Cloning of the GATA-binding Protein That Regulates Endothelin-1 Gene Expression in Endothelial Cells*

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Previously, we have identified two regions (A and B) of the endothelin-1 promoter that are important for the expression of this gene in cultured vascular endothelial cells. The cis-acting sequence in one of these regions (Region A) includes the core binding motif GATA, raising the possibility that this region of DNA mediates binding of a member of the GATA-binding protein family. In this report, we describe the use of polymerase chain reaction in conjunction with cDNA cloning to characterize the GATA-binding protein expressed in endothelial cells. The nucleotide sequence of endothelial cell cDNA clones is highly homologous to that of the chicken GATA-2 (NF-E1b) gene, indicating that we clones encode the human GATA-2 gene transcript. By RNA blot analysis, this gene is expressed in cultured cell lines derived from a number of different tissues. Transactivation experiments utilizing human GATA-2 eukaryotic expression vectors indicate that the GATA-2 protein interacts with the endothelin-1 GATA sequence to increase transcription of reporter genes in both BAEC and HeLa cells. These data provide the first evidence for a non-erythroid target gene regulated by GATA-2 and indicate that GATA-2 may have a more broad role in transcriptional regulation than the erythroid-specific GATA-1 protein.

Endothelin-1 (ET1) is a 21-amino acid peptide, originally identified as a potent vasoconstrictor produced by vascular endothelial cells in culture (1). ET1 also induces mitogenesis and increases the expression of proto-oncogenes in vascular smooth muscle cells, fibroblasts, and mesangial cells (2–5). The ability of ET1 to regulate the release of atrial natriuretic factor, aldosterone, gonadotropin, and renin from cells in culture implies a role for this peptide in a number of hormonal systems (6–9). Although the role of ET1 in the regulation of vascular function remains unclear, the effects of ET1 on vascular smooth muscle cells implicate it in the pathogenesis of hypertension and atherosclerosis. Molecular mechanisms of ET1 gene expression have been explored in an attempt to understand the role of the ET1 peptide in vascular disease.

Initial studies employed reporter gene transfection experiments. Various regions of the ET1 promoter were placed upstream of the chloramphenicol acetyltransferase (CAT) gene, and these constructs were evaluated by transient transfection into bovine aortic endothelial cells (BABC) in culture. Two regions were found to be important for constitutive expression of this gene in BAEC in culture: Region A located at base pairs (bp) −148 to −117 and Region B located at bp −117 to −98 of the ET1 gene. Region B was noted to contain an AP-1-like sequence (GTGACTAA) (10, 11). This sequence binds Fos and Jun, allows ET1 gene regulation by these transacting factors, and is essential for ET1 promoter function in BAEC in culture.2

Detailed evaluation of Region A has identified TTATCT as a cis-acting sequence that binds proteins and is essential for transcription of this gene in endothelial cells (11). This sequence has a core GATA motif which has been found to be associated with a number of other genes and recognized to mediate binding of zinc finger DNA-binding proteins. Several such proteins have been characterized in species ranging from yeast to man and provide evidence for a high degree of conservation in the zinc finger domains (12–18). The best characterized member of this family, GATA-1 (formerly Eryf1, GF-1, NF-E1a), is involved in the determination of red cell lineage (12, 13, 15, 16, 18). Using a murine GATA-1 cDNA probe, Yamamoto et al. (15) isolated two additional cDNA clones from chicken libraries. These genes have been named GATA-2 (formerly NF-E1b) and GATA-3 (formerly NF-E1c) (17). GATA-2 and GATA-3 have been implicated in the regulation of erythroid-specific genes during red cell differentiation, but little is known about their potential role as transcriptional regulators in other cell types.

In this report we describe the cloning of a cDNA which encodes the GATA-binding protein from vascular endothelial cells. The high degree of homology between the endothelial cell cDNA clones and the chicken GATA-2 gene indicates that our clones encode the human GATA-2 gene transcript. RNA blot analysis provides evidence for variable levels of human GATA-2 gene expression in a wide variety of cell culture lines in addition to endothelial cells. Finally, we have shown that transcription of reporter constructs containing the ET1 GATA motif is increased when co-transfected with a GATA-2 expression vector, thus confirming the importance of GATA-2 in the regulation of ET1 gene expression.

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) M68891.

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1 The abbreviations used are: ET1, endothelin-1; CAT, chloramphenicol acetyltransferase; BAEC, bovine aortic endothelial cells; bp, base pair(s); AP-1, activator protein-1; PCR, polymerase chain reaction; HUVEC, human umbilical vein endothelial cell.

2 M.-E. Lee, M. S. Dhadly, D. H. Temizer, J. A. Clifford, M. Yoshizumi, and T. Quertermous, unpublished observations.

3 M.-E. Lee and T. Quertermous, unpublished observations.
EXPERIMENTAL PROCEDURES

Polymerase Chain Reaction—Polymerase chain reaction (PCR) was used to amplify a cDNA sequence encoding the endothelial cell GATA-2-binding protein. Human umbilical vein endothelial cell (HUVEC) mPRA was converted to cDNA, and this cDNA was used as a template for PCR according to the method of Kawasaki (19). The first-strand HUVEC cDNA was synthesized at 37 °C for 30 min in a 20-μl reaction mixture containing 2 μg of total HUVEC RNA, 1 mM each dNTP (Pharmacia LKB Biotechnology Inc.), 1 unit of M-MLV reverse transcriptase (Promega), 0.1 μg of oligo(dT), 1 × PCR buffer (Perkin-Elmer Cetus), 10 mM MgCl2, and 200 units of Moloney murine leukemia virus reverse transcriptase (Bethesda Research Laboratories). The reverse transcriptase reaction was heated for 5 min at 94 °C, and 80 μl of 1 × PCR buffer containing 25 pmol of forward and reverse primers below) and 2.5 μl of Taq polymerase (Perkin-Elmer Cetus) were added. Thirty cycles of PCR were performed, with an annealing temperature of 55 °C. The sequences of the forward and reverse primers were CCGGAAATTCACCCGG (A + C + T/TG + A + T/CCTGGAAA C2G + A + C + A + CAG + C/A + + T/C/CAGCCCA + G/C + A + C + C(A + + C/G and CCGCATTCCAAG + G/A + A + C + G/A + G and CCGAAAACTGCA, respectively. Each primer was designed according to the amino acids of the zinc finger domain conserved between mouse GATA-1 and yeast nit-2 proteins (12, 14). cDNA Cloning and DNA Sequencing—cDNA from HUVEC (21) cDNA was prepared from HUVEC RNA using the method of Kawasaki (19). cDNA sequencing was performed using the dideoxy chain termination method with T7 DNA polymerase and alkali-denatured double-stranded plasmid DNA as template (24).

Cell Culture and RNA Blot Hybridization—BAEC were isolated and cultured in Dulbecco’s modified Eagle’s medium (Hazelton) supplemented with 10% fetal calf serum (Hyclone), 600 μg of glutamine/glutamic acid (Sigma) per ml, and cultured under recommended conditions. Human cell lines HeLa (epithelial carcinoma), HEL 92.1.7 (erythroleukemia), MOLT-4 (lymphoblastic leukemia), HL-60 (promyelocytic leukemia), U-937 (histiocytic lymphoma), JEG-3 (choriocarcinoma), Hen (human astrocytic glioma), and N17 (rat astrocytic glioblastoma) were obtained from the American Type Culture Collection (ATCC) and cultured under recommended conditions. Cell cDNAs were prepared by extraction with guanidinium isothiocyanate and centrifugation through a cesium chloride gradient (22). Each cDNA was fractionated on a 1.3% formaldehyde-agarose gel and then transferred to nitrocellulose filters. Hybridization was performed using random-primed labeled cDNA as probe (25). Filters were hybridized with a high stringency hybridization and washing conditions (0.2 X SSC, 60 °C) were employed for the RNA blot filters (22).

Plasmids—Reporter plasmids were constructed in either pPPCAT (pCAT-promoter, Promega) or pSPCAT (10). Both plasmids contained the minimal SV40 promoter 5’ to the CAT gene. The plasmid (1A)pPPCAT was constructed by cloning a synthetic DNA fragment encoding the sequence of Region 4 of the GATA-2 (26) into the EcoRI site of pSPCAT. The plasmid (1A + 3AP1)pSPCAT was generated by cloning annealed complementary oligonucleotides, encoding three of the ET1 AP-1 sequences (TGAATCTCTGACTAATTACCTGAGTT- GACTAATCCCGG) into the Xma1 site of pSPCAT, and then cloning the sequence of Region 4 in the 5’ to the sequence of three ET1 AP-1 sequences. The ET1 promoter plasmid—204CAT was constructed by cloning the ET1 5’-flanking sequence into pOCAT (10). The authenticity of each fusion plasmid was confirmed by both restriction mapping and denoloy chain termination sequencing. Plasmid DNA was isolated by alkaline lysis followed by CsCl equilibrium centrifugation (22).

Transfection and CAT Assays—The BAEC cells were transfected with the calcium phosphate method as previously described (10). Each transfection was performed with: (a) 20 μg of the appropriate reporter CAT construct (b) 10 μg of GATA-2 expression plasmid (DTA) or expression vector alone (pCDM8), and (c) 10 μg of pRSVβGAL plasmid DNA. pRSVβGAL was employed to control for the efficiency of transfection. Cell extracts were prepared 48 h after transfection and subjected to CAT and β-galactosidase assays as previously described (10). The relative CAT activity is expressed as the ratio of CAT activity to β-galactosidase activity. Each experiment was performed at least three times, and each transfection was performed in triplicate. Since there was little variation between experiments, the mean and standard error of a triplicate set of data are presented for a single representative experiment.

RESULTS AND DISCUSSION

By using deletion mutants of human ET1 5’-flanking sequence in reporter gene transfection experiments, bp -129 to -98 was found to be necessary for transcription of ET1 in BAEC (10). The sequence TTATTC in this region was found to be the functional cis-acting sequence and was shown to mediate specific protein binding (11). Since this sequence has a GATA core motif, it seemed likely that the protein binding here was a member of the GATA-binding family. With the characterization of GATA-binding proteins in yeast, it became evident that the high degree of conservation of amino acid sequence in the zinc finger region of these proteins made possible a homology cloning strategy (14, 17).

We designed oligonucleotide primers based on the conserved regions of the DNA-binding domain of two C-X5-C-X11-C-X2-C-X7-C zinc finger binding proteins, murine GATA-1 and Neurpsycia crassa nit-2 (12, 14). Using these two primers, each with a degeneracy of 256, a 239-bp DNA fragment was amplified from HUVEC cDNA by PCR. The sequence of this PCR fragment was homologous but different from the sequence of the zinc finger domain of human GATA-1 (16). Using this fragment as a probe, we screened λgt11 and pCDM8 cDNA libraries prepared from HUVEC mRNA (20, 21). Twelve independent λgt11 and pCDM8 clones were isolated and characterized. Double-stranded DNA sequencing was performed on λgt11 clone M1 and plasmid clone DT3A.

The cDNA clones contain a single long open reading frame encoding 480 amino acid residues, which would encode a protein with a predicted molecular mass of 50 kDa (Fig. 1). Nucleotides flanking the putative initiating methionine comply with Kozak’s consensus sequence for translation initiation (26). As shown in Fig. 1, the encoded protein contains two C-X5-C-X11-C-X2-C-X7-C zinc finger domains (overlined) which are the presumed binding domain for the consensus sequence ((A/T)GATA(A/G)). Several clones appeared to have a truncated 3’-untranslated region, despite having poly(A) tails. This observation plus the visualization of two bands (4.3 and 3.3 kilobases) on RNA blots indicates that this gene has alternative polyadenylation sites.

Comparison of the nucleotide sequence of the endothelial cell cDNAs to published sequences of other GATA-binding proteins indicates that these transcripts represent the human GATA-2 gene (15). There is 80% amino acid sequence homology throughout most of the coding region. Homology at the amino acid level between human GATA-2 and human GATA-1 is poor outside of the zinc finger domain. Homology between the human and chicken GATA-2 proteins is high (Fig. 2). The peptide sequence included in the two zinc fingers (amino acids 295-373) is 100% conserved between human and chicken (Fig. 2). A high degree of amino acid homology (96%) is also observed C-terminal to the second finger domain (amino acids 374-450). This conservation of amino acid sequence is in
sharp contrast to the GATA-1 gene, which is poorly conserved between chicken and human (13, 16, 25). Highly conserved domains of GATA-2 outside of the zinc finger region may mediate interaction with components of the transcriptional apparatus or other DNA-binding proteins. Work to define the structure-function relationship of the human GATA-2 protein is in progress.

RNA blot analysis was performed to investigate the cellular expression pattern of the human GATA-2 gene (Fig. 3). A radiolabeled 714-bp BamHI-Sacl fragment of the human GATA-2 cDNA was used as a probe to screen a number of cell lines and primary cultures of cells from a variety of tissues. In addition to endothelial cells, HeLa (epithelial carcinoma), HEL 92.1.7 (erythroleukemia), HL-60 (promyelocytic leukemia), JEG-3 (choriocarcinoma), HepG2 (hepatocellular carcinoma), A-172 (glioblastoma), and primarily cultured fibroblasts appeared to express the GATA-2 gene. The marked difference in level of expression of GATA-2 between HUVEC1 and HUVEC2 probably reflects the different culture conditions and time in culture. Bovine endothelial cells in culture were also shown to express the GATA-2 gene when a GATA-2 cDNA fragment was utilized as a probe in RNA blot experiments (data not shown); U-937 (histiocytic lymphoma) and MOLT-4 (lymphoblastic leukemia) cells did not express this gene under culture conditions employed in these experiments.

This broad pattern of expression is consistent with the high stringency, it is unlikely that expression of other GATA proteins was detected. Also, hybridization to GATA-2 mRNA can be excluded since this would produce a band at 1.8 kilobases (16). Hybridization to GATA-3 seems unlikely since there is no band produced in the T-cell (MOLT-4) lane (15). To verify that human GATA-2 is important in the regulation of ET-1 gene expression in vivo, we performed transactivation experiments by cotransfecting CAT reporter constructs with a human GATA-2 expression vector (clone DT3A). The best evidence for GATA-2 regulation of reporter constructs was obtained with HeLa cells. Co-transfection of...
the GATA-2 expression vector did not increase the CAT activity of the minimal SV40 promoter in pPPCAT and pSPCAT (Fig. 4, upper panel). However, the CAT activity of plasmid (1A)pPPCAT which contained one ET1 GATA motif fused to the SV40 promoter in pPPCAT was increased 6-fold in the presence of the GATA-2 expression plasmid. To mimic the native arrangement of Regions A and B in the ET1 promoter, an ET1 GATA sequence and three ET1 AP-1 consensus sequences were cloned 5' to the minimal SV40 promoter to generate the plasmid (1A+3AP1)pSPCAT. Co-transfection of the GATA-2 expression vector with this reporter plasmid increased the CAT activity by 8-fold in HeLa cells. Transactivation by GATA-2 was also observed in endothelial cells. Co-transfection of the GATA-2 expression vector increased the transcription of plasmid (1A)pPPCAT and (1A+3AP1)pSPCAT as well as that of reporter plasmids containing a native ET1 promoter which included the GATA motif (Fig. 4, lower panel). Thus GATA-2 functions as a potent trans-acting factor, acting on synthetic promoters and the native ET1 promoter, in both native and heterologous cell types.

These data contrast with that obtained from transactivation experiments with murine GATA-1. GATA-1 did not transactivate reporter constructs in erythroid cells and did not transactivate native globin promoters in any cell type (22). While murine GATA-1 was most active in 3T3 fibroblasts and COS cells, GATA-2 was ineffective at activating reporter constructs in these cell types (data not shown). The inability of GATA-2 to transactivate in these cell cannot be due simply to a high constitutive level of GATA-2, since reporter constructs transfected into 3T3 and COS cells do not have a high basal level of transcription. Transactivation experiments with both murine and chicken GATA-1 have implicated additional cellular factors in the regulation of GATA-1, and these data indicate that additional factors may be necessary for GATA-2 function as well (27, 28). Further elucidation of such issues will probably require cell-free transcription methodology.

Our data expand the potential role of the GATA-binding protein family of transcription factors. GATA-1 appears to have a restricted cellular distribution, playing a role only in the regulation of tissue-specific genes in the erythroid, megakaryocyte, and mast cell lineages (12, 13, 29, 30). In contrast, GATA-2 is expressed in a wide variety of cell types. Although the GATA motif of the ET1 gene is found within a region of the promoter which has tissue-specific characteristics in vitro, it seems unlikely that this factor can be involved in the cell-restricted pattern of expression of this gene in vivo. It seems more likely that GATA-2 functions as a general transcriptional regulatory factor. The octamer binding proteins Oct-1 and Oct-2 may provide a good comparison. Oct-2 expression is restricted to lymphoid cells where this factor is involved in the tissue-specific expression of immunoglobulin genes, while Oct-1 has a broad cellular distribution and is involved in the regulation of genes expressed in a wide variety of cell types (31).

In summary, we have cloned the GATA-binding factor from endothelial cells. The nucleotide sequences of endothelial cell cDNA clones is highly homologous to that of the chicken
GATA-2 gene. RNA blot experiments document a widespread and highly variable level of transcription in tumor cell lines as well as primary endothelial cells and fibroblasts. Co-transfection experiments indicate that GATA-2 regulates transcription from synthetic promoters containing the ET1 GATA sequence and the native ET1 promoter, thus confirming the role of this factor in the regulation of ET1 gene expression.

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