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Regulation of Mouse p45 NF-E2 Transcription by an Erythroid-specific GATA-dependent Intronic Alternative Promoter*

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The erythroid-enriched transcription factor NF-E2 is composed of two subunits, p45 and p18, the former of which is mainly expressed in the hematopoietic system. We have isolated and characterized the mouse p45 NF-E2 gene; we show here that, similar to the human gene, the mouse gene has two alternative promoters, which are differentially active during development and in different hematopoietic cells. Transcripts from the distal promoter are present in both erythroid and myeloid cells; however, transcripts from an alternative proximal 1b promoter, lying in the first intron, are abundant in erythroid cells, but barely detectable in myeloid cells. During development, both transcripts are detectable in yolk sac, fetal liver, and bone marrow. Transfection experiments show that proximal promoter 1b has a strong activity in erythroid cells, which is completely dependent on the integrity of a palindromic GATA-1 binding site. In contrast, the distal promoter 1a is not active in this assay. When the promoter 1b is placed 3′ to the promoter 1a and reporter gene, in an arrangement that resembles the natural one, it acts as an enhancer to stimulate the activity of the upstream promoter la.

NF-E2[1] is a transcription factor that was originally identified through its ability to interact, *in vitro*, with the porphobilinogen deaminase promoter and with critical functional elements of the β-globin locus control region (LCR) (1–3). NF-E2 recognizes the extended DNA sequence **G**CTGA(G/C)TCA, which includes the core symmetric AP-1 motif, as well as other non consensus sequences (4–6); these recognition motifs are present in several genes involved in the heme-synthetic pathway in erythroblasts and in genes expressed in megakaryocytes.

NF-E2 is an obligate heterodimer between a 45-kDa polypeptide (p45 NF-E2) (7), predominantly expressed in the hematopoietic system, and one of the widely expressed ≅18-kDa proteins (p18/Maf-K, Maf-G, and Maf-F), belonging to the Maf family (8–14). Both p45 and p18 subunits belong to the basic leucine zipper family of transcription factors. Five p45 NF-E2-related polypeptides have also been identified (15–21). Given the ability of large and small subunits to heterodimerize and to recognize the extended NF-E2 binding motif or variants of it, there is an enormous potential for regulation of genes containing NF-E2 sites.

Of the several large and small subunits of the NF-E2 "family," p45 NF-E2 is the only one that shows highly restricted expression, being detected primarily within the hematopoietic lineage, in erythroid, megakaryocytic, and mast cells, and possibly neutrophils (7, 9, 22–24). In erythroid cell lines, such as mouse erythroleukemia cells (MEL), NF-E2 increases when erythroid differentiation is induced (1, 25). Secondary expression sites include intestine, lung, and placenta (7, 24). The restricted expression of p45 NF-E2 suggests a critical role of this protein in at least some hematopoietic lineages. Indeed, homozygous inactivation of the p45 NF-E2 gene in the mouse (by homologous recombination) leads to a fatal hemorrhagic disease due to faulty megakaryocytic maturation and lack of platelets (4). However, in the same mice, only minor erythroid cell defects were observed (26, 27), suggesting some functional complementation in the erythroid lineage by other related genes (possibly Nrf-1, but not Nrf-2) (28, 29).

Evidence that p45 NF-E2 deficiency might affect at least a subset of erythroid cells is provided, however, by the lack of β-globin expression in a MEL cell line in which the endogenous p45 gene is inactive due to a retroviral integration; forced expression of p45-cDNA in these cells restores β-globin expression (30, 31).

So far, the transcriptional regulation of p45 NF-E2 expression has not been extensively investigated. Recently, it was reported that two cDNA isoforms of p45 NF-E2 exist in human cells, resulting from transcription from two alternative promoters and differential splicing of the transcripts into the common second exon RNA. As the transcript from the distal promoter/first exon is more abundant in adult erythroid cells, and the transcript from the proximal promoter/first exon is predominant in fetal cells, they were termed "adult" and "fetal", respectively (32).

In this work, we have investigated the presence in the mouse p45 NF-E2 gene of similar promoters and studied the regulation of their expression by RNA analysis during development and by transfection. The distal promoter/first exon (1a) corresponds to the human adult promoter (32) and drives the ex-

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[1] The abbreviations used are: NF-E2, nuclear factor erythroid 2; LCR, locus control region; MEL, mouse erythroleukemia cell; HEL, human erythroleukemia cell; RT, reverse transcription; PCR, polymerase chain reaction; TK, tymidine kinase; EKLF, erythroid Kruppel-like factor; nt, nucleotide(s).

pression of the previously described mouse mRNA (7); the proximal mouse promoter/first exon (1b), lies in the first intron, and corresponds to the human fetal promoter (32).

EXPERIMENTAL PROCEDURES

Genomic Library Screening, DNA Subcloning, and Sequencing—A mouse genomic library in λFixII (CLONTECH) was initially screened by standard procedures for NF-E2 p45 gene fragments with a mouse p45 cDNA (kindly provided by Dr. N. Andrews). DNA from positive phage clones was then analyzed by Southern blotting and hybridization using a ^{32}P-labeled 325-nt human genomic fragment of the first p45 intron, including the alternative fetal exon and its promoter (32). Hybridization was carried out at moderate stringency in the presence of 250 mM Na_2PO_4, pH 7.2, 7% SDS, 1 mM EDTA, pH 8.0, at 58 °C. Filters were washed twice for 20 min in 20 mM Na_2PO_4, pH 7.2, 5.2% SDS, 1 mM EDTA, pH 8.0, at 58 °C and finally at 65 °C for 5 min with the same solution. Positive fragments were subcloned into pGEM-7Zf+ (Promega) and sequenced to identify the proximal promoter/first exon (1b). The distal promoter/first exon (1a) was further identified by hybridization to an oligonucleotide (antisense, 5′-TCGGAGTTACCAGTGCTTCCT) from the 5′ end of the published cDNA sequence (7) and sequencing with the T7 sequencing kit (Amersham Pharmacia Biotech).

RT-PCR—For RT-PCR experiments, RNA was obtained at various developmental points from mouse yolk sac, fetal liver, and adult marrow. Adult marrow was also fractionated (exactly as in Ref. 33) into erythroid and myeloid components by separation with magnetic beads coated with antibodies against the erythroid antigen TER-119 and the myeloid antigen Mac-1, respectively. Dissected samples or fractionated cells were immediately dispersed in 0.5–1 ml of a solution containing 4 M guanidinium isothiocyanate, 25 mM sodium citrate, pH 7.0, 0.5% sarcosyl, 1 M 2-mercaptoethanol, and total RNA was extracted according to Ref. 34.

Aliquots of the RNA (1 μg) were retrotranscribed in a volume of 20 μl in the presence of 1 μl of SuperScript reverse transcriptase (200 units/μl, Life Technologies, Inc.), 1× PCR buffer (Perkin Elmer), 1.5 mM $MgCl_2$, 1 mM dNTPs 10 mM dithiothreitol, 100 pmol of random hexamers (Promega, Madison, WI), and 50 units of RNasin, at 37 °C for 1 h. One tenth of the total cDNA was first amplified with β-actin primers (sense, 5-GTGACGAGGCCCAGAGCAAGAG; antisense, 5′-AGGGGCCGGACTCATCGTACTC), as described in Ref. 33, for 15 cycles (exponential phase) to normalize for cDNA content. To determine the relative proportions of 1a and 1b transcripts, equal amounts of each cDNA sample were simultaneously amplified with specific p45 NF-E2 primer pairs: a common downstream antisense oligonucleotide, mapping in the p45 exon 2 (5′-GACATGATCTCTTGCCAAGTCAG; nt 98 to nt 76 from the ATG initiation codon, Ref. 7), was combined either with an upstream sense oligonucleotide from the previously described first exon (7) (exon 1a, 5′- CTCAGTAGTACTCAGAGGCAG) or an oligonucleotide from the putative exon 1b (Fig. 2*B*; oligonucleotide 1, 5′-CTAAGCACTCACTTCTTGGGAAG). The amplification reaction was carried out in 50 μl of 10 mM Tris-HCl, pH 9.0 at 25 °C, 50 mM KCl, 1% Triton X-100, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.5 μl of *Taq* DNA polymerase (5 units/μl Promega), 100 pmol of each primer, with a Perkin Elmer (Norwalk, CT) thermocycler (denaturing, 94 °C for 90 s; annealing, 65 °C for 1 min; primer extension, 72 °C for 90 s) and aliquots (5 μl) were withdrawn at 20, 23, 26, 30, and 35 cycles. The samples were migrated in 1.4% agarose gels, blotted onto nylon filters (Hybond-N$^+$, Amersham Pharmacia Biotech), and further probed with a radioactively labeled cloned fragment of the second exon (nt 278–431; Ref. 7).

Quantification of the radioactive bands was obtained by densitometric scanning (Image Master DTS, Amersham Pharmacia Biotech) of appropriate exposures of the radioactive filters on Kodak X-Omat AR films. Some experiments were also evaluated directly by Instant Imager Electronic Autoradiography (Packard), giving results consistent with the densitometric analysis.

To quantify the relative proportion of amplified 1b *versus* 1a promoter bands, the analysis was carried out within the exponential phase of amplification usually between cycles 20 and 26. Within this window, bands of similar intensities were compared, irrespective of the number of cycles. Correction for the number of cycles was applied on the basis of the knowledge of the amplification kinetics of the respective bands. To do this, we preliminarily purified by acrylamide gel electrophoresis the amplified fragments 1b and 1a; these fragments were diluted to 1 pg/reaction, amplified, and analyzed as described above. The kinetics of the amplifications were identical for both fragments, with a slope corresponding to a 65% increase per cycle in the exponential phase.

Ribonuclease Protection Assay—Total RNA from mouse fetal liver was extracted according to Ref. 34. Poly(A)$^+$ RNA was prepared using Message Maker mRNA isolation system (Life Technologies, Inc.). To prepare 1b and 1a riboprobes, a 335-nt 1b fragment and a 400-nt 1a fragment extending from the first 1b exon (position 651, Fig. 2*A*) or the first 1a exon (position 406, Fig. 3), respectively, into upstream flanking sequences, were cloned into the pGEM-T vector (Promega); the antisense strands were transcribed in the presence of [α-^{32}P]UTP (800 Ci/mM) with Sp6 polymerase using the MaxiScript *in vitro* transcription kit (Ambion Inc.) and purified according to the instructions of the manufacturer. Probes (≅200,000 cpm/sample) were hybridized overnight at 50 °C in 10 μl of the hybridization buffer provided in the RPA III ribonuclease protection assay kit (Ambion Inc.) with either RNA (poly(A)$^+$, 5 μg) and 20 μg of carrier *E. coli* tRNA, or with carrier tRNA alone. Following hybridization, the samples were processed at 37 °C for 30 min with a 1:100 dilution of RNase A/RNase T1 mix from the same kit, according to the instructions. The digested samples were run on 6% acrylamide-urea denaturing gels, together with Maxam and Gilbert G+A ladder of ^{32}P-5′-labeled DNA probes, and autoradiographed.

Determination of the Transcription Initiation Sites by RT-PCR—To determine the transcription initiation start sites of promoters 1b and 1a, the exon 2 antisense oligonucleotide (see above) was combined, in RT-PCR experiments, with the following sense oligonucleotides on the promoter/exon 1b and 1a regions.

For the 1b region (Fig. 2*B*), oligonuclotides were: 1, 5′- CTAAGCACTCACTTCTTGGGAAG; 2, 5′-CAACACACTCCTTGAGGATCTC; 3, 5′-CTGTCTGACTAACACTTCCCAC; 4, 5′- GGGAGGGGCTGTCTGACTAAC; 5, 5′-AGGCTTGGGGCCTATCTCAGAG; 6, 5′-GGCAGGCTTGGGGCCTATC; 7, 5′-GCTATCACCGGCAGGCTTG; 8, 5′-GGTTGCTATCACCGGCAGGC; 9, 5′- GAGGGAAGGGTTGCTATCACC; and 10, 5′- GAACCTAAAGTAAGGATGGGAGG.

For the 1a region (Fig. 3), oligonucleotides were: A1, 5′-GGAAAGGGAAGGGTTGGAGG; A2, 5′-GTTTCCTCTCTTGGCCAGAAG; A3, 5′-GAAACCACACCGGGCTCAAG; A4, 5′-GTGCAGCAGAAACCACACCG; A5, 5′-GCTGCCACAGAGAGGCGG; A6, 5′-GCTGGAGAGGGTCTGAAG; A7, 5′-GAAGTCAGCCCGAACAAACAG; A8, 5′-GCTTAGGAGTCTCTTGTGAGTG.

Electrophoretic Mobility Shift Assay (35, 36)—Nuclear extracts were prepared according to Ref. 37. The standard binding reaction (20 μl) contained 0.1–0.2 ng of ^{32}P-labeled oligonucleotide, 2–5 μg of crude nuclear protein, 1–3 μg of poly(dI-dC), 2 μg of bovine serum albumin in a buffer consisting of 4 mM spermidine, 50 mM NaCl, 1 mM EDTA, 10 mM Tris-HCl, pH 7.9, 1 mM dithiothreitol, and 0.5 mM phenylmethylsulfonyl fluoride. Unlabeled competitor oligonucleotides were added to some reactions in a 50–100-fold excess relative to the labeled oligonucleotide. The mixtures were kept on ice for 20 min. Electrophoresis was carried out in 5% acrylamide gels in 50 mM Tris borate, pH 8.2, at 20 °C. The supershifts were carried out under the conditions described above for GATA-1 binding, with 0.2 μg of anti-GATA-1 rat monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) or an anti-human GATA-1-specific antibody (38) kindly provided by Dr. Claudio Santoro. Anti-GATA-2 antibody (0.2 μg) was from Santa Cruz Biotechnology. The sequences (sense strand) of the oligomers used in gel shift experiments are shown below (GATA consensus in bold characters; mutated nucleotides underlined): wt p45 GATA, 5′-CCTGCAGC**TGATAA**ACCCC**TTATCT**GGCCCA; mut p45 GATA, 5′-CCTGCAGC**TTTTAA**ACCCC**TTAAAT**GGCCCA; 5′ mut p45 GATA, 5′-CCTGCAGC**TTTTAA**ACCCC**TTATCT**GGCCCA; 3′mut p45 GATA, 5′-CCTGCAGC**TGATAA**ACCCCC**TTAAAT**GGCCCA; HS2 GATA, 5′-CGAGTCCATC**TGATAA**GAC**TTATCT**GCTGCCCCA.

Construction of Reporter Plasmids—Fragments of the promoters/first exons 1a and 1b were amplified by PCR using specific oligonucleotides with terminally added *Kpn*I restriction enzyme sites, and cloned into the *Kpn*I site of pGL2-Basic Vector (Promega) carrying a firefly luciferase reporter gene (plasmids *1a p-luc* and *1b p-luc*, respectively).

The 1a fragment is 399 nt long and spans nt 7–405 (Fig. 3); the 1b fragment is 373 nt long and spans nt 233–605 (Fig. 2*A*). The same 1b fragment was also obtained as an *Mbo*I restriction fragment, and inserted (both in the sense and antisense orientations, relative to transcription) into the *Bam*HI site downstream of the luciferase reporter gene driven by the promoter 1a (plasmid *1a p-luc-1b enh sense* and plasmid *1a p-luc-1b enh antisense*). All the cloned fragments were sequenced to verify the absence of PCR-generated mutations.

A plasmid carrying a mutation at the GATA-tandem motif in the promoter 1b was obtained by PCR-mediated mutagenesis (plasmid *GATA-1$^-$ 1b p-luc*). Two partially overlapping oligonucleotides carrying a GA→TT and a TC→AA substitution (sense strand) and the complementary mutations (antisense strand) of the tandem repeat (nt 459–460 and 472–473, *underlined* in Fig. 2*B*) were used to separately

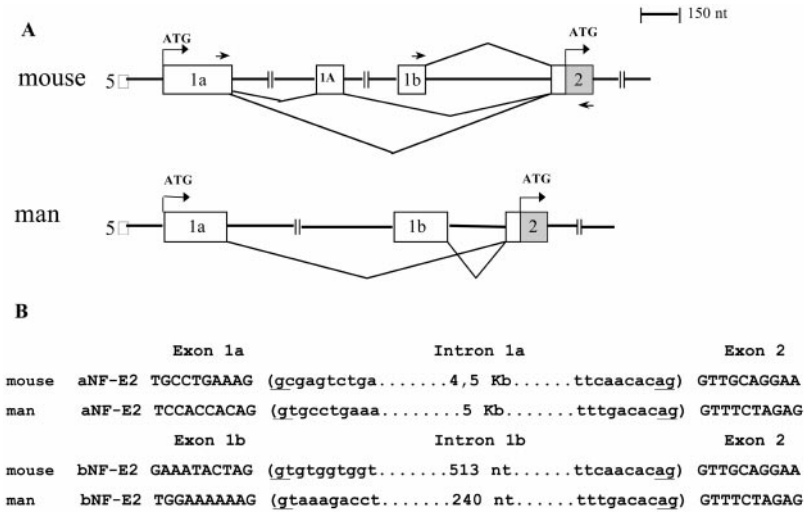

FIG. 1. **Schematic representation of the organization of alternative promoters in the murine and human p45 NF-E2- genes.** *A,* the alternative "adult" and "fetal" exons of the human p45 gene (32) are labeled 1a and 1b, both in human and mouse. Exon 1A represents an additional alternative second exon previously reported in Ref. 30. Alternative splicing events are indicated. *Arrows* represent the location of PCR primers. *B,* exon-intron junctions and intron lengths of the 1a and 1b/exon 2 introns. Data for the human sequences are from Ref. 32; data for the 1a-exon 2 mouse intron boundaries are from Ref. 39.

amplify, with external primers, the 5′ and 3′ portions of the promoter 1b. The two fragments were purified, combined, and reamplified with the external primers only, to yield the full-size mutated promoter 1b that was then cloned into the pGL-Basic plasmid. The accuracy of the sequence was verified by sequencing.

Plasmid Preparation—Each plasmid preparation was obtained by two sequential cesium chloride centrifugations; the DNA was then dialyzed against 1 mM EDTA, 10 mM Tris-HCl, pH 7.5.

Transient Transfection Experiments—Human erythroleukemic K562 and HEL cells were collected during the exponential phase of growth, washed, and resuspended in cold phosphate-buffered saline. For each transfection 10^7 cells, in 200 μl (HEL cells) or 800 μl (K562 cells), were electroporated at 200 V (HEL cells) or 400 V (K562 cells) at 960 microfarads using the Gene Pulser (Bio-Rad) electroporator in the presence of 20 μg of the luciferase reporter construct to be tested and 2 μg of the *Renilla* luciferase pRL-TK plasmid, as an internal control. The cells were further incubated for 2 days in RPMI 1640 and 10% fetal calf serum, glutamine, and antibiotics, collected by centrifugation, washed twice in phosphate-buffered saline, lysed, and processed for analysis using the Dual-Luciferase Reporter Assay System kit (Promega) exactly as specified by the manufacturer's instructions. Aliquots of the cell lysates were used to sequentially determine the activities of the firefly and *Renilla* luciferases for each sample. Each transfection was carried out with at least two independent plasmid preparations, each in duplicate or triplicate samples.

Stable Transfection Experiments—10 μg of p-luc plasmids, linearized by digestion with *Sca*I, were electroporated as above into K562 cells, both in presence (2 μg) and absence of linearized *Renilla* luciferase pRL-TK plasmid, together with the pPNT G-418 resistance plasmid. After selection in G-418 (600 μg/ml) for 10 days, populations of resistant cells were assayed for firefly luciferase (or firefly/*Renilla* luciferase) activity.

RESULTS

The Mouse p45 NF-E2 Gene Includes an Alternatively Spliced Exon Homologous to the Human p45 NF-E2 "Fetal" Exon—Using a probe corresponding to the human "fetal" first exon of the p45 NF-E2 gene, we isolated from a mouse genomic phage library three overlapping phage clones. Mapping and sequencing data identify a region with strong homology to the human "fetal" exon within the first intron of the mouse p45 NF-E2 gene. Fig. 1 summarizes the organization and transcription of the corresponding region in the human and mouse genes. The potential mouse "fetal" exon (1b) is located (as the human "fetal" exon) within the large intron separating the previously described exon 1a from exon 2. The exon 1b transcript is spliced to the second exon (Fig. 1*A*), which is thus common to both exon 1a and 1b. Fig. 1*B* shows a comparison of the exon-intron borders of the human and mouse exons 1a and 1b; in both species exon 1b lies in the most 3′ part of a large (more than 4 kilobase pairs) intron, and is separated from exon 2 by 240 nucleotides (man) or 513 nucleotides (mouse). This conclusion is based on a combination of genomic sequencing and RT-PCR data (see below). Fig. 2*A* shows the genomic sequence of part of the mouse intron 1, encompassing exon 1b (bold characters) and the most 5′ portion of the previously described exon 2. A comparison between the mouse genomic sequences and human sequences in the vicinity of exon 1b (Fig. 2*B*) identifies a ≅120-nt-long stretch of mouse sequences showing high homology (≅86%) to the region immediately upstream to the human exon 1b; the homology decreases to ≅57% in the 250 nt further upstream (data not shown), and is completely lost downstream to position 651 of the mouse sequence (data not shown). The human 1b cDNA ("fetal" cDNA in Ref. 32) has its 5′ end at position 671 (Fig. 2*B*, *arrow*); to ascertain if the homologous region in the mouse gene is part of the putative exon 1b, we performed RT-PCR on MEL cell RNA using a primer pair designed on this sequence (oligonucleotide 1, sense; Fig. 2*B*) and on the second exon (antisense, nt 98 to nt 76 from the ATG; Ref. 7), respectively. This primer pair successfully amplified a 195-nt band. Sequencing of this band (data not shown), and comparison with the genomic sequence (Fig. 2*A*), confirmed that the putative mouse exon 1b is indeed transcribed into an RNA that is spliced into the second exon at nt 1165 (Fig. 2*A*).

A similar comparison is presented in Fig. 3 for the mouse and human promoter/first exon 1a. Strong homology is detected between these sequences only in a region of ≅180 nucleotides (nt 263–445 of the mouse sequence).

Mouse 1a and 1b p45 transcripts are present at relatively low abundance; to define the start site of transcription of the mouse promoters 1a and 1b, we purified poly(A)$^+$ RNA from mouse fetal liver and performed RNase protection experiments (Fig. 4). The 1a antisense probe extended from nucleotide 406 to nucleotide 8 of the promoter/exon 1a sequence (Fig. 3), whereas the 1b antisense probe extended from nucleotide 651 (within exon 1b) to nucleotide 317 of the promoter/exon 1b sequence (Fig. 2*A*). Using probe 1a, over 10 bands of lengths ranging between 45 and 95 nt were visible, the major ones being of approximately 70 nt (Fig. 4*A*, *lane 3*). These data suggest that 1a transcription starts between nt 310 and 362, as shown in Fig. 3 (*vertical arrowheads*). Using probe 1b, we detected four bands (Fig. 4*A*, *lane 7*) ranging between approximate lengths of 38–48 nt, and several weaker bands in the range of 50–110 nt; these data indicate that transcription of exon 1b starts at several sites as shown in Fig. 2*B* (*vertical arrowheads*). To independently confirm these data, several forward PCR primers, corresponding to staggered exon 1b sequences (Fig. 2*B*, *arrows*), were used in RT-PCR experiments (in conjunction

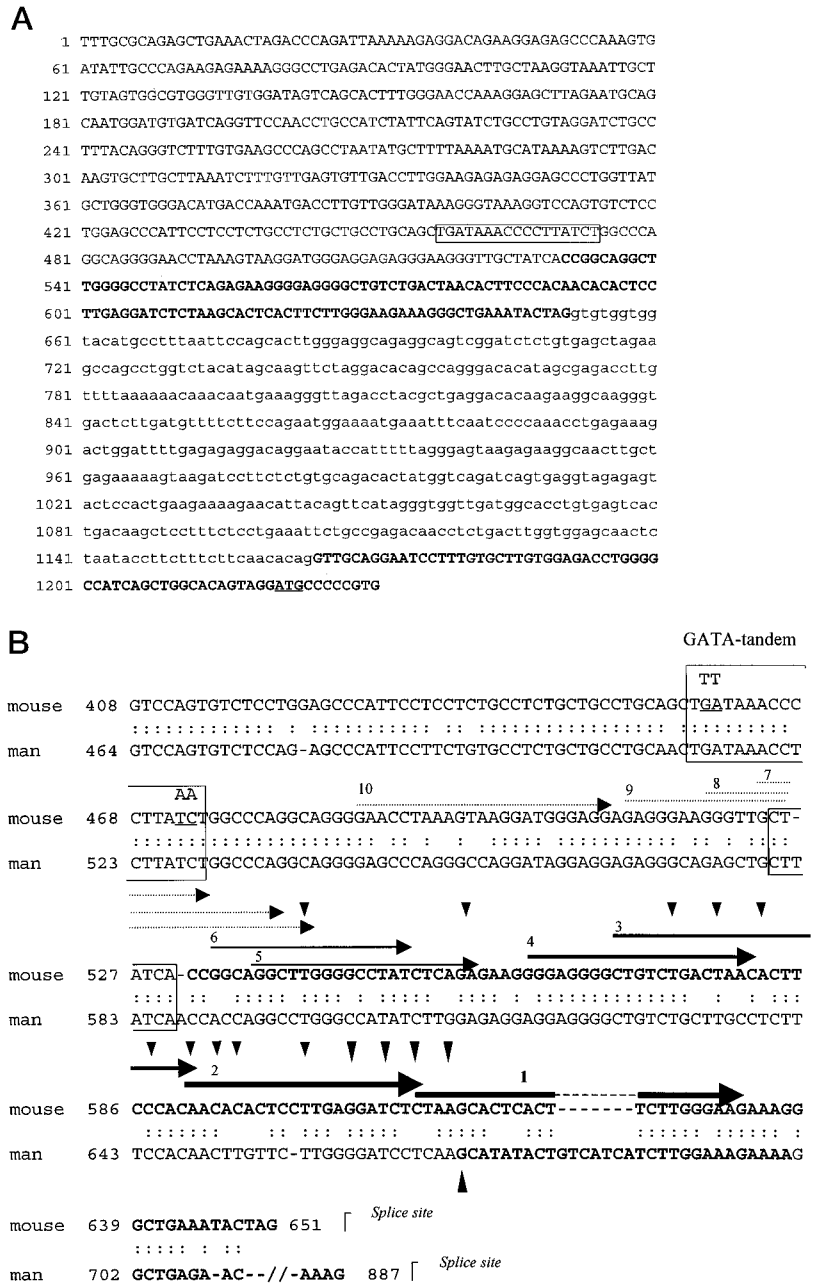

FIG. 2. **Nucleotide sequence of the mouse and human promoter/exon 1b region.** *A*, mouse promoter/exon 1b, first intron, and exon 2 up to the ATG initiation codon: *capital letters* denote promoter and exon sequences; *boldface letters* are in exons; *lowercase letters* are in the intron. A tandem GATA motif is *boxed*. *B,* homology between the human and mouse promoters and exons 1b. The transcription start points (as described under "Results") are shown by *vertical arrowheads*; *bold letters* indicate exons. Two conserved GATA motifs are *boxed*. Mutations introduced into the upstream GATA tandem repeat are indicated; sense oligonucleotide primers used for RT-PCR (see Fig. 4*B*) are indicated by *numbered horizontal arrows*.

with a downstream antisense oligonucleotide in exon 2). Using oligonucleotides 1–4, the expected amplified bands were readily obtained (Fig. 4*B*), whereas weak bands (well visible by hybridization; data not shown) were detected with oligonucleotides 5 and 6. No amplification was observed with oligonucleotides 7–10. These data are in very good agreement with the RNase protection results and indicate that most 1b transcripts start between position 605 and 614 of the 1b sequence (Fig. 2*B*), with fewer transcripts starting in the more upstream region, approximately up to nucleotides 530–540. The major transcription start sites of the promoter 1b appear to be located some 10–15 nucleotides more upstream than the human transcription start region (Fig. 2*B*). Similar experiments (data not shown) performed with promoter/exon 1a primers demonstrate a strong amplified band with the upstream oligonucleotide A1 and a weaker band with oligonucleotide A2 (Fig. 3); oligonucleotides 3–7 yield very weak bands. These data, taken together with the RNase protection experiments, indicate that most transcripts start between positions 331 and 354 (Fig. 3); the latter position corresponds to the 5′ end of the previously characterized p45 NF-E2 cDNA (7). These experiments allow us to define the promoter sequences immediately upstream to the 1b and 1a transcription start sites.

Several potential binding sites for transcription factors, including an upstream palindromic tandem GATA-1 site and a downstream GATA-1 site (Fig. 2*B*, *boxed*) are conserved 5′ to the 1b transcription start site in both the mouse and human genes. In contrast to the findings in exon 1b, no obvious binding sites for erythroid transcription factors are detectable in the mouse promoter/exon 1a sequence; this is in agreement with previous observations with the human promoter (32).

The mouse exon 1b, like the human exon, shows no translation initiation site; it is also much shorter than the human fetal exon, due to the insertion of a splice site ≅120 nt downstream to the transcription initiation site.

Differential Expression from the Promoters 1b and 1a during Development—To evaluate the relative expression of transcripts arising at promoters 1b and 1a, we developed an RT-

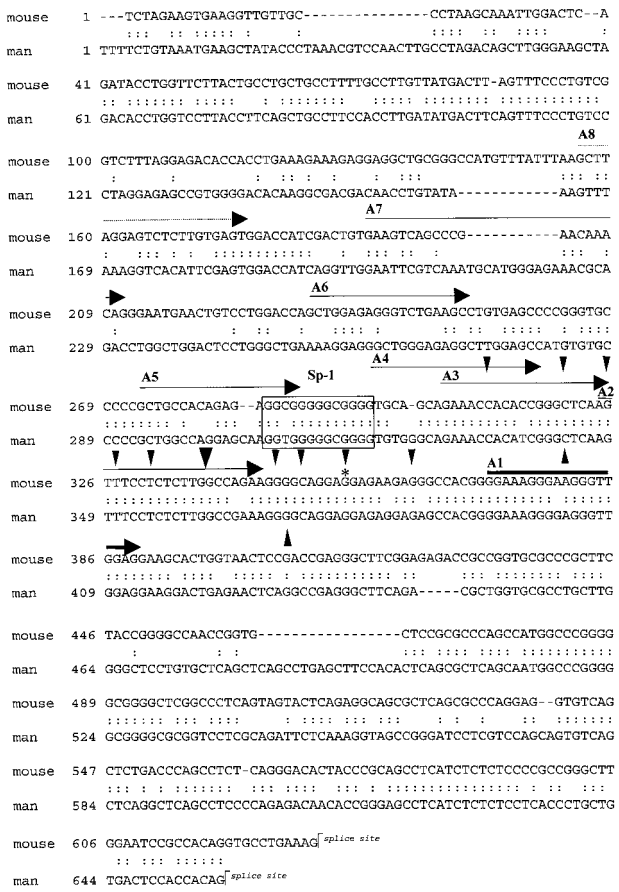

FIG. 3. **Comparison of the mouse and human promoters and exons 1a.** The transcription start sites are shown by *vertical arrowheads*. Sense oligonucleotide primers used for RT-PCR are indicated by *numbered horizontal arrows*. A conserved potential Sp1 site is *boxed*. The *asterisk* shows the start of the published mouse cDNA sequence (7); the human sequence is from Ref. 32.

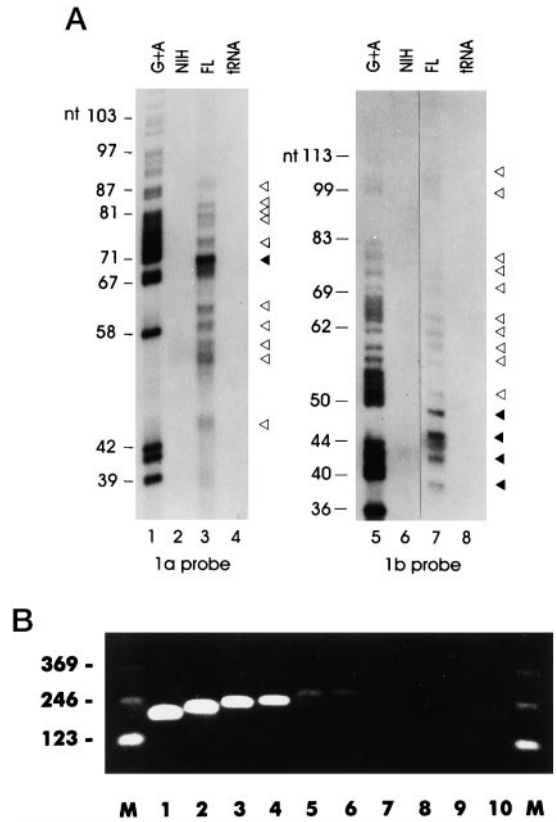

FIG. 4. **Determination of transcription start sites of 1a and 1b transcripts in mouse cells.** *A,* RNase protection assay. *Lanes 1–4*, analysis of the 1a transcription start site. *Lane 1*, G+A sequence ladder of the 1a probe; *lane 2*, NIH total RNA (10 μg) hybridized to the 1a probe; *lane 3*, fetal liver poly(A)$^+$ RNA (4 μg) hybridized to the 1a probe; *lane 4*, *E. coli* tRNA hybridized to the probe. *Lanes 5–8*, analysis of 1b transcription start site. *Lane 5*, G+A sequence ladder of the 1b probe; *lane 6*, NIH total RNA (10 μg) hybridized to the 1b probe; *lane 7*, fetal liver poly(A)$^+$ RNA (5 μg) hybridized to the 1b probe; *lane 8*, *E. coli* tRNA hybridized to the probe. *Arrows* point to the detected start sites. Lengths of bands (in nucleotides) of the sequence ladder are indicated on the *left* of the figures for probe 1a and 1b, respectively. *B,* RT-PCR analysis. Panel shows a representative RT-PCR experiment using mouse fetal liver (embryonic day 13.5) RNA. The positions of "sense" RT-PCR primers used to determine the transcription initiation site of 1b transcript, are indicated by *numbered arrows* in Fig. 2*B* (*boldface arrows* indicate strong amplification; *thin arrows*, weak amplification; *dotted arrows*, no amplification). *Numbers below* the *lanes* indicate the corresponding sense oligonucleotide used in the amplification; the molecular sizes (*nt*) of marker bands run in parallel are shown on the *left*. No high molecular weight bands (indicating amplification from genomic DNA) are detected. Similar experiments (data not shown) were carried out to determine the start site of 1a transcripts, using primers shown in Fig. 3; only primer A1 (*boldface arrow*) gives strong amplification, whereas primers A2–A7 (*thin arrows*) give weak amplification; primer A8 does not give any amplification.

PCR assay using a common antisense primer mapping in exon 2, and different sense primers mapping in exons 1b and 1a, respectively (see "Experimental Procedures").

Total RNAs were obtained from mouse yolk sac, fetal liver, and adult bone marrow. Following retrotranscription with random primers, different samples, containing equal amounts of total cDNA (based on normalization by actin RT-PCR; data not shown), were amplified with the 1b and 1a specific primer pairs. The results were evaluated in the exponential phase of the amplification by hybridization to a cloned DNA fragment including only the exon 2 common to both amplified bands.

Using the 1a-specific pair, two bands of 395 and 283 nt are detected (Fig. 5). The upper less abundant band was shown by sequencing to represent a transcript including exon 1a, the alternatively spliced exon 1A (30) and exon 2 (see Fig. 1*A*); the lower band includes only exons 1a and 2. Using the 1b-specific primer amplification pair, only one band of 195 nt is detected (1b). The results indicate that transcripts from the promoter 1b are much more abundant than those from the promoter 1a during the embryonic-fetal stage, representing over 80% of the total. On the other hand, the percentage of transcripts from 1b is somewhat lower, varying between 40% and 50% of the total in bone marrow from adult mice. Bone marrow cells were further fractionated into the erythroid and myeloid components, by separation on magnetic beads coated with antibodies against an erythroid (TER-119) or a myeloid (Mac-1) membrane antigen. In erythroid cells, the proportion of 1b transcripts was about 75% of the total p45-NF-E2 transcripts, whereas in myeloid cells it was as low as 10–15% (Fig. 5*B*). The very low expression of 1b transcripts in myeloid cells was further confirmed by analysis of myeloid interleukin-3-dependent Mac-1-positive progenitors immortalized from mice transgenic for SV40 T gene driven by GATA-1 regulatory elements (40); in these cells the proportion of 1b transcripts was even lower (≅5%) than in bone marrow myeloid cells (data not shown).

The Promoter 1b Includes a Strong GATA-1 Binding Site—As shown in Fig. 2, the promoter 1b contains a sequence resembling a tandem palindromic GATA-binding site, which is highly conserved between mouse and man (16 out of 17 nucleotides). An oligonucleotide encompassing this site (nt 450–480, Fig. 2*B* and "Experimental Procedures") generates a strong

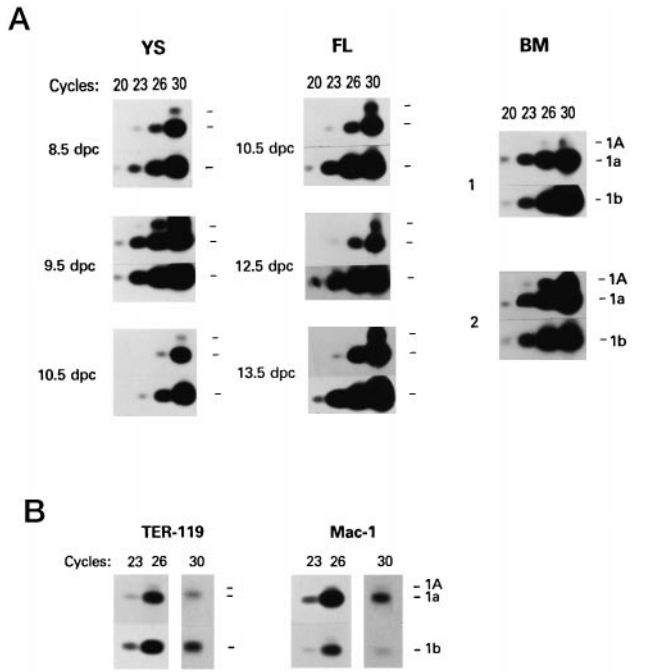

FIG. 5. **RT-PCR analysis of 1a and 1b transcripts.** The hybridization to a p45 second exon probe of a Southern blot of the amplified 1a (*top row*) and 1b (*bottom row*) transcripts is shown. The RNAs used are indicated on the *top* of each *panel*. *A, YS*, yolk sac; *FL*, fetal liver; *dpc*, days post coitum; *BM*, adult bone marrow. Cycle numbers are shown on the corresponding *lanes*. *1A*, this band includes the alternative exon 1A (30) present in the 1a transcript. The calculated percentages of the 1b band *versus* 1b+1a bands are as follows: yolk sac, 8.5–10.5 days post coitum: 72%, 52%, and 75%, respectively; fetal liver, 10.5–13.5 days post coitum: 89%, 96%, and 92%, respectively; adult bone marrow: 42% and 39%. These percentages were calculated using different exposures of the autoradiograms shown, and comparing bands of similar intensities (see "Experimental Procedures"), usually at cycle 20 or 23 (for 1b) *versus* cycle 23 or 26 (for 1a). *B, TER-119*, fractionated erythroid cells; *Mac-1*, fractionated myeloid cells from adult bone marrow. Note that the hybridization at cycle 30 was underexposed relative to those at cycles 23 and 26.

retarded complex (usually seen as a doublet consisting of a weak fast band, possibly a degradation product (see Refs. 38, 41, and 42), and a stronger slow band) in gel shift experiments using erythroid nuclear extracts from human K562 (Fig. 6) and mouse MEL cells (data not shown). Using extracts from non-erythroid T and B lymphoid and myelomonocytic cells, either no bands or weak bands of different mobilities are obtained (data not shown). These results suggest that the p45 gene GATA palindrome might bind GATA-1, the erythroid-specific member of the GATA family. Indeed, the putative GATA-1 band comigrates with a similar band obtained using an oligonucleotide (see "Experimental Procedures") from an enhancer located in the mouse GATA-1 gene hypersensitive site II (HS2) (43), which contains a well characterized GATA-1 binding site (41, 44) (Fig. 6*A*). In addition, unlabeled p45 GATA palindrome and HS2 GATA-1 oligonucleotides reciprocally compete the binding of the corresponding labeled oligonucleotides at low molar excess ratios (Fig. 6*A*, compare *lane 1* to *lanes 2* and *4* and compare *lane 5* to *lanes 6* and *8*).

A mutation (GA to TT in both halves of the palindrome; see Fig. 2*B*), destroying the GATA-1 consensus, abolished the major retarded band, leaving a weak band consistently running slightly ahead of the control GATA band (Fig. 6*B*, compare *lane 4* to *lane 1*). The weak band obtained with the mutated oligonucleotide was competed by both the unlabeled corresponding mutated and normal oligonucleotides (Fig. 6*B*, *lanes 5–8*); however, the mutated oligonucleotide did not compete the normal GATA-1 band obtained with either the p45 GATA palindrome or the HS2 GATA oligonucleotide (Fig. 6*A*, *lanes 3* and *7*). These data indicate that the weak residual band detected with the mutated oligonucleotide is not related to a GATA-1-binding protein. Interestingly, the mutation of only one (*i.e.* either the 5′ or the 3′of the two GATA-1 consensus sites) strongly decreased but did not abolish, the binding of the oligonucleotide, as shown by direct binding (Fig. 6*C*, *lanes 2* and *3*) and competition experiments (Fig. 6*C*, *lanes 6–8*). Finally, the identity of the putative GATA-1 band was confirmed by an antibody-supershift experiment. We used two anti-GATA-1 specific antibodies (provided by Dr. C. Santoro (Ref. 38) and by Santa Cruz Biotechnology); both supershifted most (at least 80%) of the putative GATA-1 band (Fig. 6*D*, *lanes 1* and *2*, and data not shown). The weak band generated by the mutated p45 GATA-1 oligonucleotide was not affected by the antibody (Fig. 6*D*, *lanes 3* and *4*), confirming that this band is not related to GATA-1.

As K562 cells contains some GATA-2 protein (44), an anti-GATA-2 antibody (Santa Cruz Biotechnology) was also used. The anti-GATA-2 antibody did not affect the binding when added to the reactions (data not shown).

The Promoter 1b Is Stronger than the Promoter 1a in Transfection Experiments in Erythroid Cells, and Its Activity Depends on the GATA-1 Binding Site—The promoter 1b shows potential binding sites for the erythroid transcription factor GATA-1, whereas the promoter 1a does not. We evaluated the activity of these promoters by transient transfection of luciferase reporter constructs in erythroid cells. Transfection was carried out in the presence of *Renilla* luciferase, driven by the herpes simplex virus thymidine kinase promoter, to normalize for transfection efficiency.

Fig. 7 indicates that the promoter 1b is much more active than the promoter 1a in both the K562 and HEL erythroleukemia cell lines (compare *lane B* to *lane C*).

As the promoter 1b is normally located within the first intron at a distance of only 4 kilobase pairs from the upstream promoter 1a, it is conceivable that sequences within 1b might act as enhancer elements for the upstream promoter 1a. To test this possibility, the promoter 1b was inserted (both in the sense and antisense orientations, relative to transcription) into the *Bam*HI site downstream of the luciferase reporter gene driven by the promoter 1a (Fig. 7, *E* and *G*). As transcripts might arise at the promoter 1b in this location and run through the circular plasmid into the reporter gene (yielding a spurious, non-enhancer-dependent activity), we also constructed two control plasmids (Fig. 7, *D* and *F*), in which the 1b enhancer was inserted into the *Bam*HI site of a pLUC plasmid lacking the promoter 1a.

As shown in Fig. 7 (compare *lanes E* and *G* to *lanes B* and *D*), the 1b element stimulates (≅5–6-fold), in both orientations, the activity of the weak promoter 1a in K562 and HEL cells.

In contrast, the control (1a-less) *luc-1b enh* plasmids showed very little activity above background, indicating that any transcripts arising at the downstream located 1b element are either unstable or efficiently stopped at the strong poly(A) addition region preceding the promoter cloning site of pLUC. These data indicate that 1b sequences have enhancer activity.

This conclusion was confirmed by stable transfection experiments in K562 cell populations in which *1b -luc* gave a ≅4-fold higher activity than *1a p-luc*, and *1a p-luc-1b enh sense* gave a ≅3.5-fold higher activity than *1a p-luc* (data not shown).

To compare the activities of promoters 1b and 1a in erythroid *versus* non-erythroid cells, a preparation of each plasmid (with firefly luciferase as reporter) was transfected into both cell types (K562 and HeLa cells), together with a fixed amount of *Renilla* luciferase plasmid (Table I). Whereas, in erythroid K562 cells, the activity of the promoter 1b is close to that of the

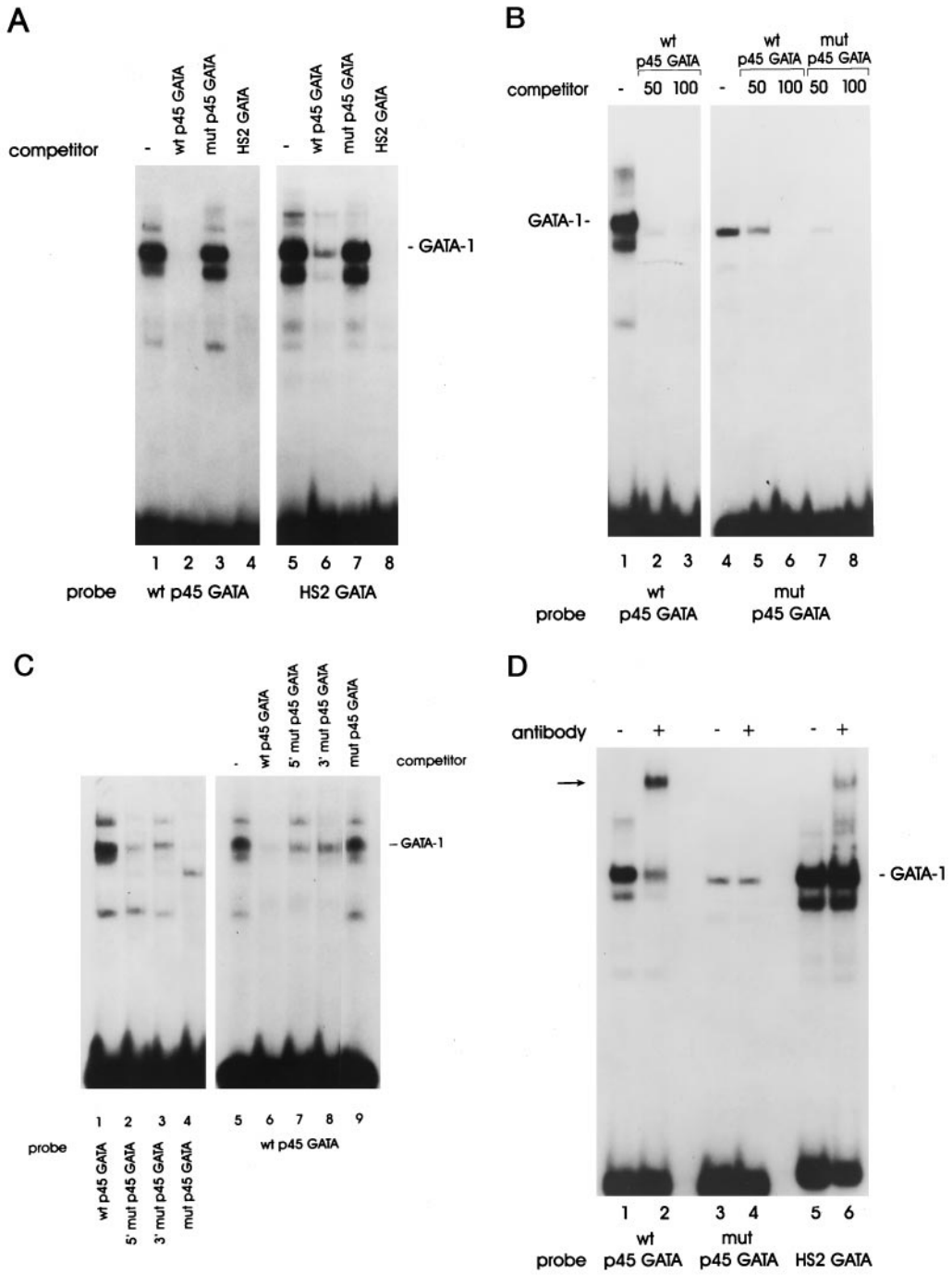

FIG. 6. **GATA-1 binds to the p45 GATA palindrome.** Electrophoretic mobility shift assays were performed with labeled oligonucleotide probes (indicated below the figures) and nuclear extracts from human erythroid K562 cells. Wild type or mutant oligonucleotides (indicated *above* the figures) were used as specific unlabeled competitors in a 50-fold molar excess (*A* and *C*) or as otherwise indicated (*B*). In *D*, an anti-GATA-1 antibody (38) was added where indicated (+). Identical results were obtained with antibodies from Santa Cruz Biotechnology. *wt p45 GATA*, *mut p45 GATA*, *5′ mut p45 GATA*, and *3′ mut p45 GATA*, the normal, doubly mutated and 5′-or 3′-mutated p45 GATA palindrome oligonucleotides (nt 450–480, Fig. 2*B* and "Experimental Procedures"), respectively; *HS2 GATA*, a GATA-1 binding oligonucleotide from the enhancer of the GATA-1 gene (see Ref. 41 and "Experimental Procedures"). In *panel D*, the *arrow* points to the antibody-supershifted GATA-1 band.

strong TK-promoter driving *Renilla* luciferase activity (Table I), in non-erythroid HeLa cells, the activity of the 1b promoter is extremely low and only slightly above that of the promoterless pGL basic vector plasmid; thus, the luciferase to *Renilla* activity ratio is greatly reduced in HeLa cells, relative to the corresponding experiment in K562 cells. Both the *1a p-luc* and *1a p-luc-1b enh* constructs in HeLa cells show the same very low activity as the *1b p-luc* construct. Therefore, neither promoter is significantly active in HeLa cells.

GATA-1 is a hematopoietic restricted transcription factor, that is particularly abundant in the erythroid system. As the promoter 1b contains a strong GATA-1 binding sequence, we destroyed it by substituting, in both sites of the palindrome, the same mutation as in the mutant p45 GATA oligonucleotide, within plasmid *1b p-luc* (Figs. 2*B* and 7). When transfected into K562 cells, this plasmid (*GATA-1*$^{-}$*1b p-luc*) showed essentially no activity (*lane H*), as compared with the wild type counterpart (*lane C*).

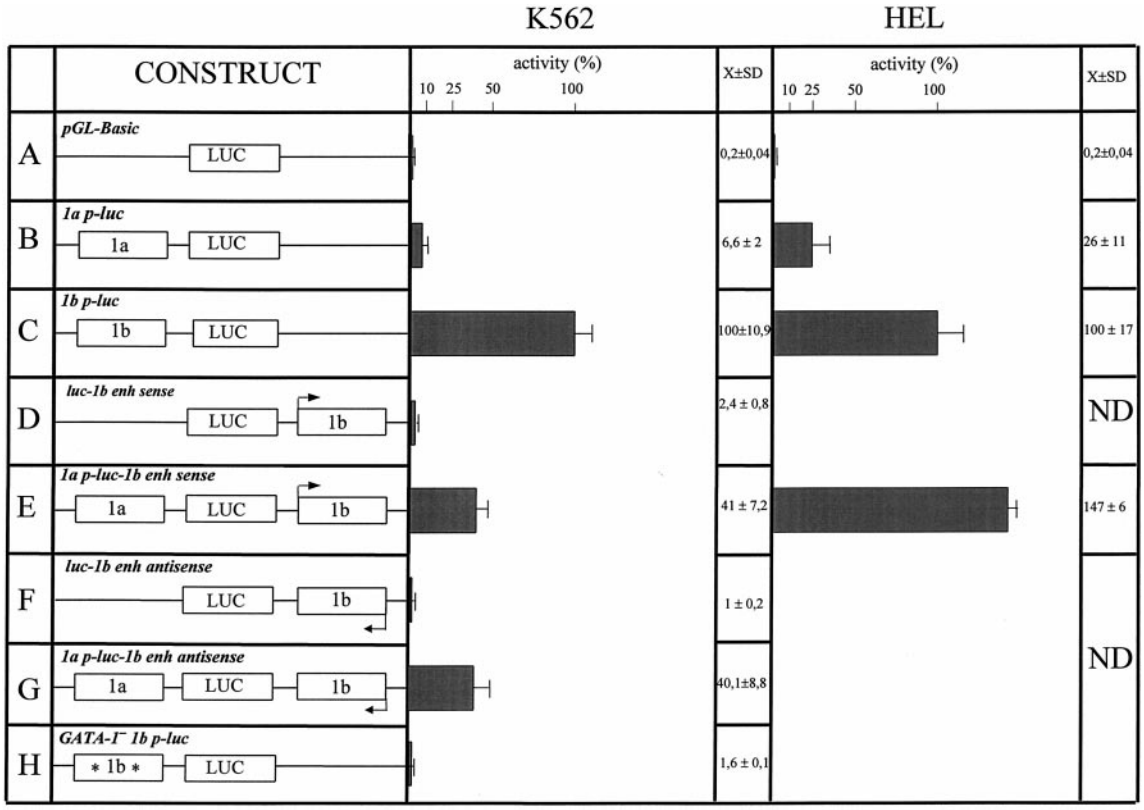

FIG. 7. **Fly luciferase activities of 1a p- and 1b p-luc reporter plasmids transfected into K562 and HEL cells.** Schematic representation of the various plasmids; their names are shown *above* each figure. The activities shown are normalized relative to cotransfected *Renilla* luciferase pRL-TK plasmid. Average and S.D. values are indicated.

TABLE I
Relative fly luciferase activities in erythroid K562 versus non-erythroid HeLa cells

The relative activities of the fly luciferase reporter constructs (test plasmid) to *Renilla* luciferase (cotransfected standard plasmid) are given. The promoterless pGL basis to *Renilla* luciferase relative activity is 0.001 in K562 and 0.01 in HeLa cells.

	K562	HeLa
	X ± S.D.	*X ± S.D.*
1a p-luc	0.014 ± 0.0013	0.033 ± 0.003
1b p-luc	0.4 ± 0.04	0.02 ± 0.002
1a p-luc-1b enh	0.13 ± 0.01	0.033 ± 0.006

DISCUSSION

Conservation of Two Alternative p45 NF-E2 Promoters between Man and Mouse—p45 NF-E2 gene expression is regulated in a complex way; in fact, p45 RNA or protein is detected in only a subset of hematopoietic cells and in few non-hematopoietic cells. The discovery in man of two alternative p45 promoters adds to this complexity (32).

In this paper, we asked whether the alternative promoter arrangement is conserved between man and mouse. As shown in Figs. 1, 2*B*, and 3, both the overall structural organization, with promoter 1b lying in the intron comprised between promoter 1a and exon 2, and significant sequence homology, are conserved between man and mouse. These observations argue in favor of important, and possibly distinct, functional roles for these promoters.

Transcripts from the Promoter 1b Are Abundant in Erythroid, but Not Myeloid, Cells—Initial analysis of the expression pattern of 1a and 1b transcripts in man, led to the suggestion that 1b and 1a transcripts be termed fetal and adult, respectively (32). The availability of early hematopoietic cells from mouse embryos allowed us to further probe this distinction. The results shown in Fig. 5 indicate that indeed transcripts from exon 1b are largely predominant in samples from the early yolk sac and fetal liver, representing 80–90% of the total p45 transcripts.

As hematopoiesis at early stages (embryonic day 8.5–9.5), particularly in the yolk sac, is mainly of the primitive type, whereas at later developmental stages definitive hematopoiesis is predominant, it might be suggested that p45 mRNA transcription from the promoter 1b is an important feature of both primitive and definitive embryonic-fetal hematopoiesis. However, p45 transcripts arising at promoter 1b are also abundant in adult bone marrow samples (Fig. 5*A*, *right panels*), representing on average approximately 40–50% of the total transcripts. This result blurs the proposed distinction (32) between a fetal (1b) and an adult (1a) promoter. In evaluating this distinction, a further note of caution should be added; bone marrow cells represent a very heterogeneous population, containing a very large proportion of myeloid cells, whereas in fetal liver and particularly in yolk sac the erythroid component is predominant. Indeed, TER-119-positive cells from adult bone marrow, representing a highly enriched erythroid population, express ≅75% 1b transcripts (relative to the total) (Fig. 5*B*), a figure that approaches that detected in fetal liver; on the other hand, the myeloid component (Mac-1-positive cells) from the same marrow expresses only 10–15% 1b transcripts relative to the total. That early myeloid progenitors also express very weakly from the promoter 1b is also demonstrated by the analysis of an immortalized population of early interleukin-3-dependent myeloid progenitors (40) in which the proportion of 1b transcripts is less than 5% (data not shown). Thus, although we cannot rigorously rule out some modulation of promoter 1b activity in erythroid cells at different developmental stages, we favor the conclusion that the greater representation of 1b *versus* 1a transcripts in yolk sac and fetal liver reflects, rather

than the developmental stage, the larger proportion of erythroid cells in these organs than in bone marrow. This conclusion is in agreement with the observation that human peripheral granulocytes abundantly express a p45 transcript, exclusively arising at the promoter 1a (23).

The Promoter 1b, but Not the Promoter 1a, Is Active in Transfected Erythroid Cells and Dependent on a GATA Site—A comparison of the sequences of the human and murine promoters 1b and 1a, shows that there is a high degree of conservation in the promoter 1b regions, upstream to the transcription initiation region (Fig. 2*B*); in the promoter 1a, the conserved region extends ≅60–80 nt upstream the major transcription starts. Pischedda *et al.* (32) had previously noticed the presence of potential binding sites for erythroid transcription factors (GATA-1, EKLF, stage-specific element binding factor) in the promoter 1b, but not in the promoter 1a. Only the GATA-1 sites, and in particular a tandem palindromic GATA-1 site (Fig. 2*B*, *boxed*) appear to be conserved between man and mouse in promoter 1b, and therefore of significant potential relevance. Several tandemly repeated GATA-1 sites are known to be strong functional elements (41, 43–46). The tandem repeat strongly binds GATA-1 as determined by gel shift analysis with erythroid (K562 and MEL) nuclear extracts (Fig. 6*A* and data not shown), competition with known GATA-1 binding oligonucleotides (Fig. 6*B*) and the almost complete supershift observed with specific anti-GATA-1 antibodies (Fig. 6*D*). As anti-GATA-2 antibodies had no effect on the gel shift pattern, we conclude that, in erythroid cell extracts, the GATA palindrome mainly, if not exclusively, binds GATA-1.

Of interest, the GATA-1 repeat also includes a CAGCTG palindromic sequence, resembling an E box, which lies 8 nt upstream to the more 3′-GATA-1 (reversed) motif. E boxes are often found in close proximity to GATA-1 sites (46, 47); the sequence arrangement found in the promoter 1b is similar to that known to bind to an at least pentameric complex including GATA-1, Tal-1, E 47, Lmo2, and Ldb-1 factors (47). Although a weak band migrating more slowly than the major GATA-1 complex is visible in Fig. 6*A*, and can be both competed by unlabeled GATA-1 oligonucleotides and supershifted by anti-GATA-1 antibodies (Fig. 6*D*), we have not been able to prove that this complex includes Tal-1, and that the p45 GATA palindrome can bind to Tal-1 in extracts from Tal-1 positive cells (data not shown).

Transient transfection experiments (Fig. 7) with promoter 1b and 1a luciferase constructs show that in erythroid cells the promoter 1b, but not the 1a, is active. This result is consistent with the presence of erythroid factor (GATA-1) binding sites in the former promoter only. Indeed, a mutation inactivating GATA-1 binding to the tandem GATA-1 site abolishes transcription from the promoter 1b; at least one precedent for a very strong effect of a mutation in a tandem GATA-1 repeat was previously reported for a site in the enhancer located in the DNase I-hypersensitive site 1 upstream to the mouse GATA-1 gene (43). Thus, we conclude that p45 NF-E2 gene expression might be regulated by GATA-1; this adds to the list of genes encoding hematopoietic transcription factors, which are likely regulated by GATA-1, such as the GATA-1 gene itself (41, 43–45), Tal-1 (47–49), and EKLF (46, 50). Although the isolated promoter 1a construct is inactive in this transient assay in erythroid cells (Fig. 7), it is possible that, *in vivo,* within the intact locus, the activity of this promoter might be stimulated by sequences in the downstream promoter 1b, acting "enhancer-wise." This is suggested by the observation that the promoter 1b, when placed (both in sense and antisense orientation, relative to transcription) 3′ to the 1a-luciferase construct (in a position that resembles the natural arrangement) is able to stimulate promoter 1a activity quite significantly (Fig. 7). It should be added that a distal enhancer, located in a 7-kilobase pair region upstream to the promoter 1a and containing GATA-1 binding sites, was shown to be strongly activated by cotransfection of GATA-1 and FOG-1 (Friend of GATA-1) expression vectors (51); this upstream site might cooperate with the 1b element in regulating either 1a or 1b or both promoter activities.

The observation that GATA-1 might contribute to p45 gene expression, has some implications for the interpretation of the phenotype of the knock-out of GATA-1 and p45 NF-E2 genes in mouse. In both instances, severe abnormalities of megakaryocytopoiesis and platelet production have been observed (26, 52, 53, 54). GATA-1 and NF-E2 are coexpressed in megakaryocytes and might have common as well as separate target genes (6, 22, 55, 56); it can now be further speculated that the deficient expression of GATA-1 in megakaryocytes from mice carrying a mutant GATA-1 locus (52) might be directly responsible for the observed down-regulation of NF-E2 in megakaryocytes (54), contributing to the severity of the phenotype.

Neither the promoter 1b nor the 1a is active in non-erythroid HeLa cells. As p45 NF-E2 expression occurs in some non-hematopoietic tissues, other regulatory elements, not included in these constructs, or non-hematopoietic tissue-specific factors may be necessary for non-hematopoietic p45 expression.

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Regulation of Mouse p45 NF-E2 Transcription by an Erythroid-specific GATA-dependent Intronic Alternative Promoter

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