Identification of the Proximal Erythroid Promoter Region of the Mouse Anion Exchanger Gene

By Kenneth E. Sahr, Brian P. Daniels, and Manjit Hanspal

The AE1 gene is expressed in erythrocytes and the A-type intercalated cells of the kidney distal collecting duct. Although the 5' end of the principal transcript expressed in murine erythroid cells has previously been mapped to a cluster of transcription start sites located immediately upstream of exon 1, the 5' end of the mouse kidney transcript has not been identified. Using the anchored polymerase chain reaction technique to analyze mouse kidney AE1 mRNA, we identified an internal transcription start site located within erythroid intron 3. This site defines an exon of 37 nucleotides that forms the 5' end of the mouse kidney AE1 transcript. AE1 transcripts beginning at this internal start site could not be detected in RNA isolated from purified erythroid progenitor cells or from erythrocytes undergoing erythropoietin-dependent terminal maturation, although transcripts derived from the upstream site were abundant, indicating that only the upstream promoter is active during erythropoiesis. Transient expression of reporter constructs in erythroid and nonerythroid cell lines identified a proximal upstream region of ~135 nucleotides that was active as a basal promoter. However, an additional ~200 nucleotides of upstream sequence was required for induced levels of activity in erythroid cells. Although our functional approach does not yet indicate the precise sequences required for erythropoietin induction, the AE1 gene upstream region contains potential GATA sites at −154, −141, and −60; an E-box at −163; CACCC or GGTGG motifs at −188, −121, and −88; and an AP-1/NF-E2-like site at −42.

© 1996 by The American Society of Hematology.

From the Department of Biomedical Research and Division of Hematology/Oncology, St. Elizabeth’s Medical Center of Boston, Tufts University School of Medicine, Boston, MA. Submitted February 26, 1996; accepted July 26, 1996. Supported by National Institutes of Health Grant HL49139 (to K.E.S.). Address reprint requests to Kenneth E. Sahr, PhD, Department of Biomedical Research, St Elizabeth’s Medical Center of Boston, Tufts University School of Medicine, 736 Cambridge St, Boston, MA 02135.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. section 1734 solely to indicate this fact.

© 1996 by The American Society of Hematology. 0006-4971/96/8812-0022$3.00/0


MATERIALS AND METHODS

Cell culture. Rauscher erythroleukemia cells42 were grown in RPMI 1640 medium (GIBCO-BRL, Gaithersburg, MD) containing...
10% fetal bovine serum (HyClone Laboratories, Inc, Logan, UT) at 37°C in 5% CO2. For analysis of erythroid induction, we isolated a subclone (Rauscher-R14) by limited dilution plating. When grown in medium containing 1 mM erythropoietin (Ortho Biotech, Raritan, NJ) and 1% (vol/vol) dimethyl sulfoxide (DMSO; Sigma Chemical Co, St Louis, MO) for 4 days, 75% to 90% of these cells became hemoglobinized, as judged by benzidine staining.

Immature erythroblasts were prepared from the spleens of BALB/c mice (Charles River Breeding Laboratories, Wilmington, MA) at 5 and 25 days after infection with the atenemia-inducing strain of Friend virus.3,26; these preparations are referred to as 15-day or 25-day FVA erythroblasts. For erythroid induction, 15-day FVA cells were grown for 24 hours with 1 mM erythropoietin.20

Preparation of RNA. Adult BALB/c mice (Charles River Breeding Laboratories) were anesthetized and then perfused by injection with physiologic saline through the left ventricle. Bone marrow (femur), spleen, and kidney tissues were removed aseptically and RNA was isolated from the remaining erythroid cells by acid precipitation.22 RNA isolated from MEL cells grown for 4 days on fibronectin-coated plates in the presence or absence of 1% (vol/vol) DMSO was provided by Drs B. GuptaRoy and C.M. Cohen.99

Transcription start sites of the mouse AE1 gene. Transcription start sites were mapped using the anchored PCR technique.3,12 Briefly, AE1 cDNA was synthesized from 1 µg of RNA using Moloney murine leukemia virus (M-MLV) reverse transcriptase (Promega Corp, Madison, WI) and antisense primer P41 (S'-CACCCATTG-TAGTTCTCGTGTCCCTCTGTCGCA) from exon 5.20 (The approximate locations of the AE1 primers are shown in Fig 1.) The 3' end of the product cDNA was tailed with dGTP using terminal deoxynucleotidyl-transferase (Promega) and then PCR amplified3 using the nested primer P44 (S'-AGTTCTCTGAAAGCTACACTAGA) and primers P43 (S'-GGAATTCGCGGCGCGGCGGAGG) and P42 (S'-GGAATTCGCGGCGCGGAGGAGGC14) specific for the 3' cDNA tail sequence and containing EcoRI and Not I adaptor sequences. The molar ratios of P44:P43:P42 were 1:1:0.1. Each reaction cycle consisted of 1 minute at 95°C, 1 minute at 55°C, and 1 minute at 72°C; 45 cycles were performed. For Southern hybridization,34 an aliquot of each reaction was separated on a 2% agarose gel and blotted to Zeta-Probe membrane (Bio-Rad Laboratories, Hercules, CA). An AE1 cDNA fragment located just upstream of oligo P44, extending from nucleotides 14 to 354 of the published sequence,3 was used for probe synthesis. This fragment was generated by PCR amplification of random primed Rauscher cell cDNA using sense primer P163 (5'-CTTCAGCCAGCAGCAGCAG) and antisense primer P209 (5'-AGCTTGGTGCAGGTTGTTGT) and subcloned into pGEM4Z (Promega). The authenticity of this insert was confirmed by sequencing. For probe synthesis, the insert was excised, gel-purified, and then labeled with 32P-dCTP (Megaprime DNA labeling system; Amersham Life Science, Arlington Heights, IL). Standard conditions were used for prehybridization and hybridization34; final washes were performed in 0.1% sodium dodecyl sulfate and 0.1× SSC at 65°C.

Anchored PCR products were subcloned into pGEM-4Z (Promega). Inserts were sequenced using Sequenase enzyme (US Biochemical Corp, Cleveland, OH), AE1 primer P44, and plasmid primers SP6 and T7.

Use of the internal transcription start site in erythroid cells. cDNA was synthesized34 from 1 µg of total RNA using M-MLV reverse transcriptase (Promega) and primer P41. An aliquot of each cDNA was then PCR-amplified3 using primers specific for truncated AE1 mRNA (sense primer P101 within exon K1: 5'-TAAGAGGCCTACCAGCTGT; antisense primer P174 spanning the exon 4 exon 5 boundary of the cDNA: 5'-GCTCACAATAGACCTGAC) or primers specific for full-length AE1 mRNA (sense primer P163; antisense primer P174). An aliquot of each PCR reaction was separated on a 2% (wt/vol) agarose gel, blotted to Zeta-Probe membrane, and analyzed by Southern hybridization.34 The probe used for Southern hybridization (described above) was internal to the primers used for PCR.

Luciferase reporter constructs. The constructs described below are diagrammed in Fig 4. Initially, a Sac I fragment (Fig 2C) of subclone pRK08 containing ~22 bp of the 5' end of exon 1 and
Fig 2. Locations of the mouse AE1 gene upstream and internal transcription start sites. (A) Anchored PCR products from bone marrow and spleen RNA (Fig 1) were subcloned, sequenced, and aligned to the mouse AE1 gene sequence of Kopito et al. The locations of the 5' ends of the longest products are indicated (V). Also shown are potential E-box (E), GATA (G), direct (C) and inverted (−C) CACCC, and AP-1/NF-E2 (AN) cis-sequences, which are conserved between the mouse and human AE1 genes. Nucleotide numbering is relative to the 5'-most anchored PCR product. The 5' ends of the luciferase reporter constructs (L4, L13, L12, L11, and L8) used in the experiments presented in Figs 4 and 5 are also indicated by the arrows below the sequence; the 3' subcloning site of each is the Sac I site in exon 1. (B) Sequence analysis of anchored PCR products from kidney RNA identified a unique 5' sequence of 37 nucleotides that was correctly spliced to RNA sequence from exon 4. Alignment of this sequence to the AE1 gene sequence gave a perfect fit to a 37-bp sequence (exon K1) within the erythroid intron 3. The sequence of exon K1 is shown with adjacent regions of intron 3, the 3' donor site of erythroid exon 3, and the 5' acceptor site of erythroid exon 4. Nucleotide numbering is according to Kopito et al. (C) The approximate locations of the upstream erythroid and internal kidney transcription start sites (−) and the location of the first exon (K1) of the kidney-truncated AE1 transcript determined by sequence analysis of subcloned anchored PCR products are shown. The approximate locations of the Bgl II (B), Pvu II (P), Sac I (S), and Sph I (Sp) restriction enzyme sites used for luciferase reporter subcloning are also indicated.
~1.8 kb of upstream sequence was subcloned into the pGL2-enhancer luciferase reporter vector (Promega) containing a downstream SV40 enhancer region. The resulting sense and antisense constructs are referred to as pLuc-1 and pLuc-2, respectively. pLuc-7 was made by subcloning the same Sac I fragment into the pGL3-basic luciferase reporter (Promega) that lacks additional regulatory elements. pLuc-3 and pLuc-4 were made by subcloning the proximal 183-bp Pvu II/Sac I and 377-bp Bal III/Sac I fragments, respectively, into pGL2-basic vector that had been prepared by digestion with Smal I and Sac I. Additional reporter constructs having 5' truncations were generated by PCR. For this approach, pLuc-7 linearized by digestion with Sal I was used as a template with a deletion-specific upstream sense primer (below) containing a Sac I adaptor and an antisense primer (K40; 5'-AGTGTGCTCTCCAGGCGGTT) extending from nucleotides 135 through 152 of the pGL2-basic plasmid. The location of the very 5' end of each of the sense primers used is given in Fig. 2. The specific primer pairs used for each construct are, for pLuc-13, K87 (5'-aaggtacCGGAGCAGGGCAAG-GGGT) and K40; for pLuc-12, K86 (5'-aaggtacCGGAGCAGGGCAAG-GGGT) and K40; for pLuc-11, K85 (5'-aaggtacctTGGGGACGG-GGCAAGATTAGGAGG) and K40; and for pLuc-8, K70 containing a Smal I adaptor (5'-ataacGGGTGGGGAGCTCCTCCCCCG) and K40. PCR products were digested with Kpn I and Sac I or Smal I and Sac I gel-purified, and ligated into pGL2-basic vector that had been prepared by digestion with the appropriate restriction enzymes. pLuc-10 was made by subcloning the upstream ~1.4-kb Sac I/Bal I AE1 gene fragment in sense orientation into the Smal I site of pLuc-8. The orientation and nucleotide sequence of each insert was verified by sequence analysis.

**Transient reporter gene expression.** All plasmid DNAs were purified by twice banding on CsCl gradients. Before electroporation, 75 μg of the appropriate pLuc construct and 50 μg of a transfection control CAT plasmid driven by an RSV promoter (pOPRSV-ICAT; Stratagene, La Jolla, CA) were combined, precipitated with ethanol, pelleted, dried, and resuspended in 180 μL of sterile dH2O. A relatively extensive analysis resulted in use of the following optimal electroporation conditions. Rauscher erythroblast cells, grown to late log density (0.8 to 1.9 × 10⁹/mL) were harvested by centrifugation at 4°C, washed once with ice-cold RPMI-1640, and resuspended in ice-cold RPMI-1640 at ~1.75 × 10⁶ cells/mL. A total of 0.8 mL of this cell suspension, 20 μL of 10× HBS (0.2 mol/L HEPES pH 7.05, 1.37 mol/L NaCl, 50 mmol/L KCl, 7.0 mmol/L Na2HPO4, and 180 mL of plasmid DNA (above) were combined in an electroporation chamber having a 0.4-cm gap (Life Technologies, Inc, Gaithersburg, MD) on ice. Cells were shocked while on ice using a BRL Cell-Portor Electroporation System I (Life Technologies, Inc) with settings of 1.180 μF and 750 V and then transferred to a culture flask containing 9 mL of prewarmed complete medium. For analysis of basal promoter activity in noninducing conditions, cells were cultured in complete medium for 36 to 40 hours and then harvested by centrifugation and lysed in 250 μL of reporter lysis buffer (Life Technologies, Inc). Luciferase enzyme activity of a constant volume of each cell lysate (10 or 20 μL) was measured using the luciferase assay system (Promega) and a Turner TD-20e luminometer (Turner Designs, Sunnyvale, CA). CAT enzyme activity of 50 μL of each lysate, measured as described by Seidman and Sheehan, was used to standardize the calculation of the total luciferase enzyme activity per sample. Experiments to analyze promoter induction were performed in the same manner except that, after electroporation, cells were grown in complete medium in the absence or presence of 1 μM erythropoietin and 1% (vol/vol) DMSO for 90 to 96 hours. Cell lysates were prepared, the protein concentration of each lysate was determined (Bio-Rad Protein Assay kit), and luciferase enzyme activity was measured using 0.1 mg of lysate protein per assay. In these experiments, the amounts of the luciferase reporter plasmids used for transfection were shown to be in a linear range of transient gene expression and not significantly affected by cotransfection with the control CAT plasmid.

NIH3T3 cells were maintained in Dulbeco's modified Eagle's medium (Life Technologies, Inc) containing 10% calf serum (Life Technologies, Inc). Cells (grown to ~75% confluency in 10-cm dishes) were cotransfected with 15 μg of the appropriate luciferase reporter construct and 5 μg of pOPRSV-ICAT using a standard calcium phosphate DNA-precipitation procedure. Cell lysates were prepared 30 to 36 hours after transfection, and luciferase and CAT enzyme activities measured as described above.

**RESULTS**

**Transcription start sites of the mouse AE1 gene.** The predominant AE1 transcript in murine erythroid cells is ~4.5 kb and encodes a protein of 929 amino acids. Using primer extension and nuclease protection approaches, Kopito et al identified a cluster of five erythroid transcription start sites upstream of exon 1 located 146 to 189 bp upstream of the initiator ATG codon in control and induced MEL cells; these same sites and an additional site at -260 were observed in RNA isolated from anemic and normal mouse spleen tissues. These experiments map a potential upstream erythroid AE1 gene promoter region. The AE1 transcript found in the mouse kidney is ~4.2 kb. This transcript lacks sequences from the first three gene exons, resulting in an AE1 protein with an N-terminal 79 amino acid truncation. The location of the 5' end of the mouse kidney transcript was not identified. In the experiments described in this section, we used anchored PCR, because of its sensitivity, to map the location of the potential promoter region that gives rise to this transcript.

As described in Fig 1, bone marrow, spleen, and kidney RNAs were first reverse transcribed using antisense primer P41 from exon 5. This primer is located ~400 bp downstream of the predicted 5' end of the full-length erythroid AE1 transcript and 62 bp downstream of the 5' end of exon 4 sequence near the predicted 5' end of the kidney transcript. The principal product obtained from bone marrow and spleen RNA was ~400 bp in size. These bone marrow and spleen cDNA products were subcloned and sequenced. The locations of the 5'-most spleen and bone marrow subclones (Fig 2A) are within the cluster of erythroid transcription start sites previously defined in detail by Kopito et al. The sites identified by these investigators are at nucleotides +10, +5, -9, -17, -34, and -102 in Fig 2. A single-product band of the expected size was obtained from spleen RNA, whereas two additional products of lower abundance were obtained from bone marrow RNA. Sequence analysis showed that these products are from incompletely processed AE1 pre-mRNA. The ~500-bp product was found to retain intron 2 sequence, whereas the ~1,000-bp products retained either intron 3 or 4 sequences.

Because AE1 protein is expressed only in the type A intercalated cells of the kidney distal collecting duct, which represent a relatively small subset of kidney cells, AE1 transcripts are predicted to be in relatively low abundance in total kidney RNA. Longer film exposures of the
Southern filter shown in Fig 1 showed hybridizing products of ~170 bp, ~400 bp, and ~500 bp (lane 3b). Using sequence analysis, the ~170-bp product was found to have a unique 5’ terminus of 37 nucleotides, which was correctly spliced to sequences contributed by exon 4. By alignment to the mouse AE1 gene, this sequence is derived from a region located within erythroid intron 3 (Fig 2B and C). This site identifies a potential internal promoter region and the first exon (exon K1) of the kidney form of the AE1 gene. The two additional products of ~400 bp and ~500 bp are identical to those obtained using erythroid RNA (above) and are probably from a small amount of contaminating RBC RNA.

Is the AE1 gene internal promoter region used in erythroid cells? Brosius et al, using Northern and S1 protection assays, reported an absence of the truncated AE1 transcript in rat and mouse spleen RNA. However, longer film exposures (data not shown) of the Southern filter presented in Fig 1 showed a faint signal at the ~170-bp position in the anchored PCR products of both bone marrow and spleen RNA. The high degree of sensitivity of the PCR reaction and the relatively low signal observed suggest that the internal promoter region might be used at very low or constitutive levels in these tissues. This possibility was studied more directly in the following manner. Bone marrow, spleen, and kidney RNA preparations were reverse-transcribed using the AE1 antisense primer P41. The resulting cDNA was then PCR-amplified using primers P174 and P101, which are specific for the 5’ end of the truncated transcript, and was analyzed by Southern hybridization. The hybridization probe used in this experiment is internal to the primers used for cDNA synthesis and PCR, and filter washing was performed in stringent conditions. Correctly spliced AE1 mRNA originating with exon K1 should yield a product of 133 bp. As shown in Fig 3A, a hybridizing product of the appropriate size (labeled T) is detected in both spleen and bone marrow RNA, in addition to kidney RNA, suggesting that the internal transcription start site is also used in these tissues. The larger PCR products were identified as incompletely processed AE1 pre-mRNA.

These results suggest two primary possibilities: (1) that both promoter regions are active in erythroid cells, but that only the upstream promoter is upregulated during RBC maturation, whereas the internal promoter is used in a constitutive manner; or (2) that the internal promoter is used at low levels in nonerythroid cells of the spleen and bone marrow. To study the first possibility further, we analyzed RNA isolated from murine erythroleukemia cell lines and from purified murine erythroid progenitor cells undergoing terminal maturation. These cell sources included progenitors at approximately the CFUe stage; Rauscher erythroleukemia cells cultured for 4 days with erythropoietin/DMSO; control MEL cells; (6) control MEL cells cultured for 4 days with DMSO; and (8) mouse reticulocytes. The cell population studied represent various stages of erythroid maturation, ranging from committed CFUe progenitor cells (15-day FVA cells, Rauscher erythroleukemia, and MEL cells) to relatively mature cells (circulating reticulocytes). Notably, erythroid induction of FVA erythroblasts, MEL erythroleukemia cells, and MEL cells results in a relatively large and rapid accumulation of AE1 mRNA. As shown in Fig 3, transcripts originating from the promoter region defined by the internal transcription start site could not be detected using purified erythroid progenitor cells or cells undergoing induced terminal erythroid maturation, although, in each, the full-length transcript derived from use of the upstream promoter region was abundant.

Transient expression analysis of the upstream promoter region. The gene region immediately upstream of exon 1 was originally cloned and sequenced by Kopito et al. The promoter activity of this region has not been previously analyzed. Initially, a ~1.8-kb Sac I fragment (Fig 2C) containing the very 5’ end of exon 1, the AE1 gene erythroid transcription start sites, and ~1.7 kb of upstream sequence was subcloned in sense (construct pLuc-1) and antisense (pLuc-2) orientations into a luciferase reporter plasmid containing a downstream SV40 early enhancer element. Transient expression of the sense construct in Rauscher erythroleukemia cells resulted in a relatively high level of luciferase gene expression, whereas the antisense construct that is otherwise identical yielded undetectable levels of luciferase gene expression (data not shown). The level of luciferase gene expression using pLuc-1 was ~50% of that obtained from a control construct containing SV40 early promoter and enhancer elements (pGL2-C; Promega). Significantly, the promoter activity of a ~1.8-kb AE1 construct identical to pLuc-1 but lacking a downstream SV40 enhancer element (construct pLuc-T) was ~80% that of pLuc-1. Subsequent analysis, therefore, was performed using reporters lacking the SV40 enhancer element. As shown in Fig 4, AE1 constructs pLuc-7, pLuc-4, and pLuc-3, which contain 1,738, 357, and 160 bp of upstream sequence, respectively, had near maximal promoter activity when transfected into Rauscher erythroleukemia cells grown in noninducing medium. Construct pLuc-13, which contains 135 bp of upstream sequence, retained ~60% of maximal promoter activity. Additional 5’ deletions of 22 and 64 nucleotides (pLuc-12 and pLuc-11, respectively) reduced promoter activity to 5% to 12% of pLuc-7, whereas construct pLuc-8 containing only the 5’ end of exon 1 and the normal AE1 gene transcription start sites was inactive.

The activities of these reporter constructs were also tested by transient expression in NIH3T3 cells. In three separate experiments, their activities relative to pLuc-7 were, for pLuc-4, 1.06 ± 0.19; for pLuc-3, 0.68 ± 0.25; for pLuc-13, 1.19 ± 0.57; for pLuc-12, 0.57 ± 0.21; for pLuc-11, 0.41 ± 0.13; and for pLuc-8, 0.03 ± 0.02. Therefore, maximal promoter activity in these cells was observed using a reporter
containing only 135 nucleotides of upstream gene sequence (pLuc-13), whereas a construct containing 113 nucleotides of upstream sequence (pLuc-12) still retained ~60% of maximal activity.

During terminal RBC maturation, the steady-state level of AE1 mRNA and the rate of AE1 gene transcription increase significantly. To determine if these changes can be ascribed in part to the upstream promoter region, the activities of the various reporter constructs described above were compared in transfected Rauscher erythroleukemia cells subsequently grown for 4 days in either control medium or medium containing 1 U/mL erythropoietin and 1% (vol/vol) DMSO. In these experiments, 75% to 90% of the cells grown in inducing medium became hemoglobinized as judged by benzidine staining, whereas less than 1% of the cells grown in control medium became benzidine positive. As shown in Fig 5, growth in inducing medium resulted in an approximately ninefold increase in luciferase gene expression using the 1,738-bp and 357-bp promoter constructs pLuc-7 and pLuc-4. Induction of the 160-bp promoter construct pLuc-3, although reduced, was still ~4.5-fold. The 135-bp promoter construct pLuc-13, although capable of near maximal levels of luciferase gene expression in cells grown in control medium, failed to yield increased levels of gene expression in cells grown in inducing medium.

**DISCUSSION**

The AE1 gene, which is expressed in erythroid cells and the type A intercalated cells of the kidney distal collecting duct, has separate upstream and internal transcription start sites. The upstream erythroid transcription start site of the mouse AE1 gene has been previously analyzed in detail.

---

**Fig 3.** Use of the AE1 gene internal transcription start site used in erythroid cells. (A) AE1 cDNA was prepared from bone marrow (BM), spleen (S), and kidney (K) RNA using primer P41 and then PCR-amplified using the nested antisense primer P174 and a truncated AE1 transcript-specific primer P101. The products were analyzed by Southern hybridization using a labeled AE1 cDNA fragment probe internal to the primers used for PCR. A PCR product produced from fully spliced truncated AE1 mRNA should be 133 bp in length (T). (B) The 5’ end of the full-length AE1 transcript contributed by exons 1 through 5 and the 5’ end of the truncated AE1 transcript contributed by exons K1, 4, and 5 are indicated. The approximate locations of the primers used in the experiments of (A) and (C) are shown. (C) cDNAs were synthesized as described in (A) from erythroid cells representing various stages of maturation: lane 1, mouse reticulocytes; lane 2, 15-day FVA cells; lane 3, 15-day FVA cells cultured for 24 hours in vitro with Epo/DMSO; lane 4, 25-day FVA cells; lane 5, control Rauscher erythroleukemia cells; lane 6, Rauscher erythroleukemia cells cultured for 4 days in vitro with Epo/DMSO; lanes 7 and 8, control MEL cells; lanes 8 and 9, and MEL cells cultured for 4 days with DMSO. cDNAs were then PCR-amplified in a multiplex reaction using oligonucleotides P163, P101, and P174 (lanes 1 through 6) or using oligonucleotides P163 and P174 (lanes 7 and 9) or P101 and P174 (lanes 8 and 10) in pairwise combinations. Aliquots of each PCR reaction were analyzed by Southern hybridization as described in (A). The expected sizes of full-length (F) and truncated (T) PCR products are shown.
Fig 4. Transient expression of AE1 promoter constructs in erythroid cells. Luciferase reporter constructs driven by the indicated AE1 gene upstream fragments were transfected by electroporation into Rauscher erythroleukemia cells. Luciferase enzyme activity was measured after 2 days of growth in noninducing medium. The results are an average of two individual experiments using separate plasmid preparations. Luciferase enzyme activity ± range is expressed relative to construct L7. The gene map on the top line indicates the approximate positions of potential E-box (E), GATA (G), direct (C) and inverted (−C) CACCC, and AP-1/NF-E2 (A/N) cis-elements of the AE1 gene upstream region (Fig 2A); the 5' end of exon 1 (solid box); and the 3' Sac I subcloning site in exon 1. The position of the 5' end of each insert is indicated to the left of each construct.

using primer extension and nuclease protection approaches and is composed of a cluster of six sites located immediately upstream of exon 1. Recent analysis of the highly conserved rat AE1 gene shows a similar cluster of sites. The results of our anchored PCR experiments using mouse bone marrow and spleen RNA described in this report are consistent with these reports. Using anchored PCR, we mapped the kidney transcription start site of the mouse AE1 gene to a unique exon (K1) of 37 nucleotides within the erythroid intron 3. The location of this site was not previously identified in the experiments of Kopito et al. or Brosius et al. Use of this site predicts a truncated AE1 transcript of 4,150 nucleotides, which is consistent with the size observed for the kidney AE1 transcript. The 3' end of exon K1 has a consensus donor sequence and, based on the results of RT-PCR of kidney RNA (Fig 3A), intron k1 appears to be spliced out of the mouse transcript. The same internal transcription start site has recently been mapped in the rat and human AE1 genes and is therefore conserved in these three different species.

Careful analysis of RNA isolated from purified erythroid cells at various stages of terminal maturation (Fig 3), ranging from CFUe progenitor cells through the reticulocyte stage, failed to detect AE1 transcripts derived from the internal transcription start site. Only transcripts originating from the upstream start site were detected in these cells, indicating that only the upstream promoter of the AE1 gene is used during terminal RBC maturation. This is in contrast to the PBGD gene, which has a structure related to that of the AE1 gene, with separate upstream and internal sequences that comprise nonerythroid and erythroid promoter regions, respectively. Although the internal erythroid promoter is upregulated during terminal erythroid maturation, resulting in elevated levels of the erythroid PBGD transcript, the upstream promoter remains constitutively active and the nonerythroid transcript is present at low levels. The reason for the complete inactivity of the internal AE1 promoter during RBC maturation is not known. Although the predominant erythroid AE1 transcript is derived from the upstream transcription start site, the internal transcription start site appears to be used at low levels in bone marrow and spleen tissues (Fig 3). This result contrasts to that reported previously by Brosius et al., who failed to detect the truncated AE1 transcript in spleen RNA using Northern hybridization and S1 nuclease protection assays, and is probably due to the high degree of sensitivity of the anchored PCR analysis. Although small amounts of the truncated AE1 mRNA and protein have recently been reported in circulating erythrocytes from the chicken, the truncated protein has not been detected in mouse, rat, or human erythrocytes; our reverse transcriptase-PCR experiments using purified mouse erythroid cells undergoing terminal maturation are consistent with these results. The origin of the truncated transcript in bone marrow and spleen RNA is not known, but could be
from nonerythroid cells present in these tissues or from erythroid progenitors less mature than the CFUe cells that we studied.

We analyzed the upstream erythroid promoter region of the mouse AE1 gene using luciferase reporter constructs transiently expressed in Rauscher erythroleukemia cells. Initial experiments were performed in noninducing medium. In these conditions, the cells do not differentiate, but grow as immature erythroblasts and synthesize low yet detectable amounts of several erythroid-specific components, including AE1 mRNA and protein. Transient reporter gene expression in these cells, therefore, allows us to identify using a functional assay the proximal upstream gene region that can function as a basal promoter. Initially, we showed that an upstream ~1.8-kb fragment could efficiently drive luciferase gene expression. Analysis of AE1 constructs having progressive 5' deletions showed that an upstream fragment of 160 nucleotides conferred ~80% of maximal promoter activity, whereas a construct containing only 135 nucleotides of upstream sequence still retained ~60% activity. Maximal promoter activity in transfected 3T3 cells was observed using a reporter containing only 135 nucleotides of upstream gene sequence, whereas a construct containing 113 nucleotides still retained ~70% of maximal activity.

The amount of steady-state AE1 mRNA increases dramatically during terminal RBC maturation. Although this change is most likely the result of transcriptional as well as posttranscriptional control mechanisms, Fraser and Curtis have reported that the rate of AE1 gene transcription is increased significantly in MEL cells induced with DMSO. To determine if part of this increase can be accounted for by erythroid induction of the upstream promoter region, luciferase reporter constructs were transfected into Rauscher cells that were subsequently grown in either control medium or in medium containing 1 U/mL erythropoietin and 1% (vol/vol) DMSO for 4 days. During this time period, 75% to 90% of cells grown in inducing medium become benzidine positive and the steady-state levels of AE1 mRNA increase greater than 50-fold. The promoter activity of construct pLuc-13 that contains 135 nucleotides of upstream sequence was not increased significantly when transfected into cells grown in inducing conditions, although, as described above and also observed in this experiment, maximal basal promoter activity was observed in control cells. In contrast, constructs driven by 357 (pLuc-4) or 1738 (pLuc-7) nucleotides of upstream sequence were increased approximately ninefold in inducing conditions, and a construct containing 160 bp of upstream sequence (pLuc-3), although reduced, was still increased approximately fivefold.

Our results suggest that the proximal ~135 nucleotides of upstream AE1 gene sequence can function as a basal promoter in transfected erythroid and nonerythroid cells. However, inducible promoter activity in erythroid cells is conferred by a gene region from approximately 135 to 357 nucleotides upstream of exon 1. Notably, the proximal 135 nucleotide sequences of the mouse, rat, and human AE1 genes are greater than 78% identical, whereas the sequences from ~135 to ~357 are greater than 70% identical. Although our functional approach does not yet indicate the precise sequence required for erythroid induction, this upstream region contains several potential cis-elements found in other erythroid promoters, including GATA sites at -154, -141, and -60; an E-box at -163; CACCC or GGTGG motifs at -188, -121, and -88; and an AP-1/NF-E2-like site at -42. The functional identification of the erythroid promoter region of the AE1 gene described in this report will allow us to analyze in more detail the transcription factors required for erythroid induction, with the goal of determining how AE1 gene expression is activated during terminal RBC maturation in coordination with that of other erythroid genes.

ACKNOWLEDGMENT

Subclone pRK108 containing the mouse AE1 gene upstream region was generously provided by Dr. R. Kopito for use in these experiments. RNA isolated from MEL cells was generously provided by Drs. B. GuptaRoy and C.M. Cohen. We thank Q. Uong for excellent technical assistance and D.M. Mironchuk for help with preparation of the illustrations. The erythropoietin used in these experiments was generously provided by Ortho Biotec.

REFERENCES

42. Raich N, Mignotte V, Dubart A, Beaupain D, Leboulic Ph,


Identification of the proximal erythroid promoter region of the mouse anion exchanger gene

KE Sahr, BP Daniels and M Hanspal