Transforming Growth Factor-β1-mediated Inhibition of the flk-1/KDR Gene Is Mediated by a 5′-Untranslated Region Palindromic GATA Site*

The angiogenic effects of vascular endothelial growth factor are mediated predominantly by the FLK-1/KDR receptor. An understanding of the transcriptional control mechanisms underlying flk-1/KDR expression should provide insight into the molecular basis of angiogenesis. In this study, we show that transforming growth factor-β1 (TGF-β1) down-regulates expression of the endogenous flk-1/KDR gene in endothelial cells. In transient transfection assays, this effect was mapped to a palindromic GATA site in the 5′-untranslated region. In electrophoretic mobility shift assays, the palindromic GATA site was shown to bind to two molecules of GATA protein. Moreover, DNA-GATA interactions were inhibited by TGF-β1. Finally, in cotransfection assays, transcription activation of the flk-1/KDR promoter by GATA-1 or GATA-2 was attenuated in TGF-β1-treated cells. Taken together, these results suggest that the TGF-β1-mediated inhibition of the flk-1/KDR gene is mediated by a 5′-untranslated region palindromic GATA site.

An understanding of the mechanisms that underlie the transcriptional regulation of the flk-1/KDR gene might provide important information about the molecular basis of endothelial cell differentiation and angiogenesis. The human and mouse flk-1/KDR promoters have been sequenced and characterized (8–12). Under in vitro conditions, the 5′-flanking region and first exon have been shown to contain information for endothelial cell-specific expression, whereas in transgenic mouse assays, intronic enhancer sequences play a critical role in mediating expression within the vasculature (13).

Although the above studies provide insight into the mechanisms of endothelial cell-specific gene regulation, they do not address the question of how the flk-1/KDR promoter is temporally controlled by positive and negative regulators of angiogenesis. Transforming growth factor-β1 (TGF-β1) has a biphasic effect on basic fibroblast growth factor- and VEGF-induced angiogenesis in vitro (14). At low concentrations TGF-β1 enhances endothelial cell response, whereas at high concentrations, TGF-β1 inhibits the effects of angiogenic factors such as VEGF or basic fibroblast growth factor on endothelial cells (14, 15). In a previous study, TGF-β1 was shown to down-regulate expression of flk-1/KDR at the level of mRNA, total protein, and 125I-VEGF binding capacity (16). These findings raised the interesting possibility that TGF-β1 exerts its anti-angiogenic effect, at least in part, through the inhibition of the VEGF signaling pathway. In this study, we extend these observations by showing that TGF-β1 suppresses flk-1/KDR expression through a palindromic GATA site in the 5′-untranslated region (5′-UTR).

EXPERIMENTAL PROCEDURES

Cell Culture—Bovine aortic endothelial cells (BAEC) (Clonetech Corp.) and human embryonic kidney (HEK) 293 cells (American Type Culture Collection CRL-1573) were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% heat-inactivated fetal bovine serum (FBS). BAEC were used within the first 10 passages.

Plasmids—For construction of the 2.2-kb flk-1-luc plasmid, a region spanning ~1940 to +296 bp of the human promoter was generated by exonuclease III digestion of the full-length flk-1/KDR promoter (generously provided by Cam Patterson, University of Texas Medical Branch, Galveston, TX) and cloned into the pGL2-basic vector (Promega). To generate the KpnI-luc construct, 2.2 kb flk-1-luc plasmid was digested with KpnI, releasing a fragment spanning region ~1940 to ~115. The remaining vector was religated, resulting in a plasmid that contained ~115 to +296 of the flk-1/KDR promoter coupled to luciferase. A series of internal deletions and point mutations were introduced into the flk-1/KDR promoter by polymerase chain reaction methodology. To generate KpnI-lucGATA-luc, two polymerase chain reaction fragments (A and B) were amplified from 2.2-kb flk-1-luc. Fragment A spanned a region between the KpnI site at ~115 and ~102, whereas fragment B spanned a region between +187 and the XhoI site of pGL2-basic. The primers were designed in such a way as to introduce an NdeI site in the deleted region. Fragment A was digested with KpnI and NdeI, whereas fragment B was digested with XhoI and NdeI.
mements A and B were then inserted into KpnI/XhoI-digested 2.2-kb flk-1-luc in a three-way ligation. The resulting plasmid contained a deletion of flk-1/KDR sequences between +103 and +187. A similar strategy was used to generate deletions of the Sp1 and NF-xB sites (KpnI/NcoI/NF-xB-luc), resulting in a plasmid that contained a deletion of flk-1/KDR sequences between +99 and +60. The same polymerase chain reaction strategy was employed to introduce the following mutations into the GATA (KpnI/GATA mut-luc), Sp1 (KpnI/Sp1 mut-luc), and NF-xB (KpnI/NF-xB mut-luc) motifs: Sp1 mut, GGGCGG → GTTCGG, GCGCCC → CCGTTC, NF-xB mut, GAGAGGCCC → AAAAAAGGCCCTT; and GATA mut, GATATCCC → GTTTAACG. All deletions and mutations were confirmed by automated DNA sequencing. To generate pGEM-bflk, a 266-bp bovine flk-1/KDR cDNA fragment was polymerase chain reaction-amplified from reverse-transcribed BAEC total RNA and subcloned into the pGEM-T-easy vector (Promega). Similarly, pGEM-bGAPDH was derived by ligating a 371-bp bovine GAPDH cDNA fragment into pGEM-T-easy.

RNA Isolation and RNase Protection Assays—BAEC were grown to confluence in 100-mm culture dishes, at which point the culture medium was replaced with serum-starved medium (Dulbecco’s modified Eagle’s medium plus 0.5% FBS). 24 h later, BAEC were incubated with 10 ng/ml TGF-β1 (Peprotec) or with serum-starved medium alone. 24 h following TGF-β1 treatment, total RNA was purified using the Trizol reagent (Life Technologies, Inc.). For in vitro transcription, flk-1/KDR- and GAPDH-specific 32P-labeled riboprobes were synthesized from pGEM-bflk and pGEM-bGAPDH, respectively. pGEM-bflk contains a 266-bp fragment of bovine flk-1, whereas pGEM-bGAPDH contains a 371-bp fragment of bovine GAPDH from +1 to +371 (see below). Both riboprobes were synthesized using T7 RNA polymerase (Ambion Inc.) and purified with a Sephadex G-50 spin column (Amersham Pharmacia Biotech). RNase protection assays were performed with an RPA III kit (Ambion Inc.) according to the manufacturer’s instructions.

Transfections and Analysis of Luciferase Activity—BAEC and HEK-293 cells were transfected using FuGENE 6 reagent (Roche Molecular Biochemicals) as instructed by the manufacturer. BAEC (1 × 105 cells/well) or HEK-293 cells (2 × 105 cells/well) were seeded in 12-well plates 18–24 h before transfection. For BAEC transfections, 0.12 pmol of the reporter gene construct and 50 ng of a control plasmid containing the Renilla luciferase reporter gene under the control of a cytomegalovirus enhancer/promoter (pRL-CMV) (Promega) were incubated with 2 μl of FuGENE 6. For HEK-293 cell transfections, 0.05 pmol of the test construct, 50 ng of the control plasmid, and 0.075 pmol of the GATA expression vector were incubated with 2 μl of FuGENE 6. 24 h later, the cells were washed with phosphate-buffered saline and cultured for 12 h in Dulbecco’s modified Eagle’s medium plus 0.5% FBS. The cells were then incubated in the presence or absence of TGF-β1, for 24 h, at which time they were lysed and assayed for luciferase activity using the dual-luciferase reporter assay system (Promega) and a Lumat LB 9507 luminometer (Berthold).

To study the effect of cell proliferation on flk-1/KDR promoter activity, BAEC (1 × 105 or 2 × 105 cells/well) were seeded in 12-well plates 15 h before transfection and assayed for luciferase activity 48 h later. To determine the effect of serum starvation on promoter activity, transfected BAEC were washed with phosphate-buffered saline 24 h following transfection, incubated in Dulbecco’s modified Eagle’s medium containing 0.5% FBS, and assayed for luciferase activity 18 h later.

Nuclear Extracts and Electrophoretic Mobility Shift Assays—Nuclear extracts were prepared as previously described (17). Double-stranded oligonucleotides were labeled with [α-32P]dCTP and Klenow fragment and purified on the spin column. 15 μg of nuclear extract was incubated with 10 fmol of 32P-labeled probe, 2 μg of poly(dI-dC), 2.5 fmol of ZnSO4, and 5 μl of 10× binding buffer (100 mM Tris HCl (pH 7.5), 50% glycerol, 10 mM dithiothreitol, and 10 mM EDTA) for 30 min at room temperature and then at 4 °C for a further 30 min. To test the effect of antibodies on DNA-protein binding, nuclear extracts were preincubated with anti-GATA-2 antibody (Santa Cruz Biotechnology) and a generous gift from Dr. Stuart Orkin) or anti-Sp1 antibody (Santa Cruz Biotechnology) for 1 h at 4 °C. In competition studies, a 10-, 50-, or 200-fold molar excess of unlabeled competitor DNA was added and the reaction continued with or without antibody for an additional 30 min at 4 °C. DNA-protein complexes were resolved on 4% native gels and visualized by autoradiography.
were transiently transfected with 0.12 pmol of 2.2-kb flk-1 exposed to increasing concentrations of TGF-β1. Similar results of unlabeled wild-type or mutant oligonucleotide was added to the reaction mixture. DNA-protein complexes were resolved on a 5% non-denaturing polyacrylamide gel containing 5% glycerol in 0.5× buffer containing 50 mM Tris, 50 mM boric acid, and 1 mM EDTA. The loaded gel was fixed with 10% methanol and 10% acetic acid and then autoradiographed.

DNAse I Footprint Analysis—DNase I footprint assays were carried out as previously described (18). A DNA fragment spanning region −111 to +296 of the human flk-1/KDR gene was isolated by digesting the 2.2-kb flk-1-luc plasmid with KpnI and XhoI. The 3′-end of the coding strand was filled in with [α-32P]dCTP and Klenow enzyme. 8 fmol of the labeled probe was mixed with 60 μg of nuclear extracts and digested with DNase I (Takara Biochemicals, Inc.) at room temperature for 2 min. The samples were loaded on 6% denaturing polyacrylamide gels. The loaded gel was fixed with 10% methanol and 10% acetic acid and then autoradiographed.

RESULTS

TGF-β1-mediated Down-regulation of flk-1/KDR mRNA—Our first goal was to confirm whether TGF-β1 down-regulates expression of flk-1/KDR in bovine aortic endothelial cells. To this end, we employed RNase protection assays with a probe that is specific for the bovine flk-1/KDR gene and total RNA derived from control and TGF-β1-treated cells. As shown in Fig. 1 (A and B), the incubation of BAEC in the presence of 10 ng/ml TGF-β1 for 24 h resulted in a 60% reduction in flk-1/KDR mRNA.

TGF-β1-mediated Down-regulation of the flk-1/KDR Promoter—We next wished to determine whether the effect of TGF-β1 on flk-1/KDR mRNA expression was mediated by the flk-1/KDR promoter. BAEC were transiently transfected with the 2.2-kb flk-1-luc plasmid, which contains a 2.2-kb region of the human flk-1/KDR promoter (between −1960 and +296) coupled to the luciferase reporter gene. Transfected BAEC were grown in the absence or presence of TGF-β1 and assayed for luciferase activity 24 h later. As shown in Fig. 2A, TGF-β1 resulted in a dose-dependent reduction in reporter gene activity, with maximal suppression occurring at a concentration of 10 ng/ml. In subsequent studies, we demonstrated that a promoter fragment spanning region −115 to +296 (KpnI-luc) contained the information for TGF-β1-mediated down-regulation (data not shown). Finally, to determine whether the effect of TGF-β1 on flk-1/KDR promoter activity was mediated by changes in the cell cycle, we compared reporter gene activity in pre-confluent and post-confluent BAEC as well as in serum-replete and serum-starved BAEC. As shown in Fig. 2 (B and C), luciferase activity did not vary between these conditions, arguing against a direct effect of cell proliferation on flk-1/KDR promoter activity.

TGF-β1-mediated Down-regulation of the flk-1/KDR Promoter Is Mediated by the GATA Motif—The human flk-1/KDR promoter contains a number of consensus binding sites, including Sp1, NF-κB, and GATA (8). Previous studies have implicated a role for the Sp1- and NF-κB-binding sites, but not the 5′-UTR GATA motif, in mediating constitutive expression of the gene (8, 12). To test whether one or more of these elements were involved in transducing the TGF-β1 signal, the Sp1, NF-κB, and reporter plasmid, which contains a 2.2-kb region of the human flk-1/KDR promoter (between −1960 and +296), was employed RNase protection assays with a probe that is specific for the bovine flk-1/KDR gene and total RNA derived from control and TGF-β1-treated cells. As shown in Fig. 1 (A and B), the incubation of BAEC in the presence of 10 ng/ml TGF-β1 for 24 h resulted in a 60% reduction in flk-1/KDR mRNA.

The results show the means ± S.D. of luciferase light units (relative to untreated cells) obtained in duplicate from three independent experiments. Luciferase light units are corrected for transfection efficiency as described under “Experimental Procedures.” BAEC were transiently transfected with the KpnI-luc plasmid and assayed for luciferase activity at pre-confluence or post-confluence as described under “Experimental Procedures.” The results show the means ± S.D. of luciferase light units (relative to pre-confluent cells) obtained in triplicate from three independent experiments. Lucif-
κB, and GATA elements were deleted and/or mutated, and the resulting plasmids were transfected into BAEC. A combined deletion of the NF-κB site and two upstream Sp1 sites (KpnI(ΔSp1/NF-κB)-luc) resulted in significant reduction (3.8-fold) in basal promoter activity (Fig. 3). A 4-bp mutation of the NF-κB site (KpnI(NF-κB mut)-luc) also resulted in a 3.7-fold reduction in promoter activity, whereas a 2-bp mutation of the two upstream Sp1 sites (KpnI(Sp1 mut)-luc) resulted in only a 10% reduction in promoter activity. These latter findings are consistent with previously published studies (12). However, to our surprise, a 4-bp mutation of the GATA site in the 5′-UTR (KpnI(GATA mut)-luc) resulted in significant reduction (4.4-fold) in reporter gene activity, suggesting that the GATA element is in fact important for basal expression (Fig. 3).

TGF-β1-mediated suppression of the flk-1/KDR promoter is associated with an inhibition of GATA binding—The above results raised the possibility that TGF-β1 inhibits flk-1/KDR expression by interfering with GATA binding to the 5′-UTR. To test this hypothesis, we performed electrophoretic mobility shift assays in which nuclear extracts derived from untreated and TGF-β1-treated BAEC were incubated with a 5′-UTR GATA probe encompassing the putative palindromic GATA site (Fig. 4A). As shown in Fig. 4 (B–D), incubation of nuclear extract from untreated BAEC with the 32P-labeled probe resulted in the appearance of two specific DNA-protein complexes (open and closed arrows). The DNA-protein complexes were inhibited by the addition of a 10–200-fold molar excess of unlabeled self-competitor (Fig. 4, B–D, lane 3; and C, lanes 3–5). Moreover, the complexes were significantly inhibited by the addition of an oligonucleotide probe competitor containing a GATA motif from the human endothelin-1 (hET-1) promoter (Fig. 4C, lanes 6–8). In contrast, DNA-protein binding was unaltered in the presence of a 200-fold molar excess of unlabeled probe containing a 4-bp mutation of the flk-1/KDR GATA sites (Fig. 4B, lane 4). Of the various members of the GATA transcription factor family, GATA-2 is believed to play the most prominent role in endothelial cells. To determine whether the DNA complex contained GATA-2 protein, the bind-
ing reactions were incubated with anti-GATA-2 antibodies. The addition of an anti-GATA-2 antibody from Dr. Stuart Orkin resulted in a supershift of the upper DNA-protein complex (Fig. 4B, lane 5, asterisk), whereas the addition of an anti-GATA-2 antibody from Santa Cruz Biotechnology inhibited formation of both DNA-protein complexes (lane 6). In contrast, incubation with an antibody against Sp1 had no effect (Fig. 4B, lane 7). Taken together, these data suggest that the 5′-UTR palindromic GATA site binds to GATA-2.

Interestingly, the 5′-UTR GATA site consists of two overlap-
HEK-293 cells were incubated with TGF-β overexpression (Fig. 6, 5). Derived from TGF-β, mobility shift assays were carried out with nuclear extracts delineated by a palindromic GATA sequence. To determine whether this palindromic sequence binds to one or two molecules of GATA-2, electrophoretic mobility shift assays were carried out with a probe containing a mutation of a single GATA site. The incubation of this mutant probe with nuclear extract resulted in a single DNA-protein complex that corresponded to the faster migrating complex of the wild-type probe and to the specific DNA-protein complex of the hET-1 probe (Fig. 4D). These results suggest that the more slowly migrating complex seen only with the wild-type 5′-UTR probe represents a complex between the palindromic GATA site and two molecules of GATA protein.

Finally, to test the effect of TGF-β on GATA binding, mobility shift assays were carried out with nuclear extracts derived from TGF-β1-treated BAEC. TGF-β1 treatment did not result in a change in mobility pattern, but rather in a significant (3-fold by densitometry) reduction in the intensity of the GATA-binding complexes (Fig. 4, B, lanes 8–13; and C, lanes 9–12). To confirm the inhibitory effect of TGF-β1 on GATA binding, we carried out DNase I footprint analyses with a labeled promoter fragment containing region −115 to +296 of the flk-1/KDR gene. As shown in Fig. 5, the region spanning the 5′-UTR GATA elements was protected by nuclear protein derived from untreated BAEC, but not from TGF-β1-treated BAEC. These results are consistent with those of the mobility shift assays and strongly support the conclusion that TGF-β1 inhibits binding of GATA protein to the 5′-UTR.

Transactivation of the flk-1/KDR Promoter by GATA Is Inhibited by TGF-β1—Having established the inhibitory effect of TGF-β1 on GATA binding in vitro, we wished to study the functional relevance of this interaction in vivo. To this end, we carried out transactivation assays in which the 2.2-kb flk-1-luc or KpnI-luc constructs were cotransfected with expression plasmids for either mouse GATA-1 (pXM-mGATA1) or human GATA-2 (pMT2-hGATA2) in HEK-293 cells. As shown in Fig. 6 (A and B), cotransfection of GATA-1 or GATA-2 induced the full-length flk-1/KDR promoter activity by 9.0- or 11.8-fold, respectively. Similar results were obtained with the shorter KpnI-luc construct, suggesting that the palindromic GATA site in the 5′-UTR is sufficient for mediating this effect (Fig. 6, A and B). Indeed, KpnI-luc constructs containing a deletion (KpnIΔ(GATA)-luc) or mutation (KpnI(GATA mut)-luc) of the 5′-UTR GATA site failed to respond to GATA-1 or GATA-2 overexpression (Fig. 6, A and B). Finally, when cotransfected HEK-293 cells were incubated with TGF-β1, transactivation of the flk-1/KDR promoter by GATA-1 and GATA-2 was attenuated by 2.3- and 4.5-fold, respectively (Fig. 7). Together, these findings support the conclusion that TGF-β1 suppression of flk-1/KDR expression is mediated by the 5′-UTR palindromic GATA sequence.

**DISCUSSION**

VEGF-mediated signaling through the FLK-1/KDR receptor is believed to play a critical role in angiogenesis both during development as well as in the adult. Temporal regulation of flk-1/KDR expression within the endothelium may represent an important mechanism for modulating activity of this signaling pathway. For example, in the embryo, flk-1/KDR is expressed in the developing vasculature, whereas in the adult, flk-1/KDR is expressed at sites of physiological and pathophysiological angiogenesis. An understanding of the transcriptional control mechanisms that regulate flk-1/KDR expression in these settings may provide important information about the molecular control of angiogenesis.

Under in vitro conditions, the flk-1/KDR promoter has been shown to direct endothelial cell-specific expression. Maximal promoter activity of the human gene resides in fragment −225 to +268 (8), whereas endothelial cell-specific expression of the mouse gene is mediated by sequences between −623 and +299 (9). There are several conserved cis-regulatory elements in the mouse and human promoters, including Sp1-, AP-2-, NF-κB-, and GATA-binding sites (8, 9). Previous studies of the human promoter have supported an important role for upstream AP-2, NF-κB, and Sp1 elements in mediating cell type-specific expression in cultured endothelial cells (8, 12).

In this study, we confirmed the importance of the upstream NF-κB site in mediating basal expression of the flk-1/KDR promoter. In addition, we demonstrated a role for an overlapping palindromic GATA sequence in mediating constitutive promoter activity. These results are in sharp contrast to a previous study in which a 3-bp mutation of the 5′-UTR GATA site was shown to have no effect on expression levels (8). The reason for this discrepancy is not clear. Both studies employed primary cultures of bovine aortic endothelial cells. In the study...
by Patterson et al. (8), the GGATATCC site was mutated to GGTCGTCC, whereas in this study, GGATATCC was mutated to GGTTAAAGC. Despite these differences, both mutations are predicted to eliminate GATA binding to the two GATA sites. It is noteworthy that the GGTCGTCC mutation was analyzed in a promoter that spanned −225 to +268 bp, whereas GTTTAAGC was studied in fragment −150 to +296. However, it is unlikely that such small differences in promoter context would account for the disparate results. In the final analysis, our findings strongly support a role for the 5′-UTR GATA motif in mediating basal expression of flk-1/KDR.

Several factors have been implicated in the temporal regulation of flk-1/KDR expression. For example, tumor necrosis factor-α has been shown to induce expression of both the endogenous flk-1/KDR gene and the upstream promoter (19, 20), whereas TGF-β1 inhibits flk-1/KDR mRNA levels in endothelial cells. To gain a better understanding of the mechanisms that control TGF-β1-mediated down-regulation of flk-1/KDR expression, we examined the effect of TGF-β1 on flk-1/KDR promoter activity. The results are consistent with a model in which TGF-β1 represses flk-1/KDR expression by inhibiting the interaction of GATA-2 with overlapping double GATA consensus sites in the 5′-UTR.

In vertebrate promoters, most GATA elements occur as single sites or as direct repeats. Rarely do GATA sequences overlap one another on opposite sides of the DNA (21). These palindromic motifs are unique in that they interact with both the C- and N-terminal domains of GATA protein, resulting in high affinity binding (21). However, to date, palindromic GATA sequences have been shown to bind only one molecule of GATA protein. In contrast, the results of this study suggest that the 5′-UTR GATA palindrome may complex with two molecules of GATA protein. It is conceivable that the two molecules of GATA protein bind to the overlapping GATA elements. Alternatively, GATA-GATA protein interactions may result in dimerization at the GATA site (22). Finally, we cannot exclude the possibility that a heterodimer involving the GATA transcription factor and another protein is binding to the double GATA motif. Regardless of the mechanism, the unique configuration and binding properties of the 5′-UTR GATA motif may be important determinants for both basal expression and TGF-β1-mediated repression of the flk-1/KDR gene.

TGF-β1 may have several effects on transcriptional pathways. TGF-β1-mediated activation of its cognate receptor results in phosphorylation of cytoplasmic transcription factors of the SMAD family. In addition, TGF-β1 may act indirectly through other families of transcription factors to attenuate gene expression. In some cases, these mechanisms serve to repress basal expression levels. For example, TGF-β1 inhibits cyclin A promoter activity by decreasing the phosphorylation and activity of activating transcription factor-1 and cAMP-responsive element-binding protein (23). In other cases, TGF-β1 signaling results in an inhibition of inducible gene expression. For example, TGF-β1 antagonizes phorbol ester-mediated transcriptional induction of matrix metalloproteinase-1 through a TGF-β inhibitory element (24). In intestinal epithelial cells, TGF-β1 attenuates glucocorticoid-mediated induction of haptoglobin mRNA by inhibiting CAAT/enhancer-binding protein binding to the proximal promoter (25).

Our report is the first to implicate the GATA family of DNA-binding proteins in mediating the inhibitory effects of TGF-β1. The transfection and DNA-protein binding assays suggest that TGF-β1 represses flk-1/KDR expression by interfering with the binding of GATA protein to its cognate binding sites in the first exon. There are several possible explanations for this effect. First, TGF-β1 may inhibit GATA expression at a transcriptional level. It is interesting to note that in hematopoietic cells, neutralization of TGF-β1 results in increased

**Fig. 6. Transactivation of the flk-1/KDR promoter by GATA-1 and GATA-2.** HEK-293 cells were transiently transfected with 50 fmol of luciferase reporter plasmids and 75 fmol of mouse GATA-1 expression plasmid (pXM-mGATA1; A), human GATA-2 expression plasmid (pMT2-hGATA2; B), or vector-alone plasmid (pXM (A) and pMT2 (B)). The expression levels were normalized to pRL-CMV activity and expressed as -fold induction relative to cotransfection with vector alone. The means ± S.D. were derived from at least three separate experiments performed in duplicate or triplicate.
TGF-β₁-mediated Suppression of the flk-1/KDR Promoter

FIG. 7 . Attenuation of GATA-1- and GATA-2-mediated transactivation of the flk-1/KDR promoter by TGF-β₁. HEK-293 cells were transiently transfected as described in the legend to Fig. 6. Cells were serum-starved, incubated with or without TGF-β₁ for 24 h, and then assayed for luciferase activity. The expression levels were normalized to pRL-CMV activity and expressed as -fold induction relative to cotransfection with vector alone. The means ± S.D. were derived from at least three separate experiments performed in duplicate or triplicate.

GATA mRNA (26). However, our observation that GATA-mediated activation of the flk-1/KDR promoter is inhibited in cotransfection assays argues against this mechanism. A second possibility is that TGF-β₁ induces the binding of other transcription factor(s) to sequences within the vicinity of the GATA site, resulting in competitive inhibition of GATA binding. This seems unlikely since TGF-β₁ does not result in a change in the pattern of mobility of the DNA-protein complexes. A final consideration is that TGF-β₁ alters GATA activity at a post-transcriptional level. Previous studies have shown that GATA binding is influenced both by its phosphorylation state (27–29) and by acetylation (30). Whether or not TGF-β₁ signaling interferes with one or more of these modifications in endothelial cells remains to be established.

Acknowledgments—We thank Stuart Orkin for providing the GATA expression vectors and for helpful suggestions. We are particularly indebted to Cecil Trainor for critical reading of this manuscript.

REFERENCES

1. Neufeld, G., Cohen, T., Gengrinovitch, S., and Poltorak, Z. (1999) FASEB J. 13, 9–22
2. Shalaby, F., Rossant, J., Yamaguchi, T. P., Gertsenstein, M., Wu, X. F., Breitman, M. L., and Schuh, A. C. (1995) Nature 376, 62–66
3. Millauer, B., Wirzmann-Voss, S., Schnurch, H., Martinez, R., Moller, N. P., Risau, W., and Ullrich, A. (1993) Cell 72, 835–846
4. Li, J., Brown, L. P., Hibberd, M. G., Grossman, J. D., Morgan, J. P., and Simons, M. (1996) Am. J. Physiol. 270, H1893–H1811
5. Hammes, H. P., Lin, J., Bretzel, R. G., Brownlee, M., and Breier, G. (1998) Diabetes 47, 401–406
6. Suzuma, K., Takagi, H., Otani, A., Suzuma, I., and Honda, Y. (1998) Microc. Res. 56, 183–191
7. Saadeh, P. B., Mehrara, B. J., Steinbrech, D. S., Dudziak, M. E., Greenwald, J. A., Luchs, J. S., Spector, J. A., Ueno, H., Gittes, G. K., and Longaker, M. T. (1999) Am. J. Physiol. 277, C628–C637
8. Patterson, C., Perrella, M. A., Haich, C. M., Yoshizumi, M., Lee, M. E., and Haber, E. (1995) J. Biol. Chem. 270, 23111–23118
9. Ronicke, V., Risau, W., and Breier, G. (1996) Circ. Res. 77, 277–285
10. Wu, Y., and Patterson, C. (1999) J. Biol. Chem. 274, 3207–3214
11. Patterson, C., Wu, Y., Lee, M. E., DeVault, J. D., Runge, M. S., and Haber, E. (1997) J. Biol. Chem. 272, 8410–8416
12. Hata, Y., Duh, E., Zhang, K., Robinson, G. S., and Aiello, L. P. (1998) J. Biol. Chem. 273, 19294–19301
13. Kappel, A., Ronicke, V., Damert, A., Flammé, I., Risau, W., and Breier, G. (1999) Blood 93, 4284–4292
14. Pepper, M. S., Vassalli, J. D., Orci, L., and Montesano, R. (1993) Exp. Cell Res. 204, 356–363
15. Gajdusek, C. M., Luo, Z., and Mayberg, M. R. (1993) J. Cell. Physiol. 157, 133–144
16. Mandriota, S. J., Menoud, P. A., and pepper, M. S. (1996) J. Biol. Chem. 271, 11500–11505
17. Dignam, J. D., Martin, P. L., Shastry, B. S., and Roeder, R. G. (1983) Methods Enzymol. 101, 562–588
18. Jones, K. A., Yamamoto, K., and Tjian, R. (1999) Cell 92, 559–572
19. Giraud, E., Primo, L., Audero, E., Gerber, H. P., Roussel, S., Klagesbrun, M., Ferrara, N., and Bussolino, F. (1998) J. Biol. Chem. 273, 22126–22135
20. Illi, B., Puri, P., Morgante, L., Capogrossi, M. C., and Gaetano, C. (2000) Circ. Res. Res. 86, E110–E117
21. Trainor, C. D., Omichinski, J. G., Vandergrun, T. L., Gruenendorn, A. M., Clare, G. M., and Felsenfeld, G. (1986) Mol. Cell. Biol. 16, 2228–2247
22. Mackay, J. P., Kowalski, K., Fox, A. H., Czolij, R., King, G. F., and Crossley, M. (1995) J. Biol. Chem. 270, 2247–2253
23. Yoshizumi, M., Wang, H., Haich, C. M., Sibinga, N. E., Perrella, M. A., and Lee, M. E. (1997) J. Biol. Chem. 272, 22059–22064
24. White, L. A., Mitchell, T. I., and Brinkerhoff, C. E. (2000) Biochim. Biophys. Acta 1490, 259–268
25. Yu, S. J., Boudreau, P., Deslites, A., Houde, M., Rivard, N., and Asselin, C. (1999) Biochem. Biophys. Res. Commun. 259, 544–549
26. Pierelli, L., Marone, M., Bonanno, G., Mozzetti, S., Rutella, S., Morosetti, R., Rumi, C., Manesu, S., Leone, G., and Scambia, G. (2000) Blood 93, 3001–3009
27. Towarity, M., May, G. E., Marais, R., Perkins, G. R., Marshall, C. J., Cowley, S., and Enver, T. (1995) J. Biol. Chem. 270, 4101–4107
28. Partington, G. A., and Patient, R. K. (1999) J. Biol. Chem. Res. 27, 1168–1175
29. Morimoto, T., Haegawa, K., Kaboragi, S., Kikita, T., Wada, H., Yanareme, T., and Sasayama, S. (2000) J. Biol. Chem. 275, 13721–13726
30. Byoys, G. Y., Byfield, P., Nakatani, Y., and Ogryzko, V. (1996) Nature 396, 594–598