Role of the Ets Transcription Factors in the Regulation of the Vascular-Specific Tie2 Gene

Antoinise Dube, Yasmin Akbarali, Thomas N. Sato, Towia A. Libermann, Peter Oettgen

Abstract—The Tie2 gene encodes a vascular endothelium-specific receptor tyrosine kinase that is required for normal vascular development and is also upregulated during angiogenesis. The regulatory regions of the Tie2 gene that are required for endothelium-specific gene expression in vivo have been identified. However, the transcription factors required for Tie2 gene expression remain largely unknown. We have identified highly conserved binding sites for Ets transcription factors in the Tie2 promoter. Mutations in 2 particular binding sites lead to a 50% reduction in the endothelium-specific activity of the promoter. We have compared the ability of several members of the Ets family to transactivate the Tie2 promoter. Our results demonstrate that 1 of 3 distinct isoforms of the novel Ets transcription factor NERF, NERF2, is expressed in endothelial cells and can strongly transactivate the regulatory regions of the Tie2 gene in comparison to other Ets factors, which have little or no effect. NERF2 can bind to the Tie2 promoter Ets sites in electrophoretic mobility shift assays. These studies support a role for Ets factors in the regulation of vascular-specific gene expression and suggest that the novel Ets factor NERF2 may be a critical transcription factor in specifying the expression of the Tie2 gene in vascular endothelial cells. (Circ Res. 1999;84:1177-1185.)

Key Words: transcription ■ vasculogenesis ■ Tie2 ■ angiogenesis

Tie1 and Tie2 are endothelium-specific receptor tyrosine kinases that have been determined to be critical for vascular development. Targeted disruption of the Tie1 gene leads to the development of leaky blood vessels, which results in edema and hemorrhage, whereas disruption of the Tie2 gene leads to dilated blood vessels and abnormal capillary networks. The growth factor ligand for the Tie2 receptor, angiopoietin-1, has been recently identified. In addition to their role during embryonic blood vessel development, expression of both the Tie1 and Tie2 genes increases during tumor angiogenesis.

Although much information has emerged concerning the role of growth factors and their receptors during vascular development, little is known of the nuclear events that orchestrate this process at a transcriptional level. The Ets transcription factors are a family of genes that share a conserved DNA-binding domain and regulate genes involved in determining tissue specificity, cellular differentiation, and proliferation. They have also been shown to play a role in the development of human cancers as a result of chromosomal translocations. Many of the target genes originally described included T- and B-cell specific genes, and it was shown that inactivation of Ets-1 lead to increased T-cell apoptosis and terminal B-cell differentiation. Recently, Ets factors have been shown to regulate a wide variety of genes and developmental processes unrelated to the immune system.

Interestingly, the regulatory elements of the Flt-1, Tie1, vascular endothelial–cadherin, and ICAM-2 genes have several conserved Ets binding sites that are critical for the transcriptional activity of the promoters and enhancers of these genes. It is unknown which of the Ets factors are critical for the transcriptional activity of these genes. One of the Ets factors, TEL, was recently shown to be involved in the development of the extra-embryonic blood vessels. Targeted disruption of the gene leads to abnormalities in vitelline vein development.

We have identified a cluster of Ets sites in the Tie2 promoter that are important for determining the basal activity of the promoter in endothelial cells. In addition, we report that 1 of the isoforms of the Ets factor NERF, NERF2, is expressed in endothelial cells and can bind to and strongly transactivate the Tie2 gene via these Ets binding sites.

Materials and Methods

Cell Culture

Human umbilical vein endothelial cells (HUVECs) and human aortic endothelial cells (HAECs) were obtained from Clonetics. The A20, HAFTL, human embryonic kidney (HEK) 293, and ECV304, a spontaneously immortalized HUVEC cell line, were grown as described previously.

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RNA Isolation and Reverse Transcription–Polymerase Chain Reaction Analysis

Total RNA was harvested from cultured cells with the Ultraspec RNA isolation kit (Biotex Laboratories, Inc; catalogue No. BL-10500), a modification of the guanidinium thiocyanate-phenol-chloroform isolation method of Chomczynski and colleagues. cDNAs were generated from 1 μg of total RNA from different cells by use of oligo(dT)12-18 priming (Gibco BRL) and the Moloney murine leukemia virus reverse transcriptase (Gibco BRL). The sequences of the oligonucleotide primers and polymerase chain reaction (PCR) conditions are as previously described.

Plasmid Constructs and Site-Directed Mutagenesis

The cDNAs that encode the selected Ets factors were cloned into the PCI CMV expression vector (Promega) and were verified by DNA sequencing. ERP (Ets-related protein) and the 3 NERF isoforms were obtained in our laboratory. Sap-1 and Elk-1 were gifts from Roger Davis (University of Massachusetts, Worcester, Mass); Ets-1 and Ets-2, Dan Tenen (Beth Israel Deaconess Medical Center, Boston, Mass); ELF-1, Jeffrey Leiden (University of Chicago, Chicago, Ill); and TEL, Todd Golub (Dana Farber Cancer Center, Boston, Mass). The mouse Flt-1 promoter luciferase construct was a gift from Bill Aird (Beth Israel Deaconess Medical Center, Boston, Mass). The urokinase promoter Ets site was constructed by ligating a double-stranded oligonucleotide that encoded the urokinase promoter ETS-binding site in a pGL3 vector that contained the cFos Δ56 minimal promoter, which we have previously used as a minimal reporter. The LacZ reporter constructs are shown in Figure 1B and include the 2.1 kb promoter and the 10.0 kb 5’ half of the first intron up to and including the 1.7-kb intronic enhancer (construct No. 1), the 2.1 kb HindIII-HindIII, the 2.1-kb promoter together with the 1.7-kb XhoI-KpnI intronic enhancer (construct No. 2), the 1.7-kb intronic enhancer with the HSV-tk minimal promoter (construct No. 3), and the promoter alone (construct No. 4). Deletion constructs of the Tie2 gene were further subcloned into the PGL3 luciferase vector (Promega) and are shown in Figure 1B and 1C. Point mutations were made with a site-directed mutagenesis kit (QuikChange, Stratagene). The expression plasmid used for overexpression of selected Ets factors was the PCI vector (Promega).

DNA Transfection Assays

Cotransfections of 1.5 to 2×10^5 endothelial cells or HEK 293 cells were performed with the use of 1.75 μg of the reporter gene construct DNA and 0.75 μg of the expression vector DNA with 6.25 μL of Lipofectamine (Gibco BRL). Cells were washed with serum-free DMEM. A total of 0.8 mL of serum-free DMEM was added per 35-mm dish with Lipofectamine (Gibco BRL). Cells were harvested 16 hours after transfection and assayed for luciferase activity and after 40 hours for β-galactosidase activity as previously described. β-Galactosidase activity was measured with the luminescent β-galactosidase genetic reporter system II (Clontech). Transfections were repeated independently in triplicate with similar results. A dual luciferase assay was used to compare transfection efficiency for different Ets factors in HEK 293 cells (Dual-Luciferase Reporter Assay System, Promega). In brief, this assay involves the additional cotransfection of 0.1 μg of the PRL vector that expressed the Renilla luciferase. After cell lysates were read for luminescence with the luminometer, 100 μL of the STOP and GLO solution (Promega) was added to the sample, which inactivates any other luciferase activity and activates the Renilla luciferase.

In Vitro Transcription-Translation

Full-length NERF2 cDNA that encoded the whole open reading frame was inserted downstream of the T7 promoter into the BlueScript vector. Coupled in vitro transcription–in vitro translation reactions were performed with a reticulocyte lysate kit (Tnt, Promega) and T7 RNA polymerase as recommended by the manufacturer. The plasmid vector without an insert was used as a control.

Figure 1. Tie2 genomic structure and reporter constructs. A, Genomic structure of the Tie2 gene, which includes the promoter (solid), and 5’ end of the first intron, which includes an intronic enhancer (gray). Bottom, Maps of the LacZ reporter constructs with the promoter and entire 5’ end of the first intron include the intronic enhancer (No. 1), promoter and intronic enhancer (No. 2), intronic enhancer with HSV-tk minimal promoter (No. 3), and the promoter alone (No. 4). B, Tie2 promoter constructs in PGL3 luciferase reporter vector. No. 5 includes HindIII-HindIII; No. 6, SacI-HindIII; and No. 7, BamHI-HindIII. C, Tie2 promoter BamHI-Styl luciferase construct (No. 8), and Bam-HindIII double Ets site point mutation (No. 9). The arrow indicates the transcription start site; Black circles, Ets sites No. 1 through 5 with nucleotide sequence shown above; and White circles, point mutations of Ets sites No. 4 and 5.
NERF2 and ELF-1 Can Transactivate the Tie2 Gene

Because Ets-binding sites are conserved in the promoters of some vascular specific genes, including the Tie1 and Flt-1 genes, and because some of these sites are also functionally important, as in the Flt-1 gene, we were interested to know whether Ets factors might regulate the expression of the Tie2 gene. A schematic diagram for constructs used in the transfection studies is shown in Figure 1 (see Materials and Methods). We first compared the ability of several members of the Ets family to transactivate the promoter and enhancer regions of the Tie2 gene (construct No. 1) in HEK 293 cells, which do not express Tie2 and are easy to transfect. We performed cotransfections with the Tie2 reporter and expression vectors that encoded several members of the Ets gene family. As is shown in Figure 2A, NERF2 and ELF-1, 2 members of the Ets gene family that are structurally similar, were able to transactivate these regulatory elements of the Tie2 gene up to 10-fold. In contrast, the other Ets factors tested, including Ets1, Ets2, SAP-1, Elk-1, TEL, and ERP had little or no ability to transactivate the Tie2 gene. To demonstrate the relative specificity of transactivation of the Tie2 promoter by NERF2 and ELF-1, we performed similar cotransfection experiments with reporter constructs from 2 other genes: the urokinase and Flt-1 genes. Ets factors, and in particular Ets-1 and Ets-2, have been shown to be important for the transcriptional regulation of these genes.22,27–29 Transactivation by both Ets-1 and Ets-2 was much stronger for both of these genes compared with NERF2 and ELF-1 (Figure 2B and 2C). ERP and ELK were able to transactivate the urokinase Ets site by 3- to 4-fold. These results support the specificity of transactivation of the Tie2 promoter by NERF2 and ELF-1.

When the Tie2 regulatory elements tested included the promoter and the intronic enhancer but lacked the remaining 5′ end of the first intron, construct No. 2, NERF2, and ELF-1 were still capable of strong transactivation, with 15- to 30-fold increases in activation (Figure 2D). This increase in transactivation by NERF2 and ELF-1 from 10-fold to 30-fold may reflect possible inhibitory domains in the additional intronic sequences contained in construct No. 1. Interestingly, only NERF2 but not NERF1a led to significant transactivation. This is similar to what we have shown for the NERF isoforms with other promoters, such as the lyn tyrosine kinase gene, in which only the NERF2 isoforms and not the NERF1 isoforms act as a positive regulator.17

NERF2 and ELF-1 Act Through the Promoter to Transactivate the Tie2 Gene

Because only NERF2 and ELF-1 significantly enhanced transcription of the Tie2 gene, we decided to further investigate their effect on Tie2 gene regulation. To determine whether the transactivation by NERF2 and ELF-1 occurred predominantly through the core enhancer or the promoter, we performed cotransfection experiments in HEK 293 cells with constructs No. 3 (core enhancer with HSV-tk promoter) and No. 4 (promoter alone). Transactivation of the core enhancer (Figure 2E) resulted in only a modest transactivation by NERF2 and ELF-1 of 2- to 3-fold. In contrast, when cotransfection experiments were performed with the promoter (Figure 2F), there was a more substantial transactivation by NERF2 and ELF-1 of 12- to 18-fold. This suggests that the ability of NERF2 and ELF-1 to transactivate the Tie2 gene occurs predominantly through the Tie2 promoter.

NERF2 but Not ELF-1 Is Highly Expressed in Endothelial Cells

To determine whether ELF-1 or the different isoforms of NERF are expressed in endothelial cells, we performed reverse transcription–PCR with RNA derived from HUVECs and HAECs and compared this to the expression in 2 B-cell lines, HAFTL and A20, in which we have previously demonstrated high levels of expression of NERF and ELF-1.17 The different isoforms of NERF are similar in size and are difficult to distinguish by Northern blot analysis.17 The results of these experiments (Figure 3) demonstrate that the predominant isoform of NERF expressed in endothelial cells is NERF2, with low levels of expression of the NERF1a isoform and no expression of ELF-1 or NERF1b. In contrast, ELF-1 is highly expressed in B-cell lines but not in endothelial cells.

Characterization of the Tie2 Promoter Elements Required for Transactivation by NERF2

To further define which regions of the Tie2 gene promoter are necessary for transactivation by NERF2, we created a variety of deletion constructs that were inserted into the PGL3 luciferase vector (Figure 1B). As shown in Figure 4A, successive deletions of the promoter down to a 500-bp BamHI to HindIII fragment (construct No. 7) in the most proximal portion of the promoter did not affect the ability of NERF2 (or ELF-1) to transactivate the Tie2 gene.

Further deletion of this fragment down to a 250-bp BamHI to Styl fragment (construct No. 8, Figure 1C) markedly diminished the ability of NERF2 to transactivate the Tie2 promoter in HEK 293 cells (Figure 4B). This suggested that the critical regulatory elements required for transactivation by NERF2 must lie within a 250-bp Styl to HindIII fragment. Sequence analysis of this region re-
revealed a cluster of putative Ets-binding sites just beyond the transcription start site (Figure 1C). Point mutations in Ets sites No. 4 and No. 5 of the cluster (construct No. 9) resulted in almost completely abolishing the transactivation of the Tie2 promoter by NERF2 in HEK 293, although point mutation of the other Ets sites had little or no effect (data not shown). Because the BamHI to HindIII fragment does not appear to exhibit endothelium-specific gene expression in transgenic experiments, we wanted to determine the effect of the same mutations on the full PsI to HindIII promoter fragment, which has been previously shown to be endothelial-cell specific in transgenic animals.26 This promoter fragment is slightly shorter than the HindIII-HindIII promoter fragment (see Figure 1A). We created a PsI to HindIII Tie2 promoter fragment that contained the Ets mutations No. 4 and 5. As is shown in

![Figure 2](image-url)
Figure 3. Expression of NERF isoforms and ELF-1 in human endothelial cells. Comparison of expression of NERF isoforms NERF1a, NERF1b, and NERF2, and ELF-1 in HUVECs, HAECs, and 2 B-cell lines (A20 and HAFTL). GAPDH is used as an internal control. Pan-NERF represents a product derived from primers designed to recognize all NERF isoforms. See Materials and Methods for PCR conditions and primer sequences.

Figure 4. Deletion analysis and point mutations of the Tie2 gene promoter. A, Comparison of ELF-1, NERF1a, and NERF2 transactivation of Tie2 gene promoter constructs in HEK 293 cells. PCI is the empty expression vector. B, Comparison of the ability of the NERF isoforms to transactivate the BamHI-HindIII (construct No. 7) compared with BamHI-StyI (construct No. 8) promoter constructs in HEK 293 cells.

Figure 5. Effect of Ets point mutations in the endothelial cell-specific Tie2 promoter. Comparison of transactivation of the wild-type Pst-HindIII Tie2 promoter (solid) with mutated Ets sites (hatched) by selected Ets factors in HEK 293 cells. PCI is the empty expression vector.

Figure 6. Inducibility by the Ets factors NERF and ELF-1. In endothelial cells, these mutations also nearly abolished the ability of NERF2 to upregulate the Tie2 promoter (Figure 6).

Ets Mutations Reduce Endothelium-Specific Tie2 Promoter Activity
We also wanted to determine whether the PstI to HindIII Tie2 promoter was endothelial cell specific in vitro and whether the Ets mutations would have any effect on the basal activity of the promoter in endothelial cells. As is shown in Figure 7, the normalized Tie2 promoter activity exhibits an endothelial specific basal expression pattern as compared with nonendothelial cells. In addition, the Ets mutations resulted in a 50% reduction of the basal activity of the promoter in endothelial cells and did not significantly alter activity in nonendothelial cells. These results suggest that the selected Ets-binding sites in the Tie2 promoter are important for both basal activity and transactivation of the Tie2 gene by selected Ets factors.

NERF2 Can Bind to the Tie2-Specific Ets Sites
We have previously shown that an Ets site in the lyn promoter is a high-affinity binding site for NERF.17 To determine whether Ets sites No. 4 and 5 in the Tie2 promoter were capable of competing with binding with NERF2, we performed competition experiments with cold oligonucleotides that encoded the 2 Ets sites in the Tie2 promoter critical for transactivation by NERF2. Lane 2 of Figure 8A demonstrates a NERF-specific complex, which corresponds to binding of in vitro translated NERF2 to the lyn Ets probe, versus control lysates in lane 1. The wild-type Tie2 oligonucleotide effectively competed with the lyn oligonucleotide for binding of NERF2 (lanes 3 and 4). In addition, NERF2 that was translated in vitro can bind directly to the Tie2 Ets sites when the wild-type Tie2 Ets oligonucleotide is used as a probe (lanes 5 and 6). To determine whether complexes that were similar in size formed in endothelial cells, we performed

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Comparison of transactivation of the wild-type Pst-HindIII Tie2 promoter (solid) with mutated Ets sites (hatched) by the NERF isoforms in ECV endothelial cells. PCI is the empty expression vector.

gel-shift assays with nuclear extracts derived from human umbilical endothelial cells and used the Tie2 oligonucleotide as the labeled probe (lanes 7 to 15). In addition to similar-sized complexes and in vitro–translated NERF2, there were also 2 higher complexes that may represent other Ets factors of higher molecular weight or additional proteins that bind to the Ets factor to form these complexes (lane 7). To investigate whether the lyn Ets site was capable of competing with the Tie2 oligonucleotide for binding, competition with the wild-type and mutated lyn oligonucleotides was performed (lanes 8 to 11). This test demonstrated that the wild-type lyn oligonucleotide was able to strongly compete with the Tie2 Ets sites for binding and that the oligonucleotide that contained the mutated lyn Ets site had no effect on binding, which supported the hypothesis that the interactions are specific for Ets sites. Finally, to investigate which of the 2 Ets sites would more strongly compete for binding with the wild-type Tie2 oligonucleotide, we synthesized oligonucleotides that encoded the wild type of either Ets site No. 4 or 5 with the other sites mutated. Of the 2 sites, Ets site No. 4 was able to compete more strongly for binding, which suggested that it may be a high-affinity binding site (lanes 12 to 15). When both Ets sites No. 4 and 5 were mutated, no competition existed with the complexes formed (data not shown). In summary, the Tie2 Ets sites appear to be capable of binding NERF2 and forming similarly sized complexes with nuclear extracts derived from endothelial cells.

We tested the ability of several antibodies of known Ets factors to determine whether they would interfere with complex formation or result in a supershift in the gel shift experiments. Antibodies that work well in gel mobility shift assays are not available for NERF2. As is shown in Figure 8B, none of the antibodies for known Ets factors affected the formation of complexes. This is further support that NERF2 may be the Ets factor that binds to the Tie2 Ets sites.

**Discussion**

The goal of this study was to characterize the transcriptional regulations of the Tie2 gene. We chose to focus on Ets transcription factors for several reasons. Ets factors have been shown to be critical regulators of developmental processes such as the development of the immune system. Targeted disruption of Ets-1 and PU.1 leads to abnormalities in B- and T-cell development. They have also been shown to regulate a wide variety of genes unrelated to the immune system. Support of a role for Ets factors in the regulation of vascular specific genes comes from several recent studies. Sequence comparison of the mouse and human Tie1 gene promoters demonstrates several conserved Ets-binding sites. Functional analysis of the Flt-1 gene has demonstrated a critical Ets site that is required for transactivation of the core promoter. Conserved binding sites for Ets factors have also been identified in the promoters of the vascular endothelial cadherin and ICAM-2 promoters. A number of studies have suggested a role for Ets-1 in the regulation of angiogenesis. Expression of Ets-1 is upregulated during angiogenesis. Recently, it has also been shown that Ets-1 can be induced by VEGF in HUVECs, and antisense oligonucleotides to Ets-1 can inhibit endothelial cell migration.

We chose to investigate the Tie2 gene because the critical regulatory elements for vascular specific gene expression have been defined in vivo in transgenic models as well as in vitro in endothelial cells. Our results demonstrate that although Ets-1 was able to weakly transactivate the Tie2 gene promoter, the effect was much weaker than with NERF2 and ELF-1, in which we obtained a 15- to 20-fold induction. Transactivation by NERF2 appears to be specific for the Tie2 gene promoter and was not observed with other vascular-specific genes regulated by Ets factors, such as the Flt-1 gene. Our results also
demonstrate that NERF2 was able to transactivate the Tie2 enhancer, albeit to a lesser extent. Because the Tie2 enhancer has been shown to lead to more complete vascular expression of the Tie2 gene, it is possible that the Ets factors may synergistically act through both the promoter and the enhancer. Mutational analysis supports an important role for Ets factors in the regulation of the basal activity of the Tie2 promoter in endothelial cells, whereas in nonendothelial cells, there was little or no effect of the mutations. Deletion of the StyI-HindIII 250-bp region that contained these Ets sites resulted in completely abolishing endothelium-directed LacZ gene expression in transgenic animals, which confirmed the importance of this region in vivo.26 Comparison of the mouse and human Tie2 promoter sequences demonstrates 100% sequence homology (Mira Puri, personal communication, 1998) in the region of the Ets sites, which supports the role of these Ets-binding sites in Tie2 gene regulation from an evolutionary standpoint. Data from the gel shift assay with endothelial nuclear extracts demonstrate that NERF can bind to the Tie2 Ets sites in endothelial cells because only the lyn oligonucleotide that contains the wild-type Ets site and not the mutant is able to competitively block the formation of this complex. Two additional higher-molecular-weight complexes that were also competitively blocked with the wild-type lyn oligonucleotide could either represent binding of other Ets factors of higher molecular weight or Ets factors that interact with additional proteins, which results in the formation of higher-molecular-weight complexes.

NERF2 and ELF-1 have been previously shown to regulate B- and T-cell specific genes.17,33,34 Our results demonstrate that only 1 isoform of NERF, NERF2, is highly expressed in endothelial cells, whereas ELF-1 does not appear to be expressed in either HUVECs or HAECs. In contrast, in other cell types and tissues, the other isoforms of NERF and ELF-1 are highly expressed. NERF and ELF-1 belong to a subfamily of Ets factors because they are 90% homologous in the DNA-binding domain. In addition, NERF2 and ELF-1 share additional homology regions at the amino terminus. In contrast, NERF1a and 1b have a shorter amino terminal end and lack these additional protein homology regions. This may in part explain the differences in the ability of the NERF isoforms to transactivate the Tie2 gene. In other promoters in which the activity of the NERF isoforms have been examined, such as the lyn and blk promoters, the NERF1 isoforms were unable to transactivate these promoters nor did they appear to downregulate the activity of these promoters. It is possible that they act as competitive inhibitors of NERF2 or become activated on phosphorylation or additional posttranslational modification.

It is interesting that NERF appears to act through a region that contains a cluster of Ets sites. The Endo A enhancer is another example in which transcription may be enhanced by clustering of Ets-binding sites, which contains 6 tandems repeats of a 22-bp unit that contains 2 Ets-binding sites.35 The Tie2 gene does not have a classical TATA box. TATA-less promoters typically initiate tran-
scription through binding of a preinitiation complex to conserved DNA-binding sites called initiator elements (Inrs). High levels of transcription can be enhanced by binding of 1 or more transcription factors or activators downstream from the transcription start site. Unlike TATA-dependent promoters, Inr-dependent promoters do not require direct binding of TFIIID but bind other factors such as YY1, TFII-I, and E2F.36–38 Although several families of initiator elements exist, such as those found in the terminal deoxynucleotidyltransferase gene, the porphobilinogen deaminase gene, and ribosomal protein genes,39–40 many initiator elements do not fit any of the consensus Inr sequences. Several of these nonconserved Inrs contain Ets-binding sites. It has been suggested that Ets factors may play a role as both transcriptional initiators and activators.41,42 Binding of specific Ets factors can also lead to tissue-specific or cell type–specific gene expression. The core promoter of the TATA-less CD4 gene binds Ets factors in close proximity to the transcription start site and leads to tissue-specific expression of the CD4 gene.43

In conclusion, these studies demonstrate the importance of the Ets genes in Tie2 gene regulation and the differences in the ability of different Ets factors to transactivate the Tie2 gene. NERF2 is a strong transcriptional activator of the Tie2 gene and is expressed in human endothelial cells and may represent one of the critical transcription factors in the regulation of Tie2 gene expression.

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