The R-ras gene encodes a small GTPase of the ras family that is closely related to H-ras and K-ras. Unlike the prototypic ras genes, the disruption of the R-ras gene in mice results in enhanced angiogenesis in tumor implants and sustained neointimal hyperplasia in response to arterial injury, indicating the in vivo role of R-ras as a negative regulator of vascular proliferation. R-ras is abundantly expressed in normal mature blood vessels but significantly down-regulated in pathologically regenerating vasculature. In this study, we investigated the roles of cis-acting elements in the transcription of the human R-ras gene, as well as the transcription factors that interact with these sequences in cultured endothelial cells and arterial smooth muscle cells. The findings from vascular cells were then compared with findings from epithelial tumor cells that aberrantly express R-ras. Deletion analyses on 5 kb of 5′-flanking DNA of the human R-ras gene revealed the functional importance of the region between −727/−476, which contains two Ets and one Sp1 consensus binding motifs. Mutation analyses of various consensus binding motifs within this region suggest both cell type-dependent and -independent regulatory mechanisms for the R-ras gene transcription. Electrophoretic mobility shift and antibody disruption assays demonstrated that an Ets transcription factor family protein, GA-binding protein (GABP), binds to the R-ras-derived sequence. Chromatin immunoprecipitation analyses determined the association of endogenous GABP as well as Sp3 proteins with the −727/−476 region of the R-ras promoter in intact cells grown in culture. Forced expression of GABP significantly enhanced R-ras mRNA expression level in endothelial cells. These results map the functional elements in the R-ras promoter sequence and suggest that the GABP may be critical for transcription of R-ras and for maintenance of normal blood vessel functions through the regulation of this gene.

R-Ras is a small GTPase of the ras family that was originally identified as a close homolog of the oncogene product v-H-Ras
RT²-PCR analyses found R-ras mRNA most abundantly in well vascularized tissues such as the lung and in smooth muscle-rich tissues such as the aorta and intestines. Low levels of R-ras mRNA were found in other tissues such as the brain. Also consistent with the immunostaining observations (10), no R-ras mRNA was detected in the epithelium of normal gastric mucosa by RT-PCR, following laser-captured microdissections of the three regions of gastric mucosa, i.e. pit, isthmus, and gland (11). In comparison, H-ras mRNA was readily detected in all three regions (11).

The abnormal up-regulation of R-ras in epithelial cell type has been reported in gastric cancer (11). The expression was detected in about half of the primary gastric tumors tested, indicating the aberrant R-ras gene induction in these tumors (11). The short interfering RNA knockdown of R-ras in these tumor cells in culture leads to significant cell death (11), suggesting that these epithelial tumor cells have adapted to be dependent on the R-Ras-mediated pro-survival pathway (3, 10). The National Institutes of Health data base of serial analysis of gene expression indicates the up-regulation of R-ras in some cancer cell lines, including those of epithelial origin (Cancer Genome Anatomy Project, NCI; cgiap.nci.nih.gov).

In the vasculature, the spatiotemporal pattern of R-Ras protein expression correlates with maturation of blood vessels and differentiation of the vascular cells; little expression is detected in the developmentally growing or pathologically regenerating vessels (e.g. hyperplastic neointimal lesions), whereas abundant expression is found in mature functional vasculatures of healthy adult tissues (10). Such an expression pattern is consistent with the inhibitory role of R-Ras in vascular proliferation. On the contrary, R-ras mRNA expression was found significantly suppressed in the endothelium of malignant breast carcinoma as well as in hemangioma (benign hyperplasia of endothelial cells) in previously reported gene expression profiling studies in humans (12). Therefore, R-ras expression is differentially regulated in the normal and disease-associated phenotypes of endothelial cells and vascular smooth muscle cells. Despite its potential significance in vascular and cardiovascular diseases, little is known about the regulatory mechanism of the R-ras gene expression at present.

In this study, we have begun to explore the molecular mechanism of transcriptional regulation of the human R-ras gene by identifying cis-regulatory elements in the 5’-flanking sequence and analyzing their activities in endothelial cells and arterial smooth muscle cells. The studies were also conducted in an ovarian cancer cell line that aberrantly expresses R-ras to gain insight into the pathologically induced R-ras expression in epithelial tumors. Furthermore, we investigated nucleoprotein interactions with the critical cis-regulatory elements as potential trans-regulatory mechanisms of the R-ras gene expression. These studies unveiled the significance of the Ets and Sp1 consensus motifs in the promoter region −727 to −476 and the interactions of an Ets family member, GA-binding protein (GABP), and Sp3 with this region of the R-ras promoter.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—Human umbilical vein endothelial cells (HUVEC) and human coronary artery smooth muscle cells were purchased from Lonza Inc. (Allendale, NJ). They were grown in EGM-2 and SmGM-2 growth medium (Lonza), respectively. The human ovarian carcinoma cell line, A2780 cells (Sigma), was grown in RPMI 1640 medium supplemented with 10% fetal bovine serum. Cells were incubated at 37 °C in a 5% CO₂ atmosphere.

**In Silico Sequence Analysis**—Human genomic DNA sequences were downloaded from the NCBI data base. Possible transcription factor-binding sites were predicated on genomic DNA sequences using the TFsearch program with a threshold of 85 and Vertebrate Matrix. CpG islands were predicted by the CpG Plot program in the R-ras genomic sequence between 3 kb upstream to 1 kb downstream from the main transcription start site. Observed/expected ratio was >0.6; percent C + percent G was >60.00, and length was >200.

**5’-Rapid Amplification of cDNA Ends (RACE)**—The 5’-end of R-Ras cDNA was amplified from human prostate RACE-Ready cDNA (Ambion) using an R-ras-specific primer, R-Ras-GSP-2 (GGACAGCCC GGACAGCTGCTA) and the 5’-RACE Out primer (Ambion). The first PCR product was further amplified with the R-Ras-nested primer (CAGGAAG TTAGGACTGGATGAACTGGAT) and the 5’-RACE inner primer (Ambion). The second PCR products were gel-purified and subcloned into the pCR-4-TOPO vector (Invitrogen) and sequenced to determine the 5’-end of the R-Ras transcript.

**Preparation of the Reporter Constructs**—Human genomic DNA was extracted from HUVEC cells using the phenol extraction. The fragments of different lengths in the 5’-flanking region of the human R-ras gene were amplified by PCR (see Table 1 for PCR primer sequences) using the human genomic DNA or cloned 5’-flanking DNA as templates. The PCR was performed using pfu DNA polymerase as follows: 95 °C for 2 min, followed by 35 cycles of 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 0.5–5 min, with a final extension at 72 °C for 7 min. The PCR products were gel-purified, digested with KpnI and XhoI, and subcloned into the pGL3-basic firefly luciferase vector (Promega). The sequences of cloned promoter regions were confirmed by DNA sequencing. To construct the dP promoter fragment, which deletes the segment between −756 and −404 from the P7 construct, two DNA fragments were amplified separately using the primers F and L-R3, and L-CR and Hs-R, respectively (see Table 1 for primer sequences). Then the two PCR products were mixed, and the dP fragments were amplified using the primers F and Hs-R.

**Antibodies**—Rabbit anti-Ets1, Elk1, GABPα, Sp1, and Sp3 polyclonal antibodies and normal rabbit IgG were purchased from Santa Cruz Biotechnology, Inc.

**Generation of Mutations in the R-ras Promoter**—Site-directed mutagenesis was carried out in the putative transcription factor-binding sites within the −727/−476 DNA fragment using the QuikChange XL site-directed mutagenesis kit (Strat-
Regulation of Human R-ras Gene Promoter

**TABLE 1**

Primer and probe sequences

Note the lowercase letters indicate mutated nucleotides.

| 5'-Deletion |  |
|---|---|
| Hs-R | CCGCTCAGTGCTCCACCGCCTGCTCCTGCTCCTGTTAG |
| Hs-F1 | CTCCTGTCAGCAGGCAAGACCGAGCAGAGAC |
| Hs-F2 | CTCCCTGTCAGCAGGCAAGACCGAGCAGAGAC |
| Hs-F3 | CTCCCTGTCAGCAGGCAAGACCGAGCAGAGAC |
| Hs-F4 | CTCCCTGTCAGCAGGCAAGACCGAGCAGAGAC |
| Hs-F5 | CTCCCTGTCAGCAGGCAAGACCGAGCAGAGAC |
| Hs-F6 | CTCCCTGTCAGCAGGCAAGACCGAGCAGAGAC |
| Hs-F7 | CTCCCTGTCAGCAGGCAAGACCGAGCAGAGAC |
| Hs-F8 | CTCCCTGTCAGCAGGCAAGACCGAGCAGAGAC |
| Hs-F9 | CTCCCTGTCAGCAGGCAAGACCGAGCAGAGAC |
| Hs-F10 | CTCCCTGTCAGCAGGCAAGACCGAGCAGAGAC |

| 3'-Deletion |  |
|---|---|
| Forward | CGATGATACCTTCTGATAGAAGAGGAGGAAAGTCTCAGAGCATC |
| R-CCR | CCTTCTCCCTTCTCTACAGATAGAAAGGAGGAAAGGAC |
| R-RACE | CGATGATACCTTCTGATAGAAGAGGAGGAAAGGAC |
| Bcl-Xp1-R | GCTTCTGATAGAAGAGGAAAGGAC |
| Bcl-Xp1-R | GCTTCTGATAGAAGAGGAAAGGAC |

| Internal deletion |  |
|---|---|
| L-R3 | CGATGATACCTTCTGATAGAAGAGGAAAGGAC |
| R-C | CCTTCTCCCTTCTCTACAGATAGAAAGGAGGAAAGGAC |

| Primers for 5'-RACE |  |
|---|---|
| GP-2 | GAAGAAGCGCGAGCGAGGAC |
| GP-nest | CGATGATACCTTCTGATAGAAGAGGAAAGGAC |

| Primers for ChIP |  |
|---|---|
| R-Ras ChIP-R | TCCACATCTGCGCTGCTGCTG |
| R-Ras ChIP-R | TCCACATCTGCGCTGCTGCTG |
| Bcl-Xp1-R | GCTTCTGATAGAAGAGGAAAGGAC |
| Bcl-Xp1-R | GCTTCTGATAGAAGAGGAAAGGAC |

| Primers for site-directed mutagenesis |  |
|---|---|
| EBSF-R | CTAGAAGCGCGAGCGAGGAC |
| EBS1-R | CTAGAAGCGCGAGCGAGGAC |
| EBS2-R | CTAGAAGCGCGAGCGAGGAC |
| EBS3-R | CTAGAAGCGCGAGCGAGGAC |
| EBS4-R | CTAGAAGCGCGAGCGAGGAC |

| Probes for EMSA |  |
|---|---|
| EBS sense | CATGGTACCGTCCGGACGCAAGAGGAC |
| EBS antisense | GCAGGAGCAGGAGAAAGTCTCAGAGCAT |
| EBS mutant sense | CGATGATACCTTCTGATAGAAGAGGAAAGGAC |

| ChIP Assay |  |
|---|---|
| Chromatin immunoprecipitation assays were performed according to the reported method (13), with some modifications. Briefly, cells were grown to more than 90% confluence in 100-mm dishes, fixed with 1% formaldehyde for 10 min at room temperature, washed with 125 mM saline (PBS) and lysed with the 1× PLB buffer (Promega) 48 h after transfection. Luciferase activities were measured with the dual luciferase assay kit (Promega). The normalized luciferase activity was expressed as the ratio of firefly luciferase activity to Renilla luciferase for each sample. Each transfection was done in triplicate, and at least three independent experiments were performed for each construct. |
plasmids by PCR (primers for GABP

was used to generate cDNA using RETROscript kit (Ambion). Real-time PCR was performed using iQ SYBR Green Supermix (Bio-Rad) with an R-Ras primer set (forward ACCATCCAGTTCACTGAGTTCTCAG and reverse GCTTGCCCACCTGTTGAAAC). PPIA was used as a reference gene for standardizing Q-PCR (forward CCCACGGTTGCTTCCTGGACAT and reverse CCGAGCTCAGACGAGCAA).

Western Blot—The cultured cells were lysed in RIPA buffer (50 mM Tris-Cl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 2 mM NaF, 1 × protease inhibitor cocktails (Sigma)). The proteins were separated by 10% SDS-PAGE and transferred onto nitrocellulose membrane. The transformed membrane was blocked for 1 h and incubated with indicated antibodies at 4 °C overnight. The membrane was washed three times with TBST for 10 min and incubated with 1:5000 diluted anti-rabbit or -mouse IgG-horseradish peroxidase (Promega) at room temperature for 1 h. The protein bands were detected using Western Lightning™ chemiluminescence reagent (PerkinElmer Life Sciences).

RESULTS


determination of R-Ras Gene Transcription Start Site—As a first step to identify the R-ras promoter, the transcription start site (TSS) of the gene was identified using an RNA ligase-mediated rapid amplification of cDNA ends method (14). This method enables amplification of cDNA only from the full-length, 5'-capped mRNA, avoiding amplification of incomplete cDNAs; therefore, the identified 5'-cDNA end will accurately represent the transcription start site of the gene. PCR amplification of human prostate RACE-ready cDNA produced DNA fragments of ~250 bp (Fig. 1A), which were cloned into pCR4-TOPO vector and sequenced. The results of sequencing 28 PCR clones indicated that there are multiple TSSs, suggesting dispersed initiation of R-ras gene transcription. The TSSs distributed between −46 and −18 bp relative to the transcription start site (ATG), with the most frequent initiation of transcription at −23 bp (Fig. 1B). For convenience, we define this site as the main transcription start site for the rest of this report. The classic basal promoter elements, TATA and CCAAT boxes, are not found in the proximal region of the putative promoter sequence. Many TATA-less promoters have been shown to initiate transcription at multiple sites (15). Such dispersed initiation typically occurs in CpG islands, GC-rich stretches of DNA that are resistant to methylation. These sequences tend to be associated with genes that are constitutively on. Accordingly, CpG islands are found in the promoter regions of most mammalian housekeeping genes (15). A sequence analysis with CpG Plot program (available on line) from 3 kb upstream to 1 kb downstream R-ras TSS indicated the presence of putative CpG islands between bases −350 and +150 and bases −2,400 and −1,750. The former confirmed the previous report identifying a CpG stretch surrounding the exon 1 (11). The −350 to +150 CpG island identified was shorter than the one described in the previous report; nonetheless, all of the observed TSSs are located in this island (Fig. 1C). The identification of the housekeeping gene-like basal promoter structure suggests that the specific regulation of R-ras transcription may be dependent
mainly on distal cis-acting elements and their associating transcription factors.

Identification of cis Regulatory Elements in the Human R-ras Promoter—To identify regulatory elements in the R-ras promoter region, we cloned 5’-flanking region upstream the translation start site of the R-ras gene. Total genomic DNA was extracted from cultured human umbilical cord vein endothelial cells (HUVEC) and used as template for generating a series of R-ras promoter fragments, initiating at various upstream positions (between −4924 and −246) and terminating just before the translation start site (position +23) by PCR amplification (Fig. 2). These fragments were inserted into the promoterless pGL3-basic vector, upstream of the luciferase reporter gene to generate promoter-reporter constructs. To determine the functional significance of regulatory domains of the promoter region and their activities in endothelial cells, these constructs were transfected into primary cultures of HUVEC, and the reporter luciferase activities were determined for each deletion promoter constructs.

The results of these analyses are shown in Fig. 2. The shortest clone, clone P1 (−246/+23) produced 15-fold higher luciferase activity than the promoterless control; however, this activity was only 26% of maximum activity exhibited by P5 or P6 clones. The second shortest clone, clone P2 (−475/+23), which extends −0.5 kb upstream from the main TSS, showed only a slight increase in the luciferase activity compared with the clone P1. These observations suggest that the activity of this 0.5-kb-long 5’-flanking DNA fragment is mainly attributed to the basal promoter activity. In contrast, the addition of the next 252 upstream bases drastically increased the basal promoter activity as shown by the clone P3 (−727/+23) producing 450% increase from the activity of the clone P1. This result demonstrated that critical positive regulatory elements are located in the −727/−476 region. Another region that substantially increases the promoter activity was found between −1455 and −1123, which conferred −70% activity increase to P5 over P4. Constructs P6 gave similar results suggesting no regulatory elements between −1455 and −1907. Both P5 and P6 produced maximal promoter activity of about 60-fold greater than the promoterless construct. Progressively decreasing activities from the P7 through P10 constructs suggest that the upstream sequence (−4924/−1908) contain multiple cis-acting elements that negatively regulate R-ras expression in endothelial cells. These results indicate that there are several regions in the R-ras 5’-flanking DNA sequence that are important for transcription in HUVEC as follows: the proximal (basal) region (−237/+23), the −727/−476 region, the −1455/−1123 region, and multiple repressor regions between −4929 and −1908.

To further characterize the proximal promoter and −727/−476 regions, we generated additional deletion constructs, RP1, dP, and RP3 (Fig. 2). The deletion of −237/+23 fragment from the clone P7 (clone RP1), which removes the basal promoter region, resulted in a significant (65%) decrease in the luciferase activity compared with the clone P7. However, this deletion did not abolish the P7 activity, reflecting the activities of positive regulatory element(s) located within −727/−476 and −1455/−1123 regions. Consistent with this observation, the internal deletion of the 350-bp DNA fragment between −756 and −404 (clone dP) reduced the promoter activity by 76%, although this clone contained the rest of the upstream
elements and the basal promoter. The deletion of this DNA segment and the basal promoter resulted in a complete silencing of the R-ras promoter. These results demonstrated the functional significance of the two DNA fragments, −727/−476 and −1455/−1123, for the R-ras gene induction in endothelial cells.

Next, we examined the same set of promoter-reporter constructs in cultured human CASMC to investigate the R-ras promoter regulation in these cells. Similar to the observation in endothelial cells, the P5 and P6 clones exhibited highest luciferase activities among all constructs in CASMC (Fig. 2). The difference between P6 and P7 was not statistically significant (p = 0.3), and P7 appears to maintain the maximum activity in arterial smooth muscle cells, in contrast to the decreased activity in endothelial cells, suggesting that the repressor activity of the −2417/−1908 fragment is not present in the CASMC (Fig. 2). However, there were decreasing activities from P7 to P10, suggesting that the upstream sequence (−4924/−2418) contains multiple negative regulators for the R-ras expression. The suppression of the promoter activity by this region was common to HUVEC and CASMC (Fig. 2).

As in HUVEC, the proximal −727/−476 region is an important region for the R-ras promoter activity in CASMC. The deletion of this region (clone dP) significantly decreased the P7 promoter activity (Fig. 2). Furthermore, the deletion of both this region and the basal promoter (clone RP3) abolished the R-ras promoter activity. The combined observations strongly suggest the functional importance of the −727/−476 DNA fragment for R-ras gene expression in both vascular endothelial and smooth muscle cells.

The −1122/−728 region showed a greater impact on transcription in CASMC than in HUVEC cells, whereas the activity of the −1455/−1123 region was more significant in HUVEC, indicating that these regions contain cis-regulatory elements that act differentially in the two vascular cell types.

Universal Requirement of the −727/−476 Fragment for R-ras Promoter Activity—The R-ras mRNA expression has been found to be up-regulated in some epithelial tumor cells (11). Therefore, we set out to determine whether transcription in these cells involves the same elements used in the normal vascular or involvement of a totally distinct mechanism. The ovarian adenocarcinoma A2780 cells express R-Ras protein at levels readily detectable by immunoblot analysis (not shown). To determine the R-ras promoter activity in epithelial tumor cells, we tested the deletion constructs in A2780 cells (Fig. 3A). As in the HUVEC and CASMC, the −727/−476 region was found to be critical for transcription in these carcinoma cells. Interestingly, the −1455/−728 region, which is responsible for more than 50% of the maximum observed R-ras promoter activities in normal vascular cells, exhibited no increase in transcription. Instead, the −1907/−1456 region, which had little or no effect in HUVEC and CASMC, was found to be important for the transcriptional activation in these tumor cells. These observations suggest that different set of cis-acting upstream elements and their interacting transcription factors control R-ras gene transcription in normal vascular cells and the aberrant transcription in certain epithelial tumors.

Despite these differences, common regulatory mechanisms were seen in all cell types tested. The clone P3 demonstrated over 60% of the maximum activity exhibited by the clone P6 in A2780 cells. This activity was more than 8- and 3-fold higher than the activities of the P1 and P2 clones, respectively. Furthermore, the analyses with the deletion clones RP1, dP, and RP3 in A2780 cells demonstrated the crucial role of the −727/−476 fragment in activating the basal promoter activity as we demonstrated in endothelial cells and arterial smooth muscle cells; the internal deletion of the 350-bp DNA fragment in the clone dP (similar to fragment 1 deletion) reduced the promoter activity by 64%, and the combined deletion of this fragment and the basal promoter (RP3) resulted in nearly complete silencing of the R-ras promoter in A2780 cells (Fig. 3A). Taken together, our results suggest a universal requirement of the −727/−476 region for the R-ras gene induction. The cell type-dependent and -independent regulatory segments in the R-ras promoter region are illustrated in Fig. 3B.
Regulation of Human R-ras Gene Promoter

The P6 promoter by 50% in HUVEC (Fig. 5). The EBS2 mutation also reduced the P6 promoter activity to a similar level in these cells. The double (EBS1 + EBS2) mutation resulted in 60% reduction. Thus, the ablation of either EBS alone diminishes the promoter activity to a similar level as the double mutation, suggesting that both sites are required for maximal activity. In CASMC, the single mutations of either EBS reduced the P6 promoter activity only by 22–23% (Fig. 5). The fact that the combined effect of the two mutations was not far greater than the effect of the single mutations suggests the cooperation of these two EBS sites in CASMC. However, both single and double mutations in these cells showed only half the effects seen in HUVEC. These observations suggest that the two EBSs play significant role in endothelial cells, but the R-ras promoter activation in arterial smooth muscle cells may depend largely on other elements in the −727/−476 region.

Alternatively, the EBSs may be blocked frequently in these cells.

The deletion analysis clearly demonstrated the importance of the −727/−476 region in the A2780 tumor cells as well (Fig. 3A); therefore, the effects of EBS point mutations were also examined in these cells. The EBS1 mutation and EBS2 mutation resulted in only 20 and 7% reductions in the promoter activity, respectively (Fig. 5). Interestingly, the EBS1 + EBS2 double mutations exhibited an additive effect of the two mutations reducing the promoter activity by 50%. Thus, unlike in normal vascular cells, the two EBSs appear to contribute to the R-ras promoter activity independently in the ovarian carcinoma cells. Overall, these results demonstrated the cell type-specific activities of the Ets-binding elements and suggest different mechanisms for R-ras induction in normal vascular cells versus transformed epithelial cells.

Roles of Sp1 and MZF1-binding Elements in R-ras Promoter Activation—Within the fragment 1 −727/−476 region, there are several other putative transcription-binding sites (Fig. 4). EBS1 shows 100% homology between all five species, and EBS2 shows a complete homology between human, dog, and bovine. Mouse and rat EBS2 sites have two nucleotides mismatches in comparison with the human sequence. To determine the significance of these putative Ets-binding sites, we introduced mutations in these sites individually or simultaneously into the P6 promoter-reporter construct. The P6 construct was chosen because it demonstrated the highest promoter activity in all cell types we tested. Two nucleotide substitutions were introduced for each mutation to ensure the inactivation of the sites (GGAATTTAA for EBS1 and TTCCCGG for EBS2). The EBS1 mutation reduced the activity of the DNA reporter construct in HUVEC by 50% (Fig. 5). The EBS2 mutation also reduced the P6 promoter activity to a similar level in these cells. The double (EBS1 + EBS2) mutation resulted in 60% reduction. Thus, the ablation of either EBS alone diminishes the promoter activity to a similar level as the double mutation, suggesting that both sites are required for maximal activity. In CASMC, the single mutations of either EBS reduced the P6 promoter activity only by 22–23% (Fig. 5). The fact that the combined effect of the two mutations was not far greater than the effect of the single mutations suggests the cooperation of these two EBS sites in CASMC. However, both single and double mutations in these cells showed only half the effects seen in HUVEC. These observations suggest that the two EBSs play significant role in endothelial cells, but the R-ras promoter activation in arterial smooth muscle cells may depend largely on other elements in the −727/−476 region.

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and A2870 tumor cells. The Sp1-binding site mutation reduced the promoter activity by 40% in both cell types (Fig. 6), demonstrating an important contribution of this element to R-ras promoter activity in both cell types. In contrast, mutation of the Myb site, which partially overlaps with the Sp1 site, did not result in statistically significant reduction in HUVEC (p = 0.55), whereas it caused a 20% reduction in A2870 cells (Fig. 6).

The analysis of the putative MZF1 sites demonstrated a distinct pattern of the mutation effects in HUVEC and A2870 cells (Fig. 6). MZF1 (myeloid zinc finger gene 1) is a zinc finger transcription factor involved in growth and differentiation of myeloid progenitor cells (19, 20). In HUVEC, the single mutation of either MZF1 site reduced the P6 promoter activity by ~15%, and the simultaneous mutation of the both sites reduced the activity by ~30%, exhibiting an additive effect of the two mutations (Fig. 6). In contrast, the single MZF1 mutation resulted in up to 70% reduction in the P6 promoter activity in A2870 cells, demonstrating a considerable effect of inactivating either MZF1 site (Fig. 6). Furthermore, the double mutation reduced the promoter activity to 10% of the wild type P6 promoter. This activity level was comparative with that of the P1, −246/+23 deletion, clone in A2870 cells (Fig. 3B), demonstrating that the silencing the MZF1-binding sites diminishes the promoter activity to that of basal promoter. This observation also indicates that the −1907/−1456 region (fragment 3), which was found responsible for 50% of the P6 promoter activity in A2870 cells (Fig. 3B), requires intact MZF1 sites to activate transcription. Notably, the deletion of the entire −756−/−404 region (similar to fragment 1) in the dP promoter clone showed >2-fold higher activity than the P6 promoter with the MZF1 double mutations (Fig. 3B and Fig. 6). This suggests that negative regulatory element(s) are also present between −756 and −404 to suppress the promoter activity in A2870 cells. In summary, our results demonstrated that the two consecutive MZF1 motifs in the −727−/−476 region are required for the R-ras promoter activation in A2870 cells, although they play a much less significant role in normal endothelial cells. Thus, the Ets-binding motifs are critical for R-ras regulation in endothelial cells, whereas the MZF1-binding sites are indispensable for the activation of the R-ras gene in A2780 tumor cells. Multiple cis-

acting elements and their associating transcription factors appear to be involved in the regulation of the R-ras gene, and these elements and transcription factors may play differential roles in endothelial cells and transformed epithelial cells.

Analyzes of Potential Interactions between Multiple cis-Acting Elements—To examine potential functional interactions between the cis-acting elements within the −727−/−476 region, we analyzed the effects of multiple simultaneous mutations on the putative transcription factor-binding sites (Fig. 7). The double mutation of Sp1 and Myb sites suggested an additive effect of the two mutations in both HUVEC and A2870 cells. The Sp1/Myb/EBS1 + EBS2 quadruple mutations, however, did not further reduce the promoter activity compared with the Sp1/Myb mutations in either cell types. The promoter activity of the quadruple mutant was also similar to that of the EBS1 + EBS2 double mutant (Fig. 7). These observations suggest that the Sp1/Myb site and the EBSs are independent on each other in activating the R-ras promoter. A functional cooperation of the transcription factors that bind to these sites is likely a key molecular basis of the R-ras promoter activation.

Ets Family Transcription Factor GABP Interacts with R-ras Promoter through the Ets Consensus Sequence—It was particularly interesting to find Ets-binding sites within the −727−/−476 region (fragment 1), which were shown to be critical for the R-ras promoter activation in endothelial cells. Ets1, a prototypical member of Ets family, is downstream of various angiogenic stimuli (21–24), and it serves a role as a transactivator of angiogenic genes such as KDR/flk-1 (21, 25–27). It is implicated in promotion of tumor angiogenesis (28, 29) and neointimal hyperplasia (30, 31). On the contrary, R-ras signaling leads to suppression of vascular proliferation and invasion (10). Therefore, it is important to identify which member of the Ets family transcription factors binds to the R-ras promoter. We conducted electrophoretic mobility shift assays (EMSA) using a 50-bp oligonucleotide, including both EBS1 and EBS2 or its double-mutated counterpart as a probe. When the labeled wild type probe was mixed with nuclear extracts from HUVEC, several shifted bands were observed, indicating the formation of multiple DNA probe-nuclear protein complexes (Fig. 8A, lanes 1 and 2). Most of these bands were efficiently competed by excess unlabeled probe (Fig. 8A, lane 3) but not by the addition of excess amount of unlabeled mutated probe (Fig. 8A, lane 4).

**FIGURE 6. Mutation analyses of the Sp1, Myb, and MZF1 motifs.** The relative positions of the putative transcription-binding motifs are illustrated at left (only the fragment 1 region is shown). A site-directed mutagenesis was carried out in each consensus motif in the P6 promoter construct. The mutations are shown as open shapes. The promoter activities of the mutants were then determined relative to the activity of the wild type P6 promoter construct in HUVEC and A2780 cells. There are two MZF1 consensus motifs in this region, MZF1(1) and MZF1(2); MZF1 (1 + 2), double mutation of both sites.

**FIGURE 7. Analysis of multiple simultaneous mutations of the consensus motifs.** Sp1 and Myb sites as well as both EBSs were simultaneously mutated in the P6 promoter construct as illustrated (left bottom, only fragment 1 region is shown). The promoter activity of this quadruple mutant was compared with the activities of single or double mutants in HUVEC and A2780 cells to determine potential functional interactions between the cis-acting elements.
Regulation of Human R-ras Gene Promoter

Figure 8. Characterization of nuclear protein bindings to the Ets consensus sequence by EMSA. Nuclear extracts were obtained from HUVEC (A) and CASMC (B). A double-stranded 50-bp oligonucleotide that includes both EBS1 and EBS2 was generated as a probe. The labeled EBS probe (lane 1) was incubated with the nuclear extracts either alone (lane 2) or in competition with 200-fold excess amounts of unlabeled probe (lane 3). Some of the specific DNA-protein complexes formed are indicated (I–IV). The double mutant (EBS1 + EBS2) probe failed to compete against the wild type probe for the specific binding (lane 4) and to form specific DNA-protein complexes (lane 5). To identify the DNA-binding proteins in an antibody-disruption assay, polyclonal antibodies specific to the Ets family members, Ets1, Elk1, GABP (α subunit), or normal rabbit control IgG, were added to the binding reactions and incubated for 20 min at room temperature before labeled probes were added (lanes 6–9).

Demonstrating the binding specificity of the HUVEC nuclear factors to the EBS probe. Moreover, when the labeled mutated probes were mixed with nuclear extracts, no band shifting was observed (Fig. 8A, lane 5). These results clearly demonstrated specific DNA-nuclear protein interactions that are dependent on the Ets consensus sequence derived from the R-ras promoter.

The TFSearch program predicted that the three members of the Ets transcription factors, Ets1, Elk1, and GABP, are the most probable candidates for the EBS binders located in the -727/-476 region of the R-ras gene. GABP is unique in that it is a heterotetramer of two α subunits with Ets-like DNA binding domain and two β subunits with Notch-like domain (32, 33). Other Ets proteins function as monomers (33). To determine whether these Ets family proteins can indeed bind to the EBS of the R-ras promoter, we performed supershift/antibody disruption assays using specific antibodies to these proteins. Our result indicated that GABP is a major binder to the EBS in the HUVEC nuclear extract (Fig. 8A, lanes 6–9). Among the specific DNA-protein bands, the bands I and II disappeared when anti-GABPα polyclonal antibodies were added to the nuclear extract prior to the addition of the probe, indicating that the antibody binding to GABPα disrupted the DNA-protein complex formation (Fig. 8A, lane 8). The band III also faded significantly, indicating that these three bands contain GABPα subunit in the DNA-protein complexes. It appears that the band II fainter and the band I disappeared with anti-Ets1 antibodies, suggesting that Ets1 is binding to the EBS probe with low affinity (Fig. 8A, lane 6). Anti-Elk1 or control normal rabbit IgG did not cause any supershift or loss of the bands (Fig. 8A, lanes 7 and 9).

Using nuclear extract of CASMC, we also demonstrated specific bindings to the EBS probe of nuclear proteins from arterial smooth muscle cells (Fig. 8B). As in the case of HUVEC, the competition and mutation analyses of the EBS probe revealed specific DNA-protein complex formations (Fig. 8B, lanes 1–5). Here, GABP was again found to be one of the specific binders to the EBS probe, which was indicated by the loss of the band IV by the anti-GABPα antibody (Fig. 8B, lane 8). However, the most prominent DNA-protein complex, indicated by the band V, was not affected by antibodies to any of the Ets members we tested (Fig. 8B, lanes 6–9). These observations suggested that GABP binds to the EBS probe, but it may not be the main CASMC nucleoprotein that binds to the EBSs of the R-ras promoter. As shown in Fig. 5, the EBS mutations exhibited less significant effect on the promoter activity in CASMC than in HUVEC. One possible explanation for this observation was that an inhibitory nuclear protein competes for the binding to the EBSs in CASMC thereby blocking the binding of the activator protein to these sites. The band V may represent such a protein that blocks either or both EBSs. Based on the results of the other supershift/antibody disruption analyses, this protein is not Ets1 or Elk1 (Fig. 8B, lanes 6 and 7).

In contrast, the HUVEC extract does not appear to contain a major EBS binder other than GABP (Fig. 8A, lane 8). The specific binding of GABPα was also found in an EMSA with A2870 tumor cell nuclear extract (data not shown). Taken together, these observations demonstrated the ability of the Ets-related transcription factor GABPα subunit to interact with one or both of the Ets1 consensus motifs within the critical -727/-476 region of the R-ras promoter. These findings were in agreement with the cell type-independent requirement of this region for the R-ras promoter activation (Figs. 2 and 3).
The association of nuclear proteins and the fragment 1 region of the promotor sequence was examined in intact cells by PCR amplification of the sequence between −823 and −459, following chromatin immunoprecipitation using specific antibodies to several candidate transcription factor proteins. A, GABPα and Sp3 were shown to be associated with this region of the R-ras promotor in HUVEC. The same protein binding profile was found for CASMC (B) and A2780 cells (C). A weak association of Sp1 was detected in A2780 cells (C). The associations of Ets and Elk with the R-ras promotor were also examined in all three cell types. Only the results of A2780 were shown. D, as positive controls, the Ets1 and Elk binding regions of Bcl-X promoter were PCR-amplified. The result shows that the Ets1 and Elk antibodies work for ChIP analyses.

**FIGURE 9. Chromatin immunoprecipitation analyses of nuclear protein associations with the R-ras promotor region.** The association of nuclear proteins and the fragment 1 region of the promotor sequence was examined in intact cells by PCR amplification of the sequence between −823 and −459, following chromatin immunoprecipitation using specific antibodies to several candidate transcription factor proteins. A, GABPα and Sp3 were shown to be associated with this region of the R-ras promotor in HUVEC. The same protein binding profile was found for CASMC (B) and A2780 cells (C). A weak association of Sp1 was detected in A2780 cells (C). The associations of Ets and Elk with the R-ras promotor were also examined in all three cell types. Only the results of A2780 were shown. D, as positive controls, the Ets1 and Elk binding regions of Bcl-X promoter were PCR-amplified. The result shows that the Ets1 and Elk antibodies work for ChIP analyses. IgG, normal rabbit IgG control; Beads-only, no antibody or IgG added; Input (1/100), 1% of pre-chromatin immunoprecipitated nuclear extract used as a PCR control.

**FIGURE 10. Up-regulation of R-ras mRNA expression in HUVEC by co-expression of GABPα and GABPβ.** Upper panels, the relative levels of R-ras mRNA expression in the cells doubly transduced by lentivirus (HUVEC) or co-transfected (A2780 cells) with GABPα and GABPβ vectors (GABPα+β) or with insert-less vector alone (control). The total RNA was extracted from the transduced cells, and the levels of R-ras mRNA were determined by real time RT-PCR. Peptidylprolyl isomerase A was used as a reference gene to normalize the quantitative RT-PCR analysis. Lower panels, the overexpression of GABPα and GABPβ was confirmed by Western blot analyses. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is shown as a loading control.

**FIGURE 10.** Up-regulation of R-ras mRNA expression in HUVEC by co-expression of GABPα and GABPβ. Upper panels, the relative levels of R-ras mRNA expression in the cells doubly transduced by lentivirus (HUVEC) or co-transfected (A2780 cells) with GABPα and GABPβ vectors (GABPα+β) or with insert-less vector alone (control). The total RNA was extracted from the transduced cells, and the levels of R-ras mRNA were determined by real time RT-PCR. Peptidylprolyl isomerase A was used as a reference gene to normalize the quantitative RT-PCR analysis. Lower panels, the overexpression of GABPα and GABPβ was confirmed by Western blot analyses. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is shown as a loading control.

The association of GABP with the R-ras promotor region in A2780 cells suggests that GABP also plays a role in certain epithelial tumors (Fig. 9C).

Because a point mutation in the Sp1 consensus motif in the fragment 1 region significantly decreased the R-ras promotor activity (Fig. 7) and the balance between Sp1/Sp3 significantly influences the regulation of other endothelial cell-associated gene expressions (16, 17), we tested the association of Sp1 transcription factor in this region by ChIP analysis. This analysis found no Sp1 association (Fig. 9, A and B) or weak Sp1 association (Fig. 9C) with the region. Instead, the association of Sp3, another member of the Sp family, with the promotor region was clearly demonstrated in all three cell types we tested (Fig. 9, A–C). The different sensitivity of the PCR bands for the Sp1 and Sp3 ChIP implies that these Sp transcription factors may compete against each other for the binding to the single Sp motif in this promotor region and that Sp3 predominantly occupies this site.

**GABP Enhances R-ras Gene Expression in Endothelial Cells**—The results of the EMSA/antibody disruption assay and ChIP analysis indicated that GABP may be one of the critical transcription factors for the R-ras gene regulation. To determine the role of GABP in R-ras regulation, CDNAs for GABPα and -β subunits were co-transduced into HUVEC. The forced expression of both subunits significantly up-regulated R-ras mRNA level in HUVEC as determined by a quantitative RT-PCR analysis. The combined observations strongly suggest that GABP is a trans-activator of R-ras promoter in endothelial cells (Fig. 10A). Interestingly, the forced expression of GABP subunits did not increase R-ras mRNA levels in A2780 cells (Fig. 10B). Instead, it resulted in a small but statistically significant reduction of R-ras mRNA expression, suggesting a negative regulatory role of GABP in these cells. These results provided supporting evidence that the induction of the R-ras gene is differentially regulated during the normal expression in endothelial cells and aberrant expression in epithelial tumor cells.

**DISCUSSION**

Previously, we reported that the small GTPase, R-Ras, is an important regulator of blood vessel regeneration (10). R-Ras signaling antagonizes pathological stimuli that induce proliferative and invasive activities of vascular cells in the vascular lesions (10). Thus, R-Ras promotes quiescence of the vascular wall. Accordingly, it is most abundantly expressed in the fully mature blood vessels of normal adult tissues and significantly
suppressed during proliferative stage of vessel regeneration and remodeling (10, 12). In addition, there is accumulating evidence that the relative abundance of the R-ras gene expression is critical for determining vascular cell phenotypes. It is therefore of great importance to determine the molecular mechanism of its transcriptional regulation. In this study, for the first time, we identified and characterized the human R-ras promoter and its activity. The roles of cis-acting elements and the nucleofactor interactions with these elements were investigated in cultured umbilical cord vein endothelial cells and coronary artery smooth muscle cells. The findings were then compared with the findings from an epithelial tumor cell line that aberrantly express R-ras. Through the series of deletion studies, we identified three crucial DNA fragments in the 5′-flanking promoter region for the gene activation as follows: fragment 1 (−727/−476), fragment 2 (−1455/−728), and fragment 3 (−1907/−1456). Fragments 2 and 3 were shown to contain cis-acting elements that differentially function in endothelial cells, arterial smooth muscle cells, and epithelial tumor cells. Fragment 1 was shown to contain key elements for the promoter activation in all cell types we tested.

Sequence analysis of the fragment 1 (−727/−476) identified several consensus transcription factor-binding sites. Mutation studies found that a transcription factor of the Ets family, GABP, binds to the R-ras promoter at the Ets1 consensus motif(s) (EBS) located within fragment 1. Because the 50-bp DNA probe we used in the EMSA/supershift study contained both EBS1 and EBS2 sites, we do not know whether GABP binds to either or both EBSs. Confirmation of GABP binding in vivo by chromatin immunoprecipitation indicated GABP is one of the nuclear proteins bound to the critical promoter region in the vascular cells, and it suggests a physiological relevance of this DNA-protein interaction for the R-ras gene regulation in blood vessels.

Notably, the effects of EBS mutations on the promoter activity were less substantial in CASMC than in HUVEC. GABP was found to be the major binder to the R-ras promoter-derived EBS probe in HUVEC extract, but not in CASMC extract where another unidentified protein (band V in Fig. 8B) appeared to bind the EBS probe. These data suggest that in CASMC, the GABP binding to EBSs may be blocked by this unknown protein. These observations imply a more decisive role of GABP in endothelial cells than in vascular smooth muscle cells and suggest differential molecular regulations between the two essential cell types that constitute the vessel wall.

GABP is unique in that its transcriptionally active complex is an obligate heterotetramer composed of two α subunits and two β subunits (32, 33). GABPα has an Ets-like DNA binding domain and binds the consensus GGA(A/T) motif (32, 33). GABPβ contains a transcriptional activation domain and Notch/ankyrin-type repeats that are necessary for the association with the α subunit (32, 33). GABPβ does not bind DNA directly but associates with GABPα and stabilizes the GABPα-DNA binding. Although numerous target genes have been identified, including many housekeeping genes, GABP is well recognized as a key transcriptional regulator of dynamically regulated, lineage-restricted genes of myeloid cells and at neuromuscular junction (33–39). For example, a critical role of GABP has been identified in the postsynaptic differentiation (40). GABP regulates transcription of the acetylcholine receptor (41, 42) and utrophin genes in the skeletal muscle at the neuromuscular junction (43–46). A number of studies suggested that the physical and functional interactions with other transcription factors and co-activators are key to the ability of GABP to regulate gene expression (33). The potential in vivo role of GABP in vascular endothelial cells and smooth muscle cells has not been determined. However, our data suggest that it may be critically involved in the vascular cell regulation through transcriptionally regulating the R-ras gene expression. Supporting this idea, we found that forced expression of GABPα and β subunits significantly up-regulates R-ras mRNA level in HUVEC (Fig. 10A). Interestingly, GABP promotes myoblast differentiation into myotubes by up-regulating the promoter activity of the tumor suppressor Rb gene. Forced expression of constitutively activated R-Ras has been shown to promote myoblast-myotube differentiation (47). As the most abundant in vivo expression of R-Ras protein is associated with differentiated smooth muscle, and quiescent endothelium as well as neonatal skeletal muscle (10), GABP is an interesting transcription regulator to explore. Further studies in vascular cell types will likely define a new biological role for GABP.

ChIP analyses also unveiled the GABP association with R-ras promoter in an ovarian cancer cell line. However, EBS single mutations caused a much smaller effect on the promoter activity in these cells than in normal endothelial cells. Furthermore, forced expression of GABP did not increase R-ras mRNA level in A2780 cells, although it showed significant effect in HUVEC (Fig. 10B). R-ras mRNA level was rather reduced slightly in A2780 cells (Fig. 10B). These observations suggest that GABP association may play a less significant or negative regulatory role in the abnormal induction of R-ras gene in these tumor cells. On the other hand, the strong activities of the putative MZF1-binding elements within the fragment 1 may represent a mechanism that leads to the aberrant R-ras expression in certain cancer cells of the nonvascular cell origin. MZF1 (myeloid zinc finger gene 1) is a transcription factor of the C2H2 zinc finger gene family, which is involved in growth and differentiation of myeloid progenitor cells (19, 20). The MZF1-binding sites are found in the promoters of several genes expressed during myeloid differentiation, and the MZF1 protein has been shown to activate the CD34 promoter (19). It is noteworthy that, although at a much lower level than endothelial cells or smooth muscle cells, macrophages also express R-ras.3 The identity of the nucleofactor that binds to the MZF1 sites of the R-ras promoter has yet to be determined. These observations suggest that some carcinoma cells may have acquired and deregulated the mechanism that is normally used for the low endogenous expression of R-ras by macrophages.

Interestingly, the mutations in the MZF1 sites did not considerably affect the R-ras promoter activity in normal endothelial cells, but these mutations were highly detrimental to the promoter activity in A2780 ovarian carcinoma cells, indicating the differential regulatory mechanisms between normal versus aberrant expressions of the R-ras gene. Nishigaki et al. (11) reported that demethylation of the CpG island within the exon 1 resulted in R-ras up-regulation in half of gastric cancer cell
Regulation of Human R-ras Gene Promoter

lines that they tested, suggesting that methylation of the CpG island silences and hypomethylation reactivates R-ras gene expression in these cells. However, another half of the gastric cancer cell lines did not show this effect, and R-ras mRNA remained undetected in these cells. Therefore, R-ras gene is not activated as default state in the gastric cancers, which can only be regulated by hyper/hypomethylation of the promoter region. Instead, abnormal activities of some cis-acting elements and transactivators are likely responsible for the up-regulation of R-ras gene in these cells. This notion is supported by our observations with A2780 ovarian cancer cells. Whether the methylation of the CpG island plays a role in R-ras regulation in normal gastric epithelial cells or other cell types is currently unknown. The sequence analysis for the consensus motifs in the fragment 1 identified additional potential transcription factor-binding sites (Brn-2, Ik-2, and AP-1) upstream from the Sp1 site. The significance of these sites for the R-ras promoter regulation is unknown.

In this study, we also found that an Sp transcription factor, Sp3, is bound to the fragment 1 region of the R-ras promoter sequence in HUVEC and CASMC as well as A2780 cells. A point mutation of the single Sp1/Sp3-binding site located in the fragment 1 decreased the R-ras promoter activity by 40% in endothelial cells. These findings are potentially very important for understanding the transcriptional regulation of the R-ras gene, which governs pathological angiogenesis. Sp3 is a bifunctional transcription factor, which can act either as a repressor of Sp1-mediated activation (by competing for the common bind site) or as a strong transcriptional activator by itself (16). The structure and the arrangement of the recognition sites appear to determine which role Sp3 takes. Promoters containing a single binding site are activated, whereas promoters containing multiple binding sites often are not activated or respond weakly to Sp3 (16, 48). It is also likely to be dependent on the cellular context (16, 49, 50). The abundance of Sp1 and Sp3 varies among different cell types. Accumulating evidence suggests that the relative abundance of these two proteins allows regulation of gene activities. Endothelial cells contain high levels of both Sp1 and Sp3, and the Sp1/Sp3 ratio is higher in endothelial cells than in other cell types (17). The endothelium-specific activation of the KDR/Flk-1 gene, which encodes vascular endothelial growth factor receptor 2, was mapped to an Sp1/Sp3-binding site of its promoter (17). The observation that Sp3 attenuates Sp1-mediated KDR/Flk-1 promoter activation suggested that KDR/Flk-1 expression may be mediated by the high Sp1/Sp3 ratio in endothelial cells (17). The KDR/Flk-1 expression is regulated during developmental and pathological angiogenesis. KDR/Flk-1 is expressed at very high levels in angiogenic vasculatures of developing embryos and tumors; however, its expression is significantly diminished to low levels in normal adult vasculature (26, 51). Interestingly, the spatiotemporal pattern of R-ras expression was found to be totally opposite; little or very low expression was found in the developing normal vasculature, in tumor vasculature, as well as in the hyperplastic vascular lesions, whereas abundant expression was found in normal mature vessels (10, 12). These combined observations suggest that Sp3 may regulate the endothelial phenotype switch between angiogenic and quiescent states by regulating the expression levels of angiogenic (KDR/Flk-1) and anti-angiogenic (R-ras) genes.

For the potential involvement of Sp3 in R-ras regulation, it is also important to note that the Sp and Ets family transcription factors are known to cooperate to regulate expression of many genes (52, 53). This raises a possibility that Sp3 and GABP cooperatively regulate the R-ras promoter activity. This possibility is supported by our observation that the Sp1/Myb/EBS1 + EBS2 quadruple mutations did not further reduce the promoter activity from the Sp1/Myb or EBS1 + EBS2 double mutations (Fig. 7). The Sp3 bound to the Sp1/Sp3-binding motif, and the GABP bound to either or both Ets1 motifs may be mutually dependent; therefore, inhibiting the binding of either one would result in the same effect as inhibiting the binding of both. The physical and functional association of GABP with Sp1 and Sp3 has been observed during the activation of utrophin promoter. Furthermore, the GABP and Sp1 are known to cooperate in the activation of several GABP target genes, including lineage-specific genes and more widely expressed genes, such as CD18 (54), heparanase-1 (52), the pem pd homeobox gene (55), and the folate receptor β (39). Whether GABP and Sp3 cooperatively or independently transactivate the R-ras promoter activity awaits further studies.

In addition, the most distal upstream sequences (−4924/−1908) from the R-Ras transcription initiation site appeared to contain multiple negative cis regulators, with the strongest inhibitors within the region −3174/−2417. In this study, we did not further characterize these upstream sequences. However, the identification of the negative regulatory elements and the nucleoproteins that bind to these elements will be crucial as they may be therapeutic targets to increase the beneficial R-ras expression and signaling in the vascular lesions. Overall, our study characterized some of the critical cis-acting elements in the R-ras promoter sequence and identified potential transactivators that may be vital for the regulation of the R-ras gene. As R-ras expression can be an excellent biomarker for the vascular quiescence, the promoter region we isolated may be of use for identifying novel intracellular signaling pathways and for drug discoveries that lead to the inhibition of abnormal vascular proliferations.

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