The Human ICAM-2 Promoter is Endothelial Cell-specific in Vitro and in Vivo and Contains Critical Sp1 and GATA Binding Sites*

(Received for publication, December 19, 1997, and in revised form, February 24, 1998)

Peter J. Cowan, Denise Tsang, Christopher M. Pedic, Lucy R. Abbott, Trixie A. Shinkel, Anthony J.F. d’Apice, and Martin J. Pearse‡

From the Immunology Research Center, St Vincent’s Hospital, Fitzroy 3065, Victoria, Australia

The expression of intercellular adhesion molecule 2 (ICAM-2) in adult tissues is restricted to vascular endothelial cells and megakaryocytes. We have previously shown that the endothelial-specific in vivo activity of the human ICAM-2 promoter is contained within a small (0.33-kilobase [kb]) 5′-flanking region of the gene. Here we describe the in vitro characterization of this region. The ICAM-2 promoter is TATA-less, and transcription in endothelial cells initiates at four sites. Reporter gene expression directed by the promoter was 125-fold greater than vector alone in bovine aortic endothelial cells but less than 2-fold vector alone in non-endothelial (COS) cells, confirming that specificity in vivo was paralleled in vitro. The addition of 2.7 kb of 5′-flanking region to the 0.33-kb fragment had no effect on promoter activity or specificity. The mutation of an Sp1 motif centered at base pair −194 or an eight-base pair palindrome at −268 each reduced promoter activity by 70%. Mutation of GATA motifs at −145 and −53 reduced promoter activity by 78 and 61%, respectively. Specific binding of bovine aortic endothelial cells nuclear proteins to the Sp1 and GATA sites was demonstrated by gel shift analysis. Promoter activity in COS cells was trans-activated 3–4-fold by overexpression of GATA-2. The results presented here suggest that transcription from the ICAM-2 promoter in endothelial cells is regulated by the interplay of several positive-acting factors and provide the basis for further analysis of endothelial-specific gene expression.

The endothelial cells that form the lining of all blood vessels perform a wide range of functions. In addition to providing a selective barrier between the bloodstream and tissues, vascular endothelial cells are critical for processes including thrombosis, angiogenesis, leukocyte trafficking, and the maintenance of vascular tone (1). The vascular endothelium also plays an important role in cancer metastasis and in the pathogenesis of non-neoplastic diseases such as rheumatoid arthritis and atherosclerosis (2, 3). To gain an understanding of the regulation of endothelial-specific gene expression and thus provide insights into these processes and conditions, a number of studies have focused on the characterization of endothelial cell-specific promoters (4–14). Promoters capable of targeting gene expression to endothelial cells in vivo as well as in vitro may be particularly useful, not only in mechanistic studies but also in medical applications ranging from transgenic modification of donor organs in xenotransplantation (15, 16) to gene therapy for diseases affecting the cardiovascular system (17).

The promoters of several genes that are expressed predominantly or exclusively in endothelial cells have been examined both in vitro in transient transfection assays and in vivo in transgenic mouse studies. The human and mouse tie1 promoters demonstrated endothelial cell specificity in vivo, with expression in the vasculature varying from organ to organ but surprisingly were not specific in vitro (7). In contrast, the human KDR/flk-1 and von Willebrand factor promoters were endothelial cell-specific in vitro, but their activity in vivo was undetectable (18) or restricted to a subset of endothelial cells (12), respectively. Unlike these promoters, which presumably lack important regulatory elements, the mouse tie2 (tek) promoter/enhancer was capable of driving endothelial cell-specific transgene expression in vitro and in vivo (4), although the strong expression in all vascular endothelium in adult tie2lacZ transgenic mice was somewhat unexpected because tie2 expression is downregulated in adult animals (19).

The first promoter shown to target uniform, high-level transgene expression to the vasculature of transgenic mice was that of the human intercellular adhesion molecule 2 gene (ICAM-2)1 (20). Human ICAM-2 is a counter receptor for lymphocyte function-associated antigen-1 (21) and may provide a costimulatory signal for T cell stimulation by alloimmune class II major histocompatibility complex (22). In human tissues, the expression of ICAM-2 is restricted largely to endothelial cells and megakaryocytes (23). Unlike the mouse tie2 promoter/enhancer, which comprises 2.1 kb of promoter and 10 kb of the first intron (4), the human ICAM-2 promoter contains all of the signals necessary for endothelium-specific transgene expression in vivo within a relatively small (<350 bp) region, presenting a novel opportunity for analysis. In this study, we report the first in vitro characterization of the ICAM-2 promoter. We demonstrate that the ICAM-2 promoter is endothelial cell-specific in vitro and identify four elements, an Sp1 site, two GATA sites, and an 8-bp palindromic sequence also present in the tie2 enhancer, that are necessary for full promoter activity. We also show that the ICAM-2 promoter is transactivated in non-endothelial cells by overexpression of two members of the GATA family of transcription factors, GATA-1 and GATA-2.

EXPERIMENTAL PROCEDURES

DNA Preparation, Sequencing, and Analysis—Plasmid DNA was prepared from bacterial cultures using Qiagen Plasmid Midi or Maxi Kits. DNA sequencing reactions were performed using Thermo Sequenase (Amersham Pharmacia Biotech). T3 and T7 primers were used to sequence inserts cloned into pBluescript II (Stratagene), and the prim-

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

‡ To whom correspondence should be addressed: Immunology Research Center, St Vincent’s Hospital, 41 Victoria Parade, Fitzroy 3065, Victoria, Australia. Tel.: 613 9288 3140; Fax: 613 9288 3151; E-mail: pearsem@svhm.org.au.

1 The abbreviations used are: ICAM-2, intercellular adhesion molecule 2; BAEC, bovine aortic endothelial cells; PCR, polymerase chain reaction; RACE, reverse amplification of cDNA ends; bp, base pair(s); kb, kilobase pair(s).
ers GL3-1 and GL3-2 (corresponding to bp 51–68 and to the complement of bp 4785–4803, respectively, in the vector) were used for inserts cloned into pGL3-Basic (Promega). Reactions were electrophoresed at either the Nucleotide Sequencing Facility, Monash University, Victoria, or the Molecular Biology Facility, Griffith University, Queensland, Australia. DNA sequencing was performed using the Sanger method. The final PAGE-purified DNA was cloned into the bacterial shuttle vector pBluescript II (Stratagene, CA). In some cases, the coding region of the plasmid was amplified by PCR and cloned into pBluescript II.

**Isolation of Human ICAM-2 5'-Flanking Sequence**—The Human GenomeWalker kit (CLONTECH) was used to walk upstream of known ICAM-2-flanking sequences. A primer complementary to the sequence +27/+50 relative to the ICAM-2 translation start site (21) was used with an outer adaptor primer in primary PCRs using five adaptornigligated human genomic libraries as templates. Samples of the primary PCRs were used in secondary PCRs with a nested ICAM-2-specific primer (−17/+5 relative to the translation start site) and a nested adaptor primer. Products of the secondary PCRs were resolved by agarose gel electrophoresis, purified using the QIAquick Gel extraction kit (Qiagen), and cloned into pBluescript II.

**Generation and Testing of Transgenic Mice**—The 5' end of the 0.33-kbp ICAM-2 promoter fragment in an ICAM-2 promoter/human CD59 microinjection construct (20) was delineated by an NcoI site. This site was employed in the precise fusion of 2.7 kbp of upstream flanking sequence (isolation and extension above) to the 0.33-kbp fragment. The resulting construct, designated PK, was used to generate transgenic mice as described (20). Peripheral blood leukocytes from the transgenic mice were examined for the expression of CD59 by flow cytometric analysis as described (20). Fresh frozen tissue sections prepared from mouse organs were stained to detect the pattern of CD59 expression as described (20).

**Rapid Amplification of cDNA Ends (RACE)**—The method used to identify the 5' end of ICAM-2 transcripts was based on the 5' RACE system (Life Technologies, Inc.). In brief, total RNA (2.5 μg) prepared from human umbilical vein endothelial cells was reverse-transcribed from an antisense primer (+27/+50 relative to the ICAM-2 translation start site) using SuperScript II (Life Technologies). The cDNA was purified and tailed with dCTP using terminal deoxynucleotidyl transferase (Promega). An aliquot of the tailed cDNA was used as template in a PCR with primers AAP (5'-GGCCACCGTGCGACTAGTACGGGIGGGI-IGGGIIGGGIIG-3') and the nested antisense primer (−17/+5 relative to the ICAM-2 translation start site) using the Advantage Thi polymerase mix (CLONTECH) to maximize fidelity. PCR was carried out in a DNA engine thermal cycler (MJ Research) using the following parameters: 94 °C for 2 min; 35 cycles of 92 °C/10 s, 55 °C/10 s, 72 °C/30 s; and 25 cycles of 92 °C/5 s, 55 °C/5 s, 72 °C/30 s. An aliquot (0.1%) of the first PCR was used directly in a second PCR with the M13/pUC reverse and the external reverse primers (P8-Rmut, etc.) were complementary to the forward primers. The forward and reverse primers were used in independent PCRs with the M13/uC reverse and forward amplification primers (Life Technologies), respectively. The two products from each of first-step PCRs (e.g., P8-Fmut/M13-R and P8-Rmut/M13-F) were purified, mixed, and used in the second-step PCR with the external primers only. Products from the second-step PCRs were cloned into pGEM3-Basic and sequenced to confirm the presence of the desired mutations.

To construct the Sp1/GATA U (upstream) double mutant, the upstream GATA-responsive M6-a promoter from an M6-a forward primer driving the expression of human GATA-2, were also provided (M6-a). Secreted alkaline phosphatase activity in the culture medium of transfected cells was measured using the Great Esape SEAP chemiluminescence detection kit (CLONTECH). Light intensity was measured using a MicroLumat LB96P plate luminometer (EG and G Berthold). The protein concentration of lysates was determined using the DC protein assay kit (Bio-Rad) and bovine serum albumin as the standard. Enzyme activities were corrected for background by subtracting the values obtained for mock-transfected cells.

**Characterization of the Human ICAM-2 Promoter**

For each well, 4 m1.5 μl of growth medium, and the cells were incubated for a further 64–72 h before harvesting and analysis. COS cells were transfected similarly except that 2 × 105 cells were plated/well, 6 μl of LipofectAMINE was used, and the cells were harvested after a 40–48 h incubation. For transient transfections, the transfection mixture contained 0.9 μg of luciferase construct, 0.2 μg of M6o-SEAP, 0.2 μg of pSVβGal, and 0.2 μg of either pXM-mGATA-1 (28), pMT2-hGATA-2 (29), or the vector pcDNA3 (Invitrogen). M6o-SEAP was constructed by cloning the GATA-responsive M6o promoter from an M6o-hGplasmid (Ref. 30; kindly provided by Dr Merlin Crossley, Biochemistry Department, University of Sydney, Sydney, Australia) into the secreted alkaline phosphatase reporter plasmid pSEAP2-Basic (CLONTECH). pXM-mGATA-1, which contains the adenovirus major late promoter driving the expression of murine GATA-1, and pMT2-hGATA-2, which contains the same promoter driving the expression of human GATA-2, were also provided by Dr. Merlin Crossley.

Luciferase and β-galactosidase activities in lysates of transfected cells were measured using the luciferase assay system (Promega) and the chemiluminescent β-Gal reporter gene assay kit (Boehringer Mannheim), respectively. Secreted alkaline phosphatase activity in the culture medium of transfected cells was measured using the Great Esape SEAP chemiluminescence detection kit (CLONTECH). Light intensity was measured using a MicroLumat LB96P plate luminometer (EG and G Berthold) and the protein concentration was determined using the DC protein assay kit (Bio-Rad) and bovine serum albumin as the standard. Enzyme activities were corrected for background by subtracting the values obtained for mock-transfected cells.

**Mutagenesis**—A two-step PCR method was used to mutagenize the 0.33-kbp ICAM-2 promoter cloned in pBluescript II such that the 5' end was cloned into the M13 forward priming site (20). In the first step, PCRs were carried out using an internal mutagenic primer with an external vector-targeted primer. The internal forward primers (mutated bases underlined) were P8-Fmut, 5'-TTCCCAAGACGGGCGGTCTTGGT-3'; P12-Fmut, 5'-GTCATATGCTGGGAATCCCATTTTG-3'; Sp1-Fmut, 5'-CATTGGCTTCGCCATTACCCCTTA-3'; GATA-U-F mut, 5'-CTCTGCTGTTGAGACGACCTT-3'; and GATA-D-F mut, 5'-CTCTGATATGAGAGGA-3'. The internal reverse primers (P8-Rmut, etc.) were complementary to the forward primers. The forward and reverse primers were used in independent PCRs with the M13/uC reverse and forward amplification primers (Life Technologies), respectively. The two products from each of first-step PCRs (e.g., P8-Fmut/M13-R and P8-Rmut/M13-F) were purified, mixed, and used in the second-step PCR with the external primers only. Products from the second-step PCRs were cloned into pGEM3-Basic and sequenced to confirm the presence of the desired mutations.

To construct the Sp1/GATA U (upstream) double mutant, the upstream GATA site mutant cloned in pGL3-Basic was used as template. The mutagenic primers were Sp1-Fmut and Sp1-Rmut, and the external primers were GL3-1 and GL3-2. The second-step PCR product was cloned and sequenced as described above.

**Characterization of the Human ICAM-2 Promoter**

The luciferase reporter plasmid pSEAP2-Basic (CLONTECH) was used to transfect COS cells transiently. Human embryonic kidney (HEK) cells and HeLa cells were transfected with the reporter constructs as described. The luciferase activities were measured using the Dual Luciferase Reporter Assay System (Promega). Transient transfections were performed at least twice, in triplicate. The values obtained for mock-transfected cells were subtracted from those of the experimental samples and expressed as a percentage of the luciferase activity observed in the pGL3-Basic control construct.

**Characterization of the Human ICAM-2 Promoter**

To construct the Sp1/GATA U (upstream) double mutant, the upstream GATA site mutant cloned in pGL3-Basic was used as template. The mutagenic primers were Sp1-Fmut and Sp1-Rmut, and the external primers were GL3-1 and GL3-2. The second-step PCR product was cloned and sequenced as described above.
RESULTS

Isolation of 3 kbp of 5‘-Flanking Region of the Human ICAM-2 Gene—Human ICAM-2 expression in the tissues is restricted largely to vascular endothelial cells and megakaryocytes (23); in the blood, ICAM-2 is expressed on platelets (31) and at a low level on resting lymphocytes and monocytes but not on granulocytes (23). We have previously shown that 0.33 kbp of 5‘-flanking region of the human ICAM-2 gene is sufficient to target high-level transgene expression to all vascular endothelium in the heart, kidney, liver, lung, and pancreas of transgenic mice (20), consistent with the tissue distribution of human ICAM-2. Unexpectedly, however, transgenic mouse lymphocytes were negative for transgene expression and granulocytes were very strongly positive. This discrepancy is unlikely to be due to differences in the pattern of expression of human and mouse ICAM-2, because flow cytometric analysis revealed that the hierarchy of ICAM-2 expression levels is similar on human and mouse leukocyte populations i.e. monocytes > lymphocytes > granulocytes. An alternative explanation is that elements regulating ICAM-2 expression on leukocytes are absent from the 0.33-kbp region. To investigate this possibility and to generate additional material for in vitro analysis of the ICAM-2 promoter, a larger 5‘-flanking region of human ICAM-2 was isolated. Nested primers targeted to known ICAM-2 promoter sequence were used with adaptor primers to amplify the 5‘-flanking sequence from five adaptor-ligated human genomic libraries. The PvuII, SspI, and ScaI libraries yielded products of 1.0, 1.8, and 3.1 kbp, respectively, which were cloned and analyzed by restriction enzyme digestion to produce the map shown in Fig. 1A. The DNA sequence obtained from the 3‘ end of the two larger clones matched that of the 0.33-kbp region (20) except for an additional G at bp 2281 and an additional C at bp 2275 (Fig. 1B). The presence of these bases, confirmed in the original 0.33-kbp clone by resequencing, revealed a consensus NF-κB motif (GGGGTTC-
Characterization of the Human ICAM-2 Promoter

Identification of the Transcription Start Sites of ICAM-2—Identification of the transcription start site(s) of the human ICAM-2 gene, 5'-RACE was performed on total RNA from human umbilical vein endothelial cells, which strongly express ICAM-2 (20). First-strand cDNA prepared using a primer targeted to the first exon was tailed with dC and used in PCR with a G-rich primer and a gene-specific nested primer. After a second PCR with nested primers, the resulting products were cloned into pBluescript II, and five clones were sequenced, revealing four transcription start sites (Fig. 1B). A thymine residue (shown in bold type) located 77 bp upstream of the ICAM-2 start codon was designated the primary transcription initiation site (+1), because the start sites for two independent clones mapped to this point and that for a third mapped immediately upstream (−1). The remaining start sites were located at −37 and −66. Thus like the promoters of several other constitutively expressed cell adhesion molecule genes including PECAM-1 (32) and CD18 (33), the ICAM-2 promoter lacks consensus TATA and CAAT boxes and contains multiple transcription start sites.

Endothelial Cell Specificity of the ICAM-2 Promoter in Vivo—The in vitro promoter activity and specificity of various lengths of ICAM-2 5'-flanking region were tested by transfecting different fragments cloned in the luciferase reporter vector pGL3-Basic into BAEC or the simian kidney fibroblast cell line COS. The constructs were cotransfected with pSVβGal to correct for differences in transfection efficiency, and luciferase activity was normalized to that of pGL3-Basic alone. The 3.0-, 1.7-, and 0.33-kbp fragments exhibited similar significant promoter activity (100–130-fold the level of vector alone) in BAEC and only minimal activity (approximately 2-fold vector alone) in COS cells (Fig. 4), indicating that the signals necessary for endothelial cell-specific ICAM-2 promoter activity in vitro are contained within the 0.33-kbp fragment. In vitro analysis of the promoter was therefore performed on this fragment.

Identification of Potential Cis-acting Elements in the ICAM-2 Promoter—The sequence of the 0.33-kbp promoter fragment was scanned for potential transcription factor binding sites and other motifs and compared with the corresponding sequence from mouse (34) to identify conservation of sites. Two sites for the GATA family of transcription factors, centered at −145 and −53 bp relative to the primary start point of transcription in the human promoter, were conserved in position and orientation in the mouse ICAM-2 gene (Fig. 1B). Four conserved sites for the Ets family were located at −135, −127, −70, and −44. An 8-bp palindrome (CAGATCTG, referred to here as P3) was detected at −268; this sequence is also present in the first intron of the endothelial-specific tek (tie2) gene and has been shown to be important in conferring endothelial specificity in vivo (4). Seven of the nucleotides in P3 were conserved in the mouse ICAM-2 sequence, although the distance between this motif and a highly conserved block containing the GATA and Ets sites was 86 bp greater in mouse than human. Another palindrome (CACGCATGCGTG, referred to as P12) present at −217 was only partly conserved (9/12 bp) in the mouse sequence, as was the NF-xB site (8/10 bp) at −276. An Sp1 motif at −194 in the human promoter was not conserved within the corresponding region in mouse. The locations of the putative regulatory elements within the human ICAM-2 promoter are indicated schematically in Fig. 1C.

Mutational Analysis of the ICAM-2 Promoter—To identify functionally important elements in the ICAM-2 promoter, several of the motifs described above were mutated, and the effect of each mutation on promoter activity in endothelial cells was determined. Mutated derivatives of the 0.33-kbp promoter fragment were constructed by a two-step PCR method, cloned into pGL3-Basic, and transfected into BAEC. The mutation introduced into the P6 site (CAGACGCGG, referred to as P6) present at −217 was only partly conserved (9/12 bp) in the mouse sequence, as the NF-xB site (8/10 bp) at −276. An Sp1 motif at −194 in the human promoter was not conserved within the corresponding region in mouse. The locations of the putative regulatory elements within the human ICAM-2 promoter are indicated schematically in Fig. 1C.
A mutation was also introduced into the P₁₂ site (−217). Although this motif does not represent the binding site for any known transcription factors, it has features suggestive of a tandem binding site for the Ah receptor nuclear translocator (35) complexed to an unknown factor. Ah receptor nuclear translocator recognizes the half-site GTG, and the mutation in P₁₂ (CACGCATGCGTGTTCGCATGCGAA) was designed to destroy the two potential half-sites. However, this mutation had no effect on ICAM-2 promoter activity in BAEC (Fig. 5).

Specific Interaction of BAEC Nuclear Proteins with Elements in the ICAM-2 Promoter—Specific binding of BAEC nuclear proteins to the functional Sp₁ and GATA sites was assessed by electrophoretic mobility shift assay. Several retarded bands were observed when BAEC nuclear extract was incubated with a labeled probe containing the Sp₁ site, but only the highest molecular weight DNA-protein complex appeared to be specific (Fig. 6A). Excess cold mutant competitor failed to prevent the formation of this complex, indicating that it is likely to contain a protein involved in the regulation of the ICAM-2 promoter in BAEC.

Incubation of BAEC nuclear extract with the upstream GATA probe resulted in the formation of two specific complexes (Fig. 6B), the smaller of which was competed out by cold wild type competitor but not by cold mutant competitor. The downstream GATA probe generated complexes of similar molecular weight to the two formed with the upstream site, in addition to a third complex of intermediate size (Fig. 6C). Although the results in this case were less clear, it appeared that only the intermediate-sized complex was not competitively inhibited by cold mutant competitor.

Transactivation of the ICAM-2 Promoter in COS Cells by Overexpression of GATA-2—GATA-2 is abundantly expressed in endothelial cells (29) and has been implicated in the regulation of endothelial expression of genes including endothelin-1 (10), eNOS (11), von Willebrand factor (36), and P-selectin (14). To determine whether either factor could activate the ICAM-2 promoter in vitro, expression plasmids for mouse GATA-1 or human GATA-2 were co-transfected with the ICAM-2 promoter-luciferase reporter into COS cells. A synthetic GATA-responsive promoter construct (M₆α-SEAP) consisting of six GATA sites linked to a minimal promoter driving the expression of secreted alkaline phosphatase was co-transfected as a control to confirm expression of the GATA factors. As shown in Fig. 7, expression of GATA-1 or GATA-2 transactivated the
ICAM-2 promoter by 7.3- and 3.4-fold, respectively. The greater activation of both the ICAM-2 and M6e promoters by GATA-1 (Fig. 7) may indicate that GATA-1 is a more potent transactivator in vitro than GATA-2 as previously suggested (37). However, since GATA-1 is not expressed in endothelial cells (28), it is likely that GATA-2 regulates ICAM-2 expression in this cell type in vivo.

**DISCUSSION**

Human ICAM-2 is constitutively expressed on all vascular endothelial cells (23), and the ICAM-2 promoter is unusual among the endothelial cell-specific promoters studied to date in that the signals necessary for specific expression in vivo reside within a very small (0.33-kbp) region of 5′-flanking sequence (20). As the first step toward understanding the mechanism of this specificity, we have characterized the ICAM-2 promoter in vitro. After confirming that the specificity of the 0.33-kbp promoter in vivo was paralleled in vitro, we showed that the addition of up to 2.7 kbp of upstream 5′-flanking region did not increase promoter activity in transfected BAEC (Fig. 4) and did not affect the pattern of transgene expression in transgenic mice (Fig. 3), suggesting that most or all of the positive-acting elements regulating ICAM-2 promoter activity in endothelial cells are indeed located in the 336-bp sequence shown in Fig. 1B.

Based on previous reports, the obvious candidates for these elements included consensus motifs for the Sp1 and GATA families of transcription factors (Fig. 1C). Functional Sp1 binding sites are required for maximum in vitro endothelial activity of the promoters of the KDR/flk-1 (18), platelet-derived growth factor (38), and eNOS (11) genes; likewise, GATA sites have been identified as positive elements in the eNOS (11), von Willebrand factor (36), P-selectin (14), and endothelin-1 (39) promoters. Mutational and gel shift analyses were used to demonstrate that the Sp1 site and two GATA sites in the ICAM-2 promoter function as cis-acting positive regulatory elements in endothelial cells in vitro (Figs. 5 and 6). Mutation of the Sp1 site abolished specific binding of a BAEC nuclear protein and reduced ICAM-2 promoter activity in BAEC by 70%. Mutation of the upstream and downstream GATA sites disrupted BAEC nuclear protein binding and reduced promoter activity in BAEC by 78 and 61%, respectively. Although the identity of the protein(s) binding the GATA sites was not determined, we believe that it is likely to be GATA-2, because overexpression of GATA-2 transactivated the ICAM-2 promoter in non-endothelial (COS) cells in vitro (Fig. 7), and GATA-2 has previously been implicated in the endothelial cell expression of several genes (11, 14, 39). GATA-1 also transactivated the promoter but is not expressed in endothelial cells (28). GATA-1 is believed to play a role in megakaryocyte gene expression (40, 41) and thus may be involved in the regulation of ICAM-2 expression in this cell type.

Another positive element identified by mutagenesis was an 8-bp palindrome that has been shown to be an important component of an intronic endothelial cell-specific enhancer in the murine tie2 gene (4). This motif, which we have termed Pm, is also present in the promoter of the endothelial-specific mouse tie1 gene (7) and (conserved in 7 out of 8 positions) in the promoters of the human tie1 (7) and mouse ICAM-2 (34) genes. Mutation of Pm reduced ICAM-2 promoter activity in BAEC by 70% (Fig. 5). Mutation of another palindrome (P12) did not affect promoter activity in BAEC, and binding of BAEC nuclear proteins to a P12 probe (bp −224 to −209) could not be clearly demonstrated by electophoretic mobility shift assay (data not shown). However, it is not possible to conclusively rule out a

![Fig. 4. In vitro promoter activity of 3.0, 1.7, or 0.33 kbp of ICAM-2 5′-flanking region.](image)

![Fig. 5. Identification of important regulatory elements in the ICAM-2 promoter by site-directed mutagenesis.](image)
Downstream GATA site probe (bp WT), mutant, or nonspecific (N/s) competitors as indicated. Specific complexes are indicated by arrowheads. A, Sp1 site probe (bp −207 to −183); nonspecific competitor, bp −285 to −269. B, upstream GATA site probe (bp −155 to −135); nonspecific competitor, bp −207 to −183. C, downstream GATA site probe (bp −61 to −43); nonspecific competitor, bp −207 to −183.

Fig. 6. Determination of BAEC nuclear protein binding to the ICAM-2 promoter by electrophoretic mobility shift assay. The indicated radiolabeled oligonucleotide probes were incubated with (+) and without (−) BAEC nuclear extract and in the presence or absence of a 100-fold molar excess of cold wild type (WT), mutant, or nonspecific (N/s) competitors as indicated. Specific complexes are indicated by arrowheads. A, Sp1 site probe (bp −207 to −183); nonspecific competitor, bp −285 to −269. B, upstream GATA site probe (bp −155 to −135); nonspecific competitor, bp −207 to −183. C, downstream GATA site probe (bp −61 to −43); nonspecific competitor, bp −207 to −183.

Fig. 7. Transactivation of the ICAM-2 promoter and a synthetic GATA-responsive promoter (M6a) in COS cells by overexpression of GATA-1 or GATA-2. Cells were cotransfected with the 0.33-kbp ICAM-2 promoter-luciferase construct, M6a-SEAP, pSVβGal, and either pXM-mGATA-1, pMT2-hGATA-2, or pcDNA3. Transactivation is expressed as the corrected luciferase (left panel) or secreted alkaline phosphatase (right panel) activity obtained when the GATA expression vectors were cotransfected relative to that obtained with pcDNA3.

Role for this site, because preliminary DNase I footprinting experiments indicated that an 18-bp region encompassing P12 is protected by a protein(s) present in BAEC nuclear extract. Further mutational analysis and identification of proteins binding to P12 are in progress.

Ets family members are involved in both endothelial (4, 38, 42) and megakaryocytic (41) gene expression. The function of four Ets motifs in the human ICAM-2 promoter, which are all largely conserved in the mouse ICAM-2 gene (Fig. 1B), was not determined in this study, but it is interesting to note the similarity between the tandem motifs (GCTTCCCCAGCTTCCCT) centered at −132 bp in the ICAM-2 promoter with tandem PEA3 motifs (GCTTCCCCAGCTTCCCTC) in the promoters of the human and mouse tie1 genes (7).

The presence of an NF-xB motif in the ICAM-2 promoter is also intriguing. NF-xB is a major regulator of the cytokine-inducible expression of various genes including the cell adhesion molecules E-selectin, VCAM-1, ICAM-1, and MadCAM-1 (reviewed in Ref. 43). NF-xB is also believed to control the constitutive level of expression of P-selectin in endothelial cells and megakaryocytes by a mechanism involving the interaction of Bcl-3 with promoter-bound p50 or p52 homodimers, and mutation of the P-selectin xB site causes a 40% reduction in promoter activity in BAEC (44). ICAM-2 is expressed constitutively at high levels on resting endothelial cells and megakaryocytes in vivo, and the level of expression in human umbilical vein endothelial cells, like that of P-selectin (44), was unchanged by treatment with a variety of inflammatory cytokines (23). Incubation of nuclear extract from resting BAEC with a probe (bp −285 to −269) containing the ICAM-2 xB site resulted in the formation of a specific DNA/protein complex. Together, these data suggest that the role (if any) of the ICAM-2 xB site in endothelial expression is similar to that of the P-selectin site, although this awaits confirmation by mutagenesis and gel supershift analysis.

Although the small size and tight specificity of the ICAM-2 promoter make it a particularly promising tool for gene therapy and other applications requiring endothelial cell targeting. It is also valuable in the study of the control of gene expression in endothelial cells, as demonstrated in this study. On the basis of the results presented here, we propose that the transcription of ICAM-2 in endothelial cells is regulated primarily by the binding of Sp1 and GATA-2 transcription factors and possibly a novel P8 binding factor to their cognate sites in the ICAM-2 promoter. Interaction of these DNA-bound proteins via their zinc fingers (45) may induce a conformational change, allowing them to interact more readily with components of the basal transcriptional machinery. For example, as suggested for GATA-1 (45), the interaction of GATA-2 with Sp1 may increase the affinity of the latter for TAF1110 or some other component of the basal complex. The involvement of other proteins, such as members of the Ets and NF-xB families, remains to be determined.

Although Sp1 and GATA-2 may be two of the major determinants of ICAM-2 promoter activity in endothelial cells, their presence alone cannot account for the specificity of the promoter, since Sp1 is ubiquitously expressed, and GATA-2 is expressed relatively widely (37). It is possible that a threshold level of GATA-2 is required for activation of the promoter. Alternatively, GATA-2 may interact with a cell-specific factor to activate transcription of ICAM-2 in endothelial cells. Such an interaction between GATA-1 and the protein FOG (Friend of GATA-1) has been proposed to explain the transcriptional regulation of hematopoietic-specific gene expression (46). It is not known whether FOG binds DNA in a sequence-specific manner (46), but if so, it is tempting to speculate that either the P12 or P13 motifs in the ICAM-2 promoter may represent the binding site of an endothelial-restricted FOG-like protein. Finally, it is conceivable that ICAM-2 expression in non-endothelial cells is repressed by a negative-acting factor such as that described for the von Willebrand factor promoter (6). The identification of

C. M. Pedic, unpublished results.
specific proteins interacting with the ICAM-2 promoter using techniques such as gel supershift and DNA footprinting will be helpful in discriminating between these possibilities.

Acknowledgments—We thank Helen Barlow, Nella Fisicaro, and Ewa Witort for assistance in the maintenance and screening of transgenic mice and Dr. Merlin Crossley for providing materials and for helpful advice and comments.

REFERENCES

1. Risau, W. (1995) FASEB J. 9, 926–933
2. Folkman, J. (1995) Nat. Med. 1, 27–31
3. Ro¨nicke, V., Risau, W., and Breier, G. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 801–809
4. Schlaeger, T. M., Bartunkova, S., Lawitts, J. A., Teichmann, G., Risau, W., Deutsch, U., and Sato, T. N. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 3058–3063
5. Rineke, V., Rисau, W., and Breier, G. (1996) Circ. Res. 79, 277–285
6. Jahrroudi, N., Ardekani, A. M., and Greenberger, J. S. (1996) Blood 88, 3801–3814
7. Khoronen, J., Lahtinen, I., HalmeekytÖ, M., Alhonen, L., Jäntti, J., Dumont, D., and Alitalo, A. (1995) Blood 86, 1828–1835
8. Weiler-Guettler, H., Aird, W. C., Husain, M., Rayburn, H., and Rosenberg, R. D. (1996) Circ. Res. 78, 180–187
9. Patterson, C., Perrella, M. A., Hsieh, C.-M., Yoshizumi, M., Lee, M.-E., and Haber, E. (1995) J. Biol. Chem. 270, 23111–23118
10. Kawana, M., Lee, M.-E., Quertermous, E. E., and Quertermous, T. (1995) Mol. Cell. Biol. 15, 4225–4231
11. Zhang, R., Min, W., and Sessa, W. C. (1995) J. Biol. Chem. 270, 15320–15326
12. Aird, W. C., Jahrroudi, N., Weiler-Guettler, H., Rayburn, H. B., and Rosenberg, R. D. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 4567–4571
13. Harats, D., Kurthara, H., Bello, P., Oakley, H., Ziober, A., Ackley, D., Cain, G., Kurthara, Y., Lawn, R., and Sigal, E. (1995) J. Clin. Invest. 95, 1335–1344
14. Pan, J., and McEver, R. P. (1993) J. Biol. Chem. 268, 22600–22608
15. Cowan, P. J., Somerville, C. A., Shinkle, T. A., Katerelos, M., Aminian, A., Romanella, M., Tange, M. J., Pearse, M. J., and d’Apice, A. F. (1996) Transplantation 62, 155–160
16. Staunton, D. E., Dustin, M. L., and Springer, T. A. (1989) Nature 339, 61–64
17. Carpenito, C., Pyszniak, A. M., and Takei, F. (1997) Scand. J. Immunol. 45, 248–254
18. de Fougereoles, A. R., Stack, A. S., Schwartz, R., and Springer, T. A. (1991) J. Exp. Med. 174, 253–267
19. Quandt, K., Prech, K., Karas, H., Wingender, E., and Werner, T. (1995) Nucleic Acids Res. 23, 4878–4884
20. Wingender, E., Kel, A. E., Kel, O. V., Karas, H., Heinemeyer, T., Dietze, P., Knueppel, R., Romaschenko, A. G., and Kolchanov, N. A. (1997) Nucleic Acids Res. 25, 265–268
21. Thompson, J. D., Higgins, D. G., and Gibson, T. J. (1994) Nucleic Acids Res. 22, 4673–4680
22. Booyse, P. M., Siodlik, B. J., and Rafelson, M. E., Jr. (1975) Thromb. Diath. Haemorrh. 34, 825–839
23. de Fougerolles, A. R., Stacker, S. A., Schwarting, R., and Springer, T. A. (1991) J. Clin. Invest. 88, 1095–1100
24. Dorfman, D. M., Wilson, D. B., Bruns, G. A. P., and Orkin, S. H. (1989) J. Biol. Chem. 264, 1279–1285
25. Yamamoto, M., Ko, L. J., Leonard, M. W., Beug, H., Orkin, S. H., and Engel, J. D. (1990) Genes Dev. 4, 1089–1109
26. Guimina, R. J., Kirschbaum, N. E., Piotrowski, K., and Newman, P. J. (1997) Blood 89, 1260–1269
27. Swanson, H. I., and Yang, J. (1996) Nucleic Acids Res. 24, 4878–4884
28. Tsai, S., Martin, D. I. K., Zon, L. I., D’Andrea, A. D., Wong, G. G., and Orkin, S. H. (1989) Nature 339, 448–451
29. Dorfman, D. M., Wilson, D. B., Bruns, G. A. P., and Orkin, S. H. (1992) J. Biol. Chem. 267, 1279–1285
30. Yamamoto, M., Ko, L. J., Leonard, M. W., Beug, H., Orkin, S. H., and Engel, J. D. (1990) Genes Dev. 4, 1089–1109
31. DiCesaro, T. G., de Fougereoles, A. R., Bainton, D. F., and Springer, T. A. (1994) J. Clin. Invest. 94, 1243–1251
32. Agura, E. D., Howard, M., and Collins, S. J. (1992) Blood 79, 602–609
33. Xu, H., Tong, I. L., de Fougereoles, A. R., and Springer, T. A. (1992) J. Immunol. 149, 2650–2655
34. Eaves, A. J. F. (1995) Mol. Cell. Biol. 15, 2650–2655
35. Yu, C., Weiss, M. J., and Orkin, S. H. (1992) Blood 80, 253–267
36. J. Biol. Chem. 267, 31657–31665
37. Jahrroudi, N., and Lynch, D. C. (1994) Mol. Cell. Biol. 14, 999–1008
38. Orkin, S. H. (1992) Blood 80, 575–581
39. Khachigian, L. M., Fries, J. W. U., Benz, M. W., Bonthron, D. T., and Collins, T. (1994) J. Biol. Chem. 269, 22647–22656
40. Lee, M.-E., Temizer, D. H., Clifford, J. A., and Quertermous, T. (1991) J. Biol. Chem. 266, 16186–16192
41. Ludlow, L. B., Schick, B. P., Budarf, M. L., Driscoll, D. A., Zackai, E. H., Cohen, A., and Konkle, B. A. (1996) J. Biol. Chem. 271, 22076–22080
42. Lemarchand, V., Ghysdael, J., Mignotte, V., Rahuel, C., and Roges, P.-H. (1993) Mol. Cell. Biol. 13, 668–676
43. Slepecky, K., Begue, A., Stachelin, D., and Shibuya, M. (1996) J. Biol. Chem. 271, 30983–30988
44. Bauerle, P. A., and Baichwal, V. R. (1994) Adv. Immunol. 55, 111–137
45. Merika, M., and Orkin, S. H. (1995) Mol. Cell. Biol. 15, 2437–2447
46. Thang, A. P., Visvader, J. E., Turner, C. A., Fujiwara, Y., Yu, C., Weiss, M. J., Crossley, M., and Orkin, S. H. (1997) Cell 90, 109–119
The Human ICAM-2 Promoter is Endothelial Cell-specific in Vitro and in Vivo and Contains Critical Sp1 and GATA Binding Sites
Peter J. Cowan, Denise Tsang, Christopher M. Pedic, Lucy R. Abbott, Trixie A. Shinkel, Anthony J.F. d’Apice and Martin J. Pearse

J. Biol. Chem. 1998, 273:11737-11744.
doi: 10.1074/jbc.273.19.11737

Access the most updated version of this article at http://www.jbc.org/content/273/19/11737

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC’s e-mail alerts

This article cites 45 references, 28 of which can be accessed free at http://www.jbc.org/content/273/19/11737.full.html#ref-list-1