The Human KDR/flk-1 Gene Contains a Functional Initiator Element That Is Bound and Transactivated by TFII-I*

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KDR/flk-1, the receptor for vascular endothelial growth factor, is required for normal vascular development. KDR/flk-1 is a TATA-less gene, containing four upstream Sp1 sites and a single transcription start site, although analysis of the start site sequence discloses only weak similarities with the consensus initiator element (Inr) sequence. In vitro transcription assays, however, demonstrate that the region from −10 to +10 relative to the start site contains Inr activity that is orientation- and position-dependent, and mutagenesis of the KDR/flk-1 Inr reduces promoter activity to 28% of the wild-type promoter in transient transfection assays. Gel shift assays confirm that nuclear proteins specifically bind the Inr, and competition experiments demonstrate that TFII-I, a multifunctional Inr-binding nuclear protein, is a component of these DNA-protein complexes. TFII-I transactivates the wild-type KDR/flk-1 promoter, but not a promoter containing a mutated Inr, in transient transfection assays. Immunodepletion of TFII-I from nuclear extracts prior to in vitro transcription assays abolishes transcription from the KDR/flk-1 Inr, an effect that can be rescued by adding back purified TFII-I, reflecting the importance of TFII-I in KDR/flk-1 Inr activity. These experiments demonstrate that the KDR/flk-1 gene contains a functional Inr that is bound by TFII-I and that both the functional Inr and TFII-I activity are essential for transcription.

Two families of transmembrane tyrosine kinase receptors that are expressed uniquely in endothelial cells and are essential for the development of the embryonic vascular system have recently been identified (1). One family includes the vascular endothelial growth factor (VEGF) receptors, flt-1 (VEGFR-1) and KDR/flk-1 (VEGFR-2) (2, 3). The other family contains the two members of the Tie receptor family, Tie1 and Tie2 (also known as Tek) (4). Of known endothelial cell markers, KDR/flk-1 is the first to be expressed during endothelial cell development, appearing as early as day 7 post-conception in the mouse embryo (5). Importantly, deletion of KDR/flk-1 by homologous recombination in mice blocks endothelial cell development; mice lacking functional KDR/flk-1 fail to form any vascular structures and die in utero between days 8.0 and 9.0 post-conception (6). These and other data indicate that KDR/flk-1 is required for the transformation of endothelial cells from their multipotent precursors and place it upstream of the other endothelial receptor tyrosine kinases in the cascade of endothelial cell development.

In addition to its developmental role, KDR/flk-1 and its ligand VEGF participate in physiologic and pathologic processes with angiogenic components, such as wound healing, bone remodeling, inflammatory arthritis, myocardial infarction, peripheral vascular disease, and tumor growth (reviewed in Ref. 7). These effects of VEGF are mediated by induction of KDR/flk-1 autophosphorylation and activation of multiple signal transduction pathways (8–10). In contrast with our increasing understanding of signaling events downstream of KDR/flk-1, there remains a paucity of information regarding the upstream events regulating the expression of this receptor during endothelial cell development and in angiogenesis.

Given the importance of KDR/flk-1 in endothelial cell biology, we have cloned the entire human KDR/flk-1 gene (11) and have sought to define the mechanisms governing its transcriptional regulation. The human KDR/flk-1 gene is TATA-less and has a core promoter containing tandemly repeated GC boxes (12). We have shown that ubiquitously expressed Sp1 binds these sites only in endothelial cells through a mechanism that involves changes in nucleosome positioning (13). The identity of nuclear factors that maintain this chromatin structure, and hence endothelial cell-restricted expression, remains elusive. Likewise, we do not yet understand how transcriptional complexes are assembled at this promoter. Even though the KDR/flk-1 promoter lacks a TATA box and contains GC-rich regions (a promoter structure that frequently produces transcripts initiated at multiple, poorly defined nucleotides), ribonuclease protection and primer extension assays have shown that the gene contains only one transcription start site (12). The mechanisms by which assembly of transcription complexes occurs at such a promoter are not completely understood.

Transcription initiation is a complex process requiring the assembly of many transcription factors on the promoter. In general, two methods exist by which basal transcription initiates from a defined site. Most commonly, mammalian protein-encoding genes contain a TATA box, which recruits TFIIID to the promoter and positions the transcription start site at a 25–30-bp downstream nucleotide (14). Less frequently, genes contain a so-called initiator element (Inr), which overlaps the transcription start site and positions the basal transcription machinery (15); many genes in the latter group, KDR/flk-1 included, lack a TATA-like upstream sequence altogether. A loose consensus sequence for Inr-dependent transcription,
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YAY[1]NTYY, has been defined (16), although exceptions to this rule have been reported (17). The mechanisms whereby Inr-mediated basal transcription occurs are at present incompletely understood, although it is clear that initiation complex formation and transcription-associated factor requirements differ between Inr- and TATA-mediated transcription (18, 19); indeed, the functional consequences of a requirement for an Inr for accurate transcription initiation are also not known, since so few Inr-dependent promoters have been adequately characterized (including TFII-I, YY1, and USF (20–23)), raising the possibility that different Inr-binding proteins regulate dependent transcription under different circumstances.

Based on our observation that the human KDR/flk-1 gene is TATA-less yet has a single transcription start site, we hypothesized that KDR/flk-1 contains a functional Inr responsible for transcription initiation, even though the KDR/flk-1 transcription start site sequence corresponds poorly to the Inr consensus sequence. In the present study, we demonstrate in vitro transcription assays that the sequence from −10 to +10 in the human KDR/flk-1 promoter contains Inr activity and that Inr activity is orientation- and position-dependent. Moreover, mutation of this element in the context of the native KDR/flk-1 promoter dramatically decreases promoter activity in vivo in transient transfection assays. This sequence has specific nuclear protein binding activity and is bound by the multi-functional transcription factor TFII-I present in nuclear extracts or by purified TFII-I. Furthermore, we show that TFII-I transactivates the KDR/flk-1 promoter in vivo and that TFII-I is necessary for transcription initiation in vitro. These studies demonstrate the importance of the Inr in KDR/flk-1 transcription and support a role for TFII-I in the regulation of this gene and hence in the developmental processes mediated by KDR/flk-1.

EXPERIMENTAL PROCEDURES

In Vitro Transcription Assays—For in vitro transcription, the reaction contained 300 ng of DNA, 50 mM KCl, 10 mM MgCl2, 0.5 mM dithiothreitol, 1 mM g/ml streptomycin, and grown as monolayers in Dulbecco's modified Eagle's medium with 10% fetal calf serum and 600 g/ml heparin, 100 units/ml penicillin, and 100 g/ml streptomycin. Nuclear extracts were prepared as described (27).

Transient Transfections—BREC from passages 4–8 were used for transient transfection experiments. BREC were used for transient transfection experiments rather than HUVEC because they can be reproducibly transfected with higher efficiency. Cells were grown to 40–60% confluence in 6-well plates and then transfected using Lipofectin (Life Technologies, Inc.), as described (28). The appropriate reporter plasmid (2 g) was transfected with 0.8 g of the secreted alkaline phosphatase expression vector pSEAP2 (CLONTECH) to correct for transfection efficiency. To determine the effect of TFII-I on reporter gene activity, 1 g of pEBG-TFII-I (25), containing the TFII-I cDNA in the expression plasmid pEBG, or pEBG alone, were cotransfected with the reporter plasmid and pSEAP2. (pEBG and pEBG-TFII-I were provided by Ananda Roy.) Extracts were prepared 48 h after transfection by a detergent lysis method (Promega). Luciferase activity was measured in duplicate for all samples with a Packard Top-count scintillation counter and the Promega Luciferase Assay system. The ratio of luciferase activity to alkaline phosphatase activity in each sample served as a measure of relative luciferase activity. All constructs were tested in at least four independent transfection experiments, and results are expressed as the mean ± S.E. Relative luciferase activity among experiments was compared by a factorial analysis of variance followed by Fisher's least significant difference test. Statistical significance was accepted at p < 0.05.

Electrophoresis Mobility Shift Assays (EMSA)—EMSA was performed as described (13). The probe consisted of annealed synthetic 20-mer complementary oligonucleotides corresponding to bp −10 to +10 of the human endothelin-1 promoter (12). A typical binding reaction contained 50,000 cpn DNA probe, 0.5 g of poly(dIdC)-poly(dIdC), 10 mM Tris-HCl (pH 7.5), 50 mM NaCl, 5 mM MgCl2, 0.5 mM dithiothreitol, 5% glycerol, and 5 g of nuclear extract in a final volume of 25 l. In reactions which produced specific TFII-I was used in place of nuclear extract, 0.1 g of poly(dIdC)-poly(dIdC) was used. The reaction mixture was incubated at room temperature for 20 min and fractionated on a 5% native polyacrylamide gel in 0.5 TBE buffer. To determine the specificity of the DNA-protein complexes, we performed competition assays using a molar excess of the unlabeled double-stranded KDR/flk-1 oligonucleotide (specific inhibitor) or excess of an unrelated double-stranded oligonucleotide of comparable length (nonspecific inhibitor) or the TdT Inr (15). To characterize specific DNA-binding proteins, we incubated nuclear extracts with anti-TFII-I or anti-YY1 antibody (Santa Cruz Biotechnology) for 3 h at 4 °C before adding probe.

RESULTS

Sequence Analysis of the KDR/flk-1 Transcription Start Site—We have previously cloned and sequenced the mouse and
human KDR/flk-1 5'-flanking regions and have characterized the proximal human KDR/flk-1 promoter in detail (12, 13). The promoters for both species are TATA-less and are characterized by the presence of multiple GC boxes. Functional characterization of the human promoter demonstrates that four tandemly repeated GC boxes are bound by Sp1 in endothelial but not in non-endothelial cells and that these GC boxes lie 39 bp upstream of a single transcription start site at the A residue of a 5'-CA-3' dinucleotide pair (Fig. 1A). The presence of a single transcription start site in a TATA-less gene led us to hypothesize that KDR/flk-1 should contain a functional Inr. Comparison of the region from -10 to +10 in the human 5'-flanking sequence with the analogous sequence in the mouse promoter (12, 29) demonstrates that there is a high degree of conservation across species (Fig. 1B). Remarkably, the A residue at +1 in the human promoter is substituted with a C residue in the mouse promoter, whereas the 8 residues downstream of +1 are identical. The human transcription start site sequence meets the minimal Inr criteria by virtue of having an A at +1 and a T at +3, since these residues are the most critical for determining the level of Inr activity (16). However, these residues, although in a relatively pyrimidine-rich context, are not accompanied by a sequence meeting the consensus for Inr activity, YYA+NTTY, which has been determined empirically (16) and by comparison with other well characterized Inrs (15, 21, 30).

**The Human KDR/flk-1 Promoter Contains a Functional Inr—**To address whether the sequence surrounding the KDR/flk-1 transcription start site contains a functional Inr, we measured its effect on transcript initiation in vitro using a soluble cell-free system (15). The plasmid pSp1, which contains two 21-bp GC box repeats but no TATA box upstream of its multiple cloning site, was used in these assays. Transcription from pSp1 is dependent on the insertion of sequences with functional Inr activity. We synthesized oligonucleotides from -10 to +10 (5'-CCCACCGTTCGACTGACCC-3') and inserted the annealed oligonucleotides into the EcoRI and SmaI sites of vector pSp1 in two orientations (Fig. 2A). The recombinant plasmids were named according to the insertion sites and orientation. Plasmid pSp1/TdT, containing the TdT Inr, was used for comparison as a positive control.

We were able to detect pSp1 transcripts by in vitro transcription, and we mapped the transcription start sites by primer extension. (We used HeLa nuclear extract in these experiments because we were not able to consistently produce nuclear extracts from primary culture endothelial cells with sufficient transcriptional activity. As mentioned below, binding activity to the KDR/flk-1 Inr is broadly expressed, and therefore studies from cell types such as HeLa should be relevant to the analysis of this promoter under these circumstances.) As expected, the TdT Inr directed transcription predominantly from a single start site (lane 1, Fig. 2B), whereas in the absence of the Inr no transcription occurred (lane 1). Plasmid pSp1-KDRE (+), containing the sequence -10 to +10 in the forward orientation with the transcription start site located 38 bp downstream of the last GC repeat (similar to the distance from the GC boxes in the endogenous KDR/flk-1 promoter), was able to initiate transcription from a single site. This site corresponded to the +1 nucleotide of the endogenous KDR/flk-1 promoter, producing a fragment slightly larger than that produced by pSp1/TdT after gel electrophoresis (lane 3). Most interestingly, transcription initiated from this site rather than from 5'-CA-3' dinucleotides, produced in the cloning process, located 7 bp upstream or 9 bp downstream (Fig. 2A). If the transcription start site was situated 52 bp downstream of the GC boxes (plasmid pSp1-KDRE (-), lane 4) transcription still occurred; however, the ability to initiate from a single site was lost. If the oligonucleotide was inserted in the reverse orientation relative to the GC boxes, either 38 bp (pSp1-KDRE (-)) or 52 bp (pSp1-KDRE (-)) downstream, no appreciable transcription was evident (lanes 5 and 6). In every case, primer extension
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Characterization of the KDR/flk-1 Inr by Mutagenesis—Having identified a functional Inr in the KDR/flk-1 gene, we sought to determine the nucleotides necessary for Inr activity, with the expectation that these experiments might help to understand why the KDR/flk-1 Inr departs from the typical Inr consensus sequence. A series of constructs were created by introducing mutations between bps −5 to +5 in the Inr in plasmid pSp1-KDRE (+) (Fig. 3A). Introduction of two point mutations at −5 and −4 (both from C to A) to create plasmid pSp1-KDRE (+)-M1 did not affect Inr activity (Fig. 3B, lane 3) compared with the activity of pSp1-KDRE (+) (lane 2). Mutation of residues −3 and −2 (from T to G and G to T, respectively, to create pSp1-KDRE (+)-M2) consistently increased Inr activity modestly (lane 4), an effect that may be explained by introducing a pyrimidine at position −2 and therefore establishing concordance with the Inr consensus at this position (16). Surprisingly, mutations at positions −1 and +1 (from C to A and A to C, respectively, pSp1-KDRE (+)-M3), which increase divergence from the consensus sequence, also increased the level of transcription slightly and did not change the start site position (lane 5). As discussed below, this mutation provides one line of evidence indicating that KDR/flk-1 Inr activity is not simply the result of a 5′-CA-3′ nucleation event. Mutations at positions +2 and +3 (from C to A and T to G, respectively; pSp1-KDRE (+)-M4) not only decreased transcription but also influenced transcription start site usage (lane 6); this was to be expected by removing the two pyrimidines at these positions. We had anticipated that changing residues +4 and +5 to pyrimidines (from G to T and A to C, respectively, pSp1-KDRE (+)-M5) would enhance Inr activity by improving homology with the Inr consensus at these positions. Surprisingly, both the rate of transcription and start site selectivity were decreased by these changes. The significance of this result is discussed below. Finally, complete corruption of the core Inr sequence from positions −1 to +3 (from CACT to GTGC, pSp1-KDRE (+)-M6) resulted in loss of all Inr activity in the assay (lane 8). Since this last mutation had the most profound effect on Inr activity, we utilized it as a test of the role of Inr activity in in vitro assays of transcriptional activity.

Effect of Inr Disruption on KDR/flk-1 Promoter Activity in in Vivo Transient Transfection Assays—The transcription assays discussed above demonstrate that the sequences spanning the human KDR/flk-1 transcription start site increase initiation in vitro. In order to demonstrate a functional role for these sequences in vivo, in the context of the native promoter, we compared mutant and wild-type KDR/flk-1 Inr sequences in transfection assays. We created the luciferase reporter construct pGL2INR-MUT by incorporating the nonfunctional mutations (CACT to GTGC between −1 to +3) into the luciferase reporter plasmid pGL2−225+268; this plasmid contains a fragment of the human KDR/flk-1 promoter from −225 to +268, which is the smallest fragment of this promoter with complete activity in transient transfection assays (12). Reporter constructs were transiently transfected into BAEC, and luciferase activity was measured. Mutagenesis of the KDR/flk-1 Inr significantly reduced promoter activity to 28 ± 5.5% of pGL2−225+268 (p < 0.05, data not shown; see also Fig. 5). These results are consistent with previous reports demonstrating that mutation of functional Inrs reduces, but does not abolish, transcription in transient transfection assays (16, 22, 25), and they indicate that the wild-type KDR/flk-1 Inr sequence is required for maximal transcriptional activity of the KDR/flk-1 promoter in vivo, as it is in vitro.

Identification of Nuclear Protein Complexes Interacting with the KDR/flk-1 Inr—A variety of nuclear proteins, including USF, YY1, TFII-I, and members of the transcription-associated factor family, have been shown to interact directly with Inrs of various genes (reviewed in Ref. 19). As a first step to determine whether specific nuclear proteins interact with the KDR/flk-1 Inr, we examined whether primary culture HUVEC contain a binding activity that will associate with the fragment from −10 to +10 by EMSA, since HUVECs are known to express KDR/flk-1 mRNA and protein at high levels (12, 31). Incubation of the labeled Inr fragment with nuclear extract resulted in the formation of two retarded bands, A and B (Fig. 4A). Formation of both complexes was efficiently abolished by as little as 10-fold molar excess of unlabeled Inr fragment. Complex B was partially competed by an unlabeled nonspecific competitor, whereas complex A was not efficiently competed. We should note that the binding activity of complex B was variably present in HUVEC extracts (Fig. 4C and data not shown). On the basis of this finding and the competition experiments, we favor the interpretation that this complex represents nonspecific binding to the Inr; however, we cannot exclude the possibility that it represents a specific complex with lower affinity for the
TFII-I in the presence of a specific TFII-I antibody in order to compare complexes produced by unfractionated nuclear extract and TFII-I alone. Components of the DNA-protein complexes formed were assayed by incubating nuclear extracts with antibodies (Ab) recognizing TFII-I or YY1 prior to binding reactions. The specificity of complex A formation (denoted by an arrow) was tested by addition of unlabeled specific (Sp) or nonspecific (NS) oligonucleotides or with an oligonucleotide encoding the TdT Inr (TdT). Components of the DNA-protein complexes formed were assayed by incubating nuclear extracts with antibodies (Ab) recognizing TFII-I or YY1 prior to binding reactions. C, the KDR/flk-1 Inr was incubated with either HUVEC nuclear extract (NE), with affinity purified TFII-I, or with TFII-I in the presence of a specific TFII-I antibody in order to compare complexes produced by unfractionated nuclear extract and TFII-I alone.

Inr in comparison with complex A. Similar retarded bands were also observed when EMSA was performed with nuclear extracts from a variety of nonendothelial cell types, indicating that the protein or proteins involved in their formation are broadly expressed among cell types.

In an effort to identify proteins that participate in complex A, we assayed for the presence of known Inr-binding proteins. Our initial experiments in this regard focused on well described Inr-binding proteins, TFII-I and YY1 (20, 32). We first tested the ability of unlabeled fragments to compete away the formation of complex A (Fig. 4B). As shown previously, complex A could be competed by 10-fold excess of a specific but not 100-fold excess of a nonspecific competitor. This complex could also be efficiently competed by 10-fold excess of the TdT Inr, which can compete for binding by TFII-I in these assays (21), although this Inr may bind and compete for other general transcription factors as well. By comparison, a YY1 element did not compete for formation of complex A (not shown). As a more precise test of the components in complex A, we preincubated nuclear extracts with specific antibodies prior to binding reactions. When nuclear extract was incubated with an anti-TFII-I antibody (which is known to block TFII-I binding without producing a supershifted band (30)) before the binding reaction, formation of the specific DNA-protein complex A was significantly reduced, instead enhancing formation of complex B. In contrast, anti-YY1 antibody had no effect on complex A formation (Fig. 4B) nor did preimmune serum (data not shown).

The competition and immunodepletion experiments strongly suggested that TFII-I was present in HUVEC nuclear extracts and participated in nuclear protein complex formation with the KDR/flk-1 Inr. Consistent with these observations, we detected TFII-I mRNA and protein expression in HUVEC (not shown). To prove more definitively that TFII-I bound the KDR/flk-1 Inr, we examined the interaction of purified native TFII-I to the KDR/flk-1 Inr, and we compared complex formation with that of unfractionated HUVEC nuclear extract by EMSA (Fig. 4C). Under the conditions employed, a stable complex could form between TFII-I and the KDR/flk-1 Inr, which was identical in size to a complex formed by HUVEC nuclear extract alone. (The strong band migrating just above the probe in this experiment was variably present in our nuclear extract preparations and may represent a proteolytic fragment with DNA binding activity.) The TFII-I-Inr complex could be blocked by preincubation of purified native TFII-I with an anti-TFII-I antibody. These experiments do not determine the stoichiometry of TFII-I binding to the KDR/flk-1 Inr, nor do they preclude the possibility that other nuclear proteins may also interact with this sequence. However, taken together, these binding studies indicate that TFII-I participates in complex formation with the KDR/flk-1 Inr, and they provide a rationale for examining whether TFII-I regulates KDR/flk-1 promoter activity in vivo and Inr activity in vitro.

**TFII-I Specifically Transactivates the Human KDR/flk-1 Promoter in Transient Transfection Assays**—To demonstrate the possible role of TFII-I in activating the KDR/flk-1 promoter in vivo, the reporter plasmids pGL2 Promoter, pGL2-Et1, or pGL2—225—268 were cotransfected into BAEC with either the eukaryotic expression plasmid pEBG-TFII-I or pEBG alone as a control (25). Cotransfection of TFII-I had no effect on activity of the SV40 promoter present in pGL2 Promoter nor on the endothelin-1 promoter (Fig. 5), another endothelial cell-restricted promoter (33). In contrast, TFII-I significantly increased KDR/flk-1 promoter activity by approximately 3-fold in comparison with empty vector (p < 0.05). This induction may actually underestimate the ability of TFII-I to transactivate this promoter, since activity of this construct is already very high (equivalent to the potent SV40 promoter plus enhancer (12)) and since endothelial cells express endogenous TFII-I constitutively (data not shown). Since TFII-I can bind to the KDR/flk-1 Inr, we asked whether an intact and functional Inr was necessary for TFII-I-mediated transactivation. Consistent with our previous experiments, mutation of the Inr resulted in a decrease in promoter activity to 28% of the wild-type vector. It is significant to note that this level, while low, was 2 orders of magnitude greater than the activity of the promoterless vector pGL2 Basic and therefore was well above the limits of detection for this assay. In contrast with its effect on the wild-type promoter, TFII-I did not transactivate a promoter with a mutated Inr. Whereas we cannot exclude the possibility that TFII-I interacts with other sequences within the KDR/flk-1 promoter (and, indeed, TFII-I has been shown to interact with E box sequences in addition to the Inr in the adenovirus major late (AdML) promoter (34)), these data indicate that an intact and functional Inr is necessary for KDR/flk-1 transactivation by TFII-I.

KDR/flk-1 Inr Activity Is Dependent on TFII-I—By having shown that TFII-I bound the KDR/flk-1 Inr and transactivated the promoter in vivo in an Inr-dependent fashion, we addressed whether TFII-I was necessary for the Inr activity observed in our in vitro studies. By using the in vitro transcription/prim
Fig. 5. Transactivation of the KDR/flk-1 Inr by TFII-I. The indicated reporter constructs (2 μg) and either a mammalian expression vector expressing TFII-I or the same vector alone (1 μg) were transfected into BAEC. After 48 h, transfected cells were lysed and luciferase activity was measured. Results were normalized to secreted alkaline phosphatase activity to correct for transfection efficiency and expressed as a percentage of pGL2−225+268 alone. *p < 0.05, compared with transfection with pEBG.

extension system, we examined the effect of immunodepletion of nuclear extracts with an anti-TFII-I antibody on Inr activity (Fig. 6). In comparison with reactions performed with untreated extracts (lane 2) or with extracts treated with preimmune serum (lane 5), extracts depleted of TFII-I prior to addition of template failed to support transcription (lane 3). Adding back purified native TFII-I to immunodepleted extracts prior to transcription rescued transcription, providing further evidence that this effect was due specifically to depletion of TFII-I (lane 4). This effect was similar to that observed with the TdT Inr (lanes 6–8). We cannot exclude the possibility that the TFII-I fraction used in these experiments, although purified to homogeneity as assessed by silver staining, contains one or more factors other than TFII-I that may account in part for the ability of this fraction to rescue transcript initiation after TFII-I immunodepletion in this assay. However, the results of these experiments are consistent with our in vivo observations that TFII-I regulates KDR/flk-1 transcriptional activity through the Inr.

DISCUSSION

In this report, we demonstrate that the gene encoding KDR/flk-1, a receptor for VEGF that is essential for vascular endothelial development and for endothelium-dependent processes such as angiogenesis, contains a functional Inr, although important differences exist between the nucleic acid sequence of this Inr and the well established general consensus Inr sequence. In addition, we demonstrate that the DNA-protein complexes that form on this Inr in EMSA experiments contain the Inr-binding protein TFII-I and that TFII-I, through interactions with the Inr sequence described here, is necessary for KDR/flk-1 promoter activity both in vitro and in vivo.

In order to define and characterize the Inr function of the KDR/flk-1 promoter, we have employed a well established in vitro transcription assay in which transcriptional activity is totally dependent on Inr activity (15). This system allows for the simultaneous analysis of transcriptional activity and start site selection. Based on several lines of evidence, the data in Fig. 2 support our hypothesis that KDR/flk-1 contains a functional Inr and that this assay is measuring specific Inr activity rather than, for instance, nonspecific nucleation of transcription by the introduction of a 5′-CA-3′ dinucleotide. First, transcription initiates from an A residue within the KDR/flk-1 Inr in plasmid pSp1-KDRE (+), rather than from 5′-CA-3′ sequences located immediately upstream or downstream (Fig. 2).

Second, disruption of the 5′-CA-3′ sequence in plasmid pSp1-KDRE (+)-M3 does not affect transcriptional activity or start site selection (Fig. 3). Third, insertion of 5′-CA-3′ in a nonspecific or purine-rich context, which occurs fortuitously in plasmids pSp1-KDRE (−) and pSp1-KDRS (−) (Fig. 2A; see also Ref. 16), does not support transcription in this assay. Therefore, we believe these data indicate the existence of a specific Inr function in the KDR/flk-1 promoter. Similar to other Inrs (24, 35), KDR/flk-1 Inr activity is orientation-dependent and inhibitable by α-amanitin. Transcript initiation is also influenced in our studies by Inr position in relation to upstream Sp1 sites. (Unfortunately, these positional effects prevent us from making quantitative comparisons between the activity of the KDR/flk-1 and TdT Inrs in our experiments, since the start sites are positioned differently in relation to upstream sequences.) In the case of KDR/flk-1, placing the Inr 52, rather than 38, bp downstream of Sp1 sites results in transcription initiating at multiple sites, although overall transcriptional activity seems preserved. Such findings have been reported for other Inrs (15) and suggest that, at least in this assay, the transcriptional activation and start site selection properties of an Inr may be separable and that start site selection may be more susceptible than transcriptional activation to minor perturbations in promoter structure. The necessity for, and the mechanisms for maintaining, a single start site remain enigmas in the general understanding of Inr function.

Perhaps the most striking feature of the KDR/flk-1 Inr is the difference in its nucleic acid sequence compared with the previously reported and well characterized consensus sequence for Inrs, YYA′−1NTYY (16). The human KDR/flk-1 gene differs from this degenerate consensus at 3 out of 6 sites (G at −2, G at +4, and A at +5, see Fig. 1). We have performed mutagenesis at each of these sites in an effort to gain insight into what differences, if any, exist between the KDR/flk-1 Inr and Inrs with more typical sequences (Fig. 3). A change in the G residue...
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TFII-I was originally purified (21) and subsequently cloned (22) based on its ability to interact with and support transcription from the AdML Inr. TFII-I is a 120-kDa, uniquely structured transcription factor containing six directly repeated 90-residue elements, each of which possesses potential helix-loop-helix protein-protein interaction motifs. A functional role for TFII-I has been demonstrated in transcription of the Inr-containing T cell receptor Vβ and AdML promoters (21, 25, 30, 32) through interactions with their Inr sequences, and TFII-I physically associates with the HIV-1, TdT, and ribonuclease I activity. R1 Inrs as well (17, 43). In addition to Inr binding activity, TFII-I can associate with the E box motif, CACGTT, and with serum response element sequences (44, 45). TFII-I interacts functionally with several transcription factors, including helix-loop-helix proteins USF (21) and Myc (34), homeodomain protein Phox 1 (44), MADS box protein serum response factor (44, 45), and STAT1 and STAT3 (45), in order to transactivate gene expression. In addition, TFII-I is bound and phosphorylated by Bruton’s tyrosine kinase (46) and is also phosphorylated in response to epidermal growth factor stimulation (45). These diverse activities suggest either that TFII-I has multiple independent molecular functions or that TFII-I integrates signaling pathways at both basal and regulatory levels to influence gene expression.

TFII-I has previously been shown to bind to at least three different types of DNA sequences: typical Inrs, E box motifs, and serum response elements (21, 34, 44, 45). Notably, the serum response elements with which TFII-I interacts overlap typical Inr motifs, although they do not have Inr function in vivo. In contrast, the E box motif bound by TFII-I, CACGTT, does not match the Inr consensus, and as pointed out above, the KDR/flk-1 Inr matches poorly with the Inr consensus. The nature of TFII-I-binding site selectivity is not well understood. On the basis of our results and those of others, it is possible that either TFII-I contains multiple DNA binding domains or that TFII-I-binding site selection is influenced by factors such as DNA structure that may not be readily apparent by analysis of primary nucleic acid sequence.

The precise role of Inr-binding proteins such as TFII-I in Inr function and transcriptional regulation is only recently being clarified. A persistent concern in previous analyses has been the possibility that these proteins are actually transcriptional activators with binding sites near or overlapping functional Inrs, rather than true Inr-binding proteins with the capacity for recruitment of the basal transcriptional machinery. In the case of YY1 and E2F, data suggest this may be the case (16). The close correlation between in vitro KDR/flk-1 Inr activity and TFII-I-mediated transcriptional activation in vivo, as well as the dependence of Inr activity on TFII-I in vitro argue against this possibility. At present, KDR/flk-1 is the only gene aside from the T cell receptor Vβ promoter for which both in vitro and in vivo requirements have been met for TFII-I Inr function (25).
Since TFII-I-independent pathways have also been described for transcription initiation (47), we favor the hypothesis that TFII-I mediates Inr function in a subset of Inr-containing genes, possibly as the result of common transcriptional requirements. One shared characteristic of the Vβ and KDR/flk-1 promoters is that they mediate exquisitely cell-type-specific, developmentally regulated expression of functionally indispensable proteins. Although not itself cell-type-specific, TFII-I may integrate signals from other nuclear proteins to maintain appropriate promoters is that they mediate exquisitely cell-type-specific, cell type-restricted transactivators, such as yet to be characterized homeodomain or MADS box proteins, to initiate transcription under the appropriate circumstances. In this regard, an understanding of the role of TFII-I in KDR/flk-1 gene expression may help to unravel the complexities of endothelial cell development and cell type-specific gene expression.

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