Genomic Cloning and Promoter Analysis of Aortic Preferentially Expressed Gene-1

IDENTIFICATION OF A VASCULAR SMOOTH MUSCLE-SPECIFIC PROMOTER MEDIATED BY AN E BOX MOTIF*

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Aortic preferentially expressed gene-1 (APEG-1) was originally identified as a 1.4-kilobase (kb) transcript preferentially expressed in differentiated vascular smooth muscle cells (VSMC). Its expression is markedly down-regulated in de-differentiated VSMC, suggesting a role for APEG-1 in VSMC differentiation. We have now determined that APEG-1 is a single-copy gene in the human, rat, and mouse genomes and have mapped human APEG-1 to chromosome 2q34. To study the molecular mechanisms regulating its expression, we characterized the genomic organization and promoter of mouse APEG-1. APEG-1 spans 4.5 kb in the mouse genome and is composed of five exons. Using reporter gene transcription analysis, we found that a 2.7-kb APEG-1 5′-flanking sequence directed a high level of promoter activity only in VSMC. Its activity was minimal in five other cell types. A repressor region located within an upstream 685-base pair sequence suppressed the activity of this 2.7-kb promoter. Further deletion and mutation analyses identified an E box motif as a positive regulatory element, which was bound by nuclear protein prepared from VSMC. In conjunction with its flanking sequence, this E box motif confers VSMC-specific enhancer activity to a heterologous SV40 promoter. To our knowledge, this is the first demonstration of an E box motif that mediates gene expression restricted to VSMC.

The de-differentiation of vascular smooth muscle cells (VSMC) from a quiescent and contractile phenotype to a proliferative and synthetic phenotype is one of the most prominent features of atherosclerosis, the leading cause of death in developed countries (1). These phenotypic changes may result from vascular injuries caused by smoking, hypercholesterolemia, hyperhomocysteinemia, hypertension, or trauma (1–6). Owing to, at least in part, a lack of markers specific for differentiated VSMC, the molecular mechanisms regulating VSMC differentiation are largely unknown (2).

Although several gene products have been used as specific markers for differentiated smooth muscle cells (SMC), such as smooth muscle α-actin, smooth muscle myosin heavy chain, calponin, SM22α, and caldesmon (7–12), their expression in vivo is not restricted to VSMC. However, a 0.4-kb segment of the SM22α promoter, which contains two CArG elements, has been shown recently to confer expression only in the arterial SMC of transgenic mice (13, 14). Because mutation of the proximal CArG element eliminates all SM22α promoter activity in transgenic animals, this element appears to be necessary and sufficient for high-level expression restricted to the SMC lineage (13). The CArG element binds to nuclear proteins such as serum response factor and YY1. Serum response factor and YY1 are both expressed ubiquitously; thus it is unclear how the CArG element regulates arterial SMC-specific expression conferred by the 0.4-kb segment of the SM22α promoter. This puzzle could be explained by the presence of arterial SMC-specific transacting factors or co-factors that have yet to be identified.

Aortic preferentially expressed gene-1 (APEG-1) was cloned in our laboratory by virtue of its preferential expression in VSMC (15). It is a 1.4-kb message and encodes a 12.7-kDa protein (15). APEG-1 is expressed in differentiated VSMC in vivo and is down-regulated rapidly in de-differentiated VSMC in vitro and in injured arteries in vivo (15). These data suggest that APEG-1 may serve as a sensitive marker for VSMC differentiation, and that it may play a role in regulating growth and differentiation in this cell type.

In this report we show that APEG-1 is a single-copy gene. The APEG-1 transcription unit spans 4.5 kb in the mouse genome and contains five exons. Using reporter gene transcription analysis, we found that 2.7 kb of the APEG-1 5′-flanking sequence contains potent VSMC-specific promoter activity. Further deletion and mutation analyses identified an E box motif as a positive regulatory element, which was bound by nuclear protein prepared from VSMC. In conjunction with its flanking sequence, this E box motif confers VSMC-specific enhancer activity to a heterologous promoter.

EXPERIMENTAL PROCEDURES

Cell Culture and Reagents—Bat aortic SMC (RASMC) were harvested from the thoracic aortas of adult male Sprague-Dawley rats (200–250 g) by enzymatic digestion (16). Bovine aortic endothelial cells were harvested from bovine aortic endothelium as described (17). U-2 OS cells were kindly provided by Dr. T.-P. Yao (Dana-Farber Cancer Institute, Boston, MA) and were harvested from the thoracic aortas of adult male Sprague-Dawley rats (200–250 g) by enzymatic digestion (16). These phenotypic changes may result from vascular injuries caused by smoking, hypercholesterolemia, hyperhomocysteinemia, hypertension, or trauma (1–6). Owing to, at least in part, a lack of markers specific for differentiated VSMC, the molecular mechanisms regulating VSMC differentiation are largely unknown (2).

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Institute, Boston, MA). HeLa, HepG2, C2C12, and NIH 3T3 cells were obtained from the American Type Culture Collection. All cells were grown in Dulbecco’s modified Eagle’s medium (JRH Biosciences, Lenexa, KS) supplemented with 10% fetal bovine serum (HyClone, Logan, UT), 4 mM l-glutamine, penicillin (100 units/ml), and streptomycin (100 

**Southern Blot Analysis and Chromosomal Localization of APEG-1**

Genomic DNA was extracted from cultured mouse embryonic stem cells, RASM, and human umbilical vein endothelial cells. Genomic DNA (10 μg) was digested with 50 units of the restriction enzyme XhoI, EcoRI, BamHI, HindIII, or SacI, separated on a 0.8% agarose gel, denatured, and transferred to NitroPure filters (MSI, Westboro, MA) by using a standard protocol (19). The filters were hybridized with [α-32P]dCTP-labeled APEG-1 cDNA probes from the appropriate species (the cDNA probes contain exons 2 to 5). After hybridization, filters were washed at 55 °C in 0.2 × SSC (30 mM sodium chloride, 3 mM sodium citrate), 0.1% sodium dodecyl sulfate and exposed to Kodak BioMax films with intensifying screens. To locate APEG-1 on a specific human chromosome, we hybridized a genomic Southern blot membrane containing an EcoRI-digested human monochromosomal somatic cell hybrid panel (BIOS Laboratories, New Haven, CT) with a human APEG-1 cDNA probe under the same hybridization and washing conditions.

**Genomic Library Screening and Sequence Analysis**—A 129/SvJ mouse genomic library in a FIX II vector (Stratagene, La Jolla, CA) was screened with a mouse APEG-1 cDNA probe. Four positive clones were purified and subcloned into pUC 18 (Promega, Madison, WI) or pBlue-Script II SK (Stratagene) plasmid vectors. The genomic DNA and exon-intron junctions were sequenced by the dideoxy chain termination method with primers designed from the mouse APEG-1 cDNA. Intron sizes were determined by polymerase chain reaction (PCR) with flanking exon primers, and by direct sequencing. The DNA sequences were assembled and analyzed with Sequencer 3.0 software (Gene Codes Co., Ann Arbor, MI) running on a PowerMacintosh (Apple Computer, Cupertino, CA).

**RNAse Protection Assay**—A 305-bp mouse APEG-1 fragment (−122 to +183) was amplified by PCR from genomic DNA using the forward primer 5'-CGTTCgcagTCACCACTACcAGG-3' (the lowercase sequence indicates a SacI site introduced for cloning purposes) and the reverse primer 5'-GAGGGTTGCACACGcAC-3' and subcloned into the pCR-Script vector (Stratagene). After linearization this construct was used to produce an [α-32P]UTP-labeled antisense riboprobe with T3 RNA polymerase. Gel-purified antisense riboprobe was hybridized overnight with 20 μg of total RNA from mouse aorta, heart, or skeletal muscle. Yeast tRNA was also hybridized as a negative control. RNAse A (0.1 unit) and RNAse T1 (4 units) were added subsequently to digest single-strand RNA, and the remaining riboprobe was separated on an 8% sequencing gel along with a sequencing ladder using the linearized template and reverse primer. The gel was exposed to Kodak X-OMAT film tight with an intensifying screen at −80 °C.

**5' RACE (Rapid Amplification of cDNA Ends) Experiment**—A 5' RACE system (Life Technologies, Inc., Manassas, VA) was used according to the protocol provided by the manufacturer. The nested primers were R592, 5'-CCATATTGGTTGACGCCGC-3' for reverse transcription; R354, 5'-TTGGGGGTGCTCCTGGAAgAGATC-3' for the initial PCR; and R309, 5'-TGCAgCTGCTCCTCCTGCTC-3' for the nested PCR. One microgram of total RNA from mouse aorta was used. Chloramphenicol acetyltransferase (CAT) RNA and primers provided by the manufacturer were used as positive controls. The 5' RACE product was cloned into the pCR-II vector (Invitrogen, Carlsbad, CA) for sequence analysis.

**Cloning of the Mouse APEG-1 Promoter and Construction of Reporter Plasmids**—A 2.7-kb mouse APEG-1 5'-flanking genomic sequence (bp −2663 to +76) was amplified from genomic DNA by PCR using the forward primer −2663 (5'-GGCGTAATAAGCCCCCTGGATAC-3') and the reverse primer +76 (5'-CTACGCTGCCCCAGCCCCCTACAAC-3') and the reverse primer +76 (5'-TACGGCTGGCCAGCCCCCTACAAC-3'). The reporter plasmid p(−2663/+76) was made by inserting this 2.7-kb fragment into the SacI and SalI sites of the pGL3-Basic vector (Promega). Additional 5'-end deletion constructs were generated by PCR with pGL3-Basic (Promega) and the following primers: B3 (5'-CTGGAGCtccGAAATCTAAATC-3'), −479 (5'-CCCTGAGAcATCTCTTGGTTCTC-3'), −355 (5'-GCGGcGTCATGTTGGAACAC-3'), and −122 (5'-CGTGCAGcTCCACCACCTGCGG-3'). (The nucleotides in lowercase in each primer show mutations introduced for cloning purposes.) To generate 3' deletion and mutation constructs, we paired the −479 or −122 forward primer with the +38 (5'-TAGCCaaGACTGAGG-3'). Hybridization was carried out with APEG-1 cDNA probes. The positions of DNA size markers are indicated in kb. monochromosomal somatic cell hybrid Southern analysis. Each lane contained the EcoRI-digested genomic DNA of a single human chromosome (1-22, X, and Y) and mouse or hamster background genomic DNA (asterisk). Human, mouse, hamster, and an equal mixture of human and mouse (Mix) genomic DNAs were included as controls. Arrow marks a signal specific to human APEG-1, present in the positive control lanes (Human and Mix) and in the lane containing the genomic DNA of human chromosome 2.

**Detecting Transfection and Reporter Activity Assays**—p(−2663/+76) and pGL3-Control (equal moles) were used to transfect growing cells on 60-mm dishes with DEAE-dextran for RASM (20) or LipofectAMINE (Life Technologies, Inc.) for other cell types. To correct for variability in transfection efficiency, we co-transfected 1 μg of pCAT3-Control in all experiments. Luciferase and CAT activities were measured 48–60 h after transfection as described (20, 21). To compare APEG-1 promoter activity in different cell types, we expressed the normalized activity of each construct was expressed as a percentage of the activity of p(−2663/+76). All data are presented as the mean ± S.E.

**Luciferase Protein Extraction and Electrophoretic Mobility Shift Assay**—RASM and NIH 3T3 nuclear extracts were prepared from cells grown on 150-mm dishes essentially as described (22). C2C12 cells were grown in 10% fetal bovine serum then kept in 2% horse serum for 3 days to induce differentiation into myotubes. Protein concentration was measured by the Bradford dye-binding method (23) with the Bio-Rad protein assay system (Bio-Rad). For the electrophoretic mobility shift assay, oligonucleotide probes were synthesized according to the mouse APEG-1 exon 1 sequences E (5'-GGGCCCCTACGTTGGTACG-3') and Emut (5'-GGGCCCTACGCGGTGTCAG-3'). An 18-bp E box-containing site.
probe was also synthesized according to the mouse muscle creatine kinase (MCK) enhancer sequence (5'-CCCAACCTGGCCTGCT-3'). An unrelated Oct-1 sequence (5'-TTAGGAAAAATTAAATAGTTATTGAATG'-3') was made as a nonspecific competitor. Double-stranded oligodeoxynucleotide (50 pmol) was end-labeled with [γ-32P]ATP by using polynucleotide kinase (New England Biolabs, Beverly, MA) and purified on a Sephadex G-25 column (Roche Molecular Biochemicals, Indianapolis, IN). A typical binding reaction consisted of 8 ng of nuclear extract, DNA probe (20,000 cpm), 250 ng of poly(dI-dC), 25 mM HEPES (pH 7.9), 40 mM KCl, 0.1 mM EDTA, 1 mM dithiothreitol, and 10% glycerol. A molar excess (100-fold) of unlabeled, double-stranded oligodeoxynucleotide was added to the reaction to compete for DNA binding. For mobility supershift experiments, 4 μg of an anti-E2A protein (E12 and E47) monoclonal antibody (Nue antibody, Santa Cruz Biotechnology, Santa Cruz, CA) was included in the binding reaction and incubated on ice for 20 min before the probe was added. Binding reactions were incubated on ice for 15 min and resolved by 5% nondenaturing polyacrylamide gel electrophoresis in 0.5 X TBE buffer (44.5 mM Tris base, 44.5 mM boric acid, 1 mM EDTA) at 4 °C.

RESULTS AND DISCUSSION

APEG-1 Is a Single-copy Gene and Is Located on Human Chromosome 2q—We performed Southern analysis with a full-length APEG-1 cDNA probe to determine whether APEG-1 is a single-copy gene. Hybridization of EcoRI-, BamHI-, HindIII-, or SacI-digested genomic DNA from human, rat, and mouse revealed a single pattern that suggests that APEG-1 is a single-copy gene in all three species (Fig. 1A). To test the possibility that APEG-1 associates with certain genetic disorders that have been mapped, we determined the chromosomal location of the human APEG-1 gene. Southern hybridization to an EcoRI-digested human/rodent monochromosomal somatic cell hybrid panel revealed that APEG-1 is located on human chromosome 2 (Fig. 1B). This result was confirmed by using an independent human/rodent somatic cell hybrid panel (data not shown). To determine the subchromosomal localization of human APEG-1, we carried out a genomic PCR analysis by using the GeneBridge 4 radiation hybrid panel with specific primers from the human APEG-1 cDNA sequence (24). The PCR results were then screened against a sequence-tagged site database for the human genome (25). APEG-1 mapped to human chromosome 2q44 between markers D2S360 and D2S353 (data not shown). Despite the preferential expression of APEG-1 in VSMC, a search of the human genome database did not reveal an association with known inherited vascular diseases or with a particular gene cluster (26).

Genomic Cloning and Organization of APEG-1—Using a full-length mouse APEG-1 cDNA probe, we identified several clones from a 129/SvJ mouse genomic library. These APEG-1 genomic sequences assembled into a 4.5-kb organization that contained five exons and four introns (Fig. 2, A and B). The open reading frame for the APEG-1 protein began in the second exon and terminated at the 5' end of the fifth exon (Fig. 2B). The identified exon-intron junctions (Fig. 2B) were all in agreement with the consensus 5' GT and 3' AG sequences (27).

Identification of the APEG-1 Transcription Start Site—To determine the APEG-1 transcription start site, we performed RNase protection assays. A 305-bp (122 to +183) APEG-1 antisense riboprobe encompassing the 5'-untranslated region and most of the first exon was hybridized with RNA from mouse aorta, brain, heart, and undifferentiated and differentiated Monc-1 cells (Monc-1 cells are transformed neural crest cells that can be differentiated into VSMC) (18). After RNase
mouse aorta, brain, heart, and undifferentiated and differentiated (5 days) Monc-1 cells was hybridized to an \( \text{a} \) transcription start site is shown as a percentage of the intensity of the aorta signal. The transcription start site was identified by running a sequencing ladder with the riboprobe as described under “Experimental Procedures.” The transcription start site was identified by the RNase protection assays.

A 363-bp fragment was obtained from mouse aortic RNA and cloned for sequencing. We had removed sequences introduced by the 5'-end sequence of the 363-bp product contained a 309-bp 9-mer (Fig. 3B, bent arrow) corresponded to downstream CA nucleotide pairs. Asterisk indicates a fully protected probe that does not contain the vector sequence. It is the result of a riboprobe hybridizing with a 4-kb APEG-1 isoform whose transcript shares genomic sequence with APEG-1 (see text). An RNA size marker is included to indicate the length of the nucleotide sequence. Riboprobe hybridized with yeast tRNA was used as a negative control, and the lane without RNase treatment (no RNase) shows the input of riboprobe in the hybridization (2.5 \( \times \) 10^4 cpm/reaction). One additional probe lane (Probe) (2.5 \( \times \) 10^4 cpm) was loaded to indicate the size of the riboprobe in the absence of hybridization and RNase treatment. The signal intensity of the primary transcription start site is shown as a percentage of the intensity of the aorta signal. B, 5'-RACE was used to locate the 5'-end of mouse APEG-1. A 363-bp fragment was obtained from mouse aortic RNA and cloned for sequencing. C, 5'-end sequence of the 363-bp product is shown on the right. The poly(G) sequence was introduced during the 5’ RACE procedure. The 5’-end of mouse APEG-1 is indicated by an arrow.

To confirm the transcription start site of APEG-1, we performed a 5’ RACE experiment. We amplified and cloned a 363-bp cDNA fragment from mouse aortic RNA (Fig. 3B). After we had removed sequences introduced by the 5’-RACE procedure, this 363-bp product contained a 309-bp APEG-1 cDNA sequence whose 5’ end (Fig. 3C, bent arrow) was the same as the primary transcription start site (Fig. 3A, long arrow) identified by the RNase protection assays.

APEG-1 Promoter Region Contains a CArG Motif Specific to Smooth Muscle Gene Expression—To identify potential cis-acting elements that may be important in the regulation of APEG-1 promoter activity, we cloned and sequenced the 3.3-kb 5'-flanking region (Fig. 4). There are no upstream TATA-like sequences in the APEG-1 5'-flanking sequence; however, there is a consensus initiator motif (5'-YYCAYYYYY-3') at the APEG-1 transcription start site (Figs. 3A and 4, bent arrow) (29, 30). We identified several cis-acting elements (by their consensus sequences) that have the potential to regulate APEG-1 promoter activity (Fig. 4). Among them are two CArG (or CArG-like) elements known to regulate expression of SMC-specific genes (13, 31–33). In addition, we identified two Sp1 sites in the proximal promoter region (bp -52 and bp -143 relative to the transcription start site) that may potentiate transcription from the TATA-less APEG-1 promoter (34).

The APEG-1 5'-Flanking Region Contains a Potent VSMC-Specific Promoter—To see whether the APEG-1 5’-flanking sequence contains a VSMC-specific promoter, we constructed the reporter plasmid p(-2663/+76) by cloning a 2.7-kb APEG-1 5’-flanking region and 76 bp of exon 1 into the pGL3-Basic vector. In VSMC (RASM), the 2.7-kb APEG-1 5’-flanking region directed a high level of promoter activity that was similar (86%) to the activity of pGL3-Control, which contains the potent SV40 promoter/enhancer (Fig. 5). In contrast, this 2.7-kb APEG-1 sequence directed low levels of promoter activity in the five other non-SMC that we tested (bovine aortic endothelial cells, HeLa, U-2 OS, NIH 3T3, and HepG2). Thus, the 2.7-kb APEG-1 5’-flanking region contains potent VSMC-specific promoter activity.

The 2.7-kb APEG-1 Promoter Is Inhibited by a 5' Repressor—We reported elsewhere that expression of APEG-1 is down-regulated in de-differentiated VSMC both in vivo and in vitro (15). Thus although we had expected VSMC-specific APEG-1 promoter activity, we were surprised that 2.7 kb of the APEG-1 5’-flanking region directed high levels of promoter activity in cultured, and therefore de-differentiated, RASMC.

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2 C.-M. Hsieh and M.-E. Lee, unpublished data.
One explanation for this discrepancy would be the presence of negative DNA regulatory elements outside the 2.7-kb APEG-1 5'-flanking sequence. To test this possibility, we constructed p(23336/1176) and p(23336/1176)Rev by cloning an additional 685 bp of APEG-1 5'-flanking sequence into p(22663/176) in both orientations. In comparison with that of p(22663/176), the promoter activity of p(23336/1176) and p(23336/1176)Rev was reduced markedly (Fig. 6). An additional (upstream) 4-kb DNA sequence did not decrease promoter activity further (data not shown). Taken together, these results indicate that an orientation-independent transcription repressor is located between bp 23336 and 22663 5' of the APEG-1 transcription start site. This APEG-1 transcriptional repressor may explain, at least in part, the decrease in expression of APEG-1 in dedifferentiated VSMC (15).

An E Box Motif Mediates High Levels of APEG-1 Promoter Activity in RASMC—To identify the cis-acting element in the APEG-1 promoter responsible for its potent activity in RASMC, we made three deletion constructs from the 5' end of p(22663/176). In transient transfection experiments, the three deletion constructs, p(−1073/+6), p(−479/+6), and p(−355/+6), had promoter activity similar to that of p(−2663/+6). A fourth deletion construct, p(−122/+76), showed a 20% reduction in promoter activity (Fig. 7). These data suggest that most of the APEG-1 promoter activity is contained between bp −122 and +76.

To further localize the positive cis-acting element, we generated a series of 3' deletion constructs based on p(−479/176) and p(−122/176). These allowed us to determine whether the presence of the 76-bp exon 1 sequence was important for promoter activity. In comparison with p(−479/176), p(−479/38) and p(−122/38) both had much lower promoter activity (16% and 4%, respectively) (Fig. 7). These results indicated that the sequence between bp −38 and +76 in exon 1 was essential for APEG-1 promoter activity. When we inspected the sequence between bp −38 and +76 for potential transcription factor-binding sites, we identified an E box motif (CAGCTG) at bp −43 to −48 (Fig. 4). To determine the importance of this E box motif, we mutated its sequence from CAGCTG to CAGCAC in p(−479/176) and p(−122/176). As demonstrated by transfection experiments with p(−479/176)Emut and p(−122/176)Emut, mutation of the exon 1 E box motif caused a dramatic reduction in APEG-1 promoter activity (Fig. 7). These data indicate that this E box motif located at the 5' untranslated region of the APEG-1 gene is critical for its expression in RASMC.
of that of pGL3-Control.

APEG-1 promoter constructs, p(-2663/+76) were each transfected into the indicated cell types, and reporter luciferase activity was measured as a representation of promoter activity. The difference in transfection efficiency was corrected by the CAT activity from a co-transfected pCAT3-Control vector. For each cell type, the corrected p(-2663/+76) promoter activity is shown as a percentage of that of pGL3-Control.

The E Box in APEG-1 Exon 1 Is Not Bound by E12 and E47

To see if E47 or E12 was present in the DNA-protein complex, we performed mobility shift assays with the Yae monoclonal antibody specific to E47 and E12 (48). Addition of the Yae antibody to the binding reaction did not, however, cause a mobility supershift or a disappearance of the DNA-protein complex (Fig. 8A). This DNA-protein complex was specific because only an excessive amount (100-fold) of unlabeled E box oligonucleotide could compete with it for binding (Fig. 8A); the mutated E box oligonucleotide and an unrelated Oct-1 oligonucleotide could not compete with it.

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Next we tested the specificity of the APEG-1 E box-binding protein in two additional cell types and found that the same binding complex also existed in NIH-3T3 fibroblasts and C2C12 myotubes (Fig. 8C). This result and the deletion/mutation analyses of the APEG-1 promoter by transfection experiments would suggest that the E box is essential for APEG-1...
promoter activity but not sufficient to confer SMC specificity. A similar scenario is found in CARG box-mediated SMC-specific gene expression (13, 31–33). By electrophoretic mobility shift assay, the CARG box is bound by serum response factor, whose protein complex with the E box-binding specificity. A monoconal anti-E2A protein antibody (α-E2A) was added to the binding reaction but was not able to change the mobility of the E box-binding complex in RASMC (SM) nuclear extract (N.E.). As a positive control, a mouse MCK enhancer E box (MCK E box) was used as probe to incubate with differentiated C2C12 myotubes nuclear extract (C2) in the presence or absence of α-E2A. The MCK E box probe was bound weakly by RASMC nuclear extract. C, nuclear extracts (N.E.) from RAMSC (SM), C2C12 myotubes (C2), and NIH-3T3 cells (3T3) were incubated with the APEG-1 exon 1 E box to demonstrate that the E box-binding protein is present in all three cell types.

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