 RNA isoforms. Whereas fNF-E2 promoter has a CAAT and a TATA box-like sequence, aNF-E2 has an SP1 binding site replacing the TATA box and a CRE binding site at −167 bp. Moreover, fNF-E2 has GATA-1, EKLF, SSP, and YY-1 protein binding sites, all of which are frequently found in erythroid promoters. The richness of G+C content and the presence of an SP1 binding site in the aNF-E2 promoter suggest less restricted tissue expression (21) and explain the multiplicity of the cap sites, given the reduced accuracy of SP1 promoters in the positioning of the transcription start site (22). Thus, the organization of the NF-E2 gene is reminiscent of the structure of the porphobilinogen deaminase gene (PBGD) (23), in association with which NF-E2 was first described (24, 25). PBGD mRNA isoforms are also transcribed from two alternative promoters,

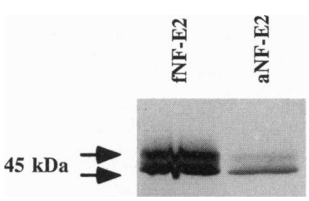

FIG. 5. *In vitro* transcription and translation of fNF-E2 and aNF-E2 cDNAs.

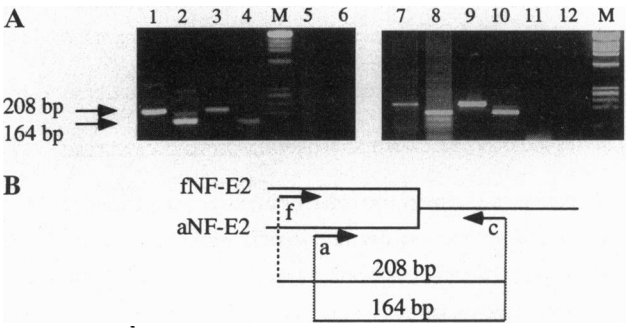

FIG. 6. (*A*) Detection of fNF-E2 and aNF-E2 splicing isoforms by RT-PCR analysis of selected tissues. Nusieve agarose gels (2%) stained with ethidium bromide. Lanes were loaded with amplified cDNA derived from fetal liver (lanes 1 and 2), adult bone marrow (lanes 3 and 4), fetal muscle (lanes 5 and 6), erythroid colonies (lanes 7 and 8), K562 (lanes 9 and 10), K562 control RNA without RT addition (lanes 11 and 12), and fX markers (lanes M). RT-PCR products done with primer sets specific for fNF-E2 (f and c) and for aNF-E2 (a and c) were loaded in the odd- and even-numbered lanes, respectively. (*B*) Schematic representation of DNA fragments generated by amplifying the mRNA through the first exon junction of NF-E2 with the indicated primers.

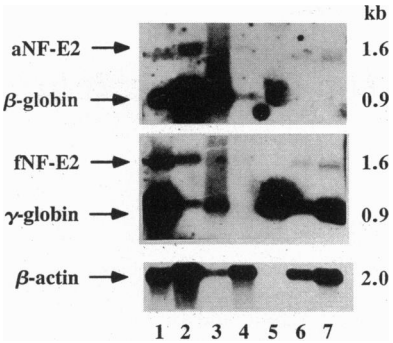

FIG. 7. Expression of NF-E2 isoforms in selected tissues. Northern blot analysis of poly(A) RNA derived from fetal liver, adult bone marrow, fetal lung, adult lung, placenta, K562 uninduced, and K562 hemin induced (lanes 1–7). The same filter was hybridized sequentially with fNF-E2 and γ-globin, aNF-E2 and β-globin, and β-actin probes.

producing two primary transcripts, a long one of ≈10 kb and a shorter one that originates in the 3′ end of the first intron. However, the two promoters of the PBGD gene appear to be more tissue specific as one form is expressed in erythroid tissues while the other is ubiquitous. A similar gene organization has also been found in the GATA-1 gene where, transiently during embryonic development, a GATA-1 isoform is transcribed from a distal promoter in the embryonic testis (26). In contrast, the two alternatively spliced forms of NF-E2 are produced in the erythroid tissues, although in different amounts. The presence of a GATA-1 DNA binding site in the fNF-E2 promoter suggests that NF-E2 resides downstream of the GATA proteins in the transcription factor hierarchy that governs the differentiation and regulation of erythroid tissues. The presence of sites for EKLF and SSP, which are both thought to play a role in the regulation of hemoglobin switching, suggests a causative role for them in developmental regulation of the NF-E2 gene.

Even though it is not presently clear what might be the functional relevance of the alternative transcription and splicing of the NF-E2 gene, the development in humans of such a relatively complex mechanism suggests that it must serve an evolutionarily important function. The recent report of an alternative splicing isoform in the same first intron in the NF-E2 mouse gene (27) and in the human NF-E2-related factor 1 gene (Nrf1) (28) suggests that such gene organization is evolutionarily conserved and underscores an important function. Even though they do not alter the ORF, the 5′ untranslated region may regulate the amount of protein product in different ways. The presence of initiation codons followed immediately by termination codons in the fNF-E2 isoform, as reported in other gene systems, could determine reduced protein output through a mechanism of abortive translation. Although the *in vitro* translation of both forms appears to be efficient, the 5′ untranslated region could support *in vivo* a differential regulation through preferential interaction with proteins that affect mRNA stability or processivity as postulated for the Tat–Tar interaction during human immunodeficiency virus replication (18, 29).

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