Identification and Characterization of an Angiogenin-Binding DNA Sequence That Stimulates Luciferase Reporter Gene Expression†

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ABSTRACT: Angiogenin undergoes nuclear translocation in endothelial and smooth muscle cells where it accumulates in the nucleolus and binds to DNA. Nuclear translocation of angiogenin is necessary for its biological activity and is mediated by an endocytotic pathway that is independent of the microtubule system and lysosomal processing. Because the nucleolus is a subnuclear organelle containing clusters of transcriptionally active ribosomal RNA genes, we studied the binding of angiogenin to the intergenic spacer of the ribosomal RNA gene where many of the transcription regulatory elements are located. Here we report that angiogenin binds to CT repeats that are abundant in the nontranscribed region of the ribosomal RNA gene. An angiogenin-binding DNA sequence (CTCTCTCTCTCTCTCTCCCTC) has been identified and designated *a*ngiogenin-*b*inding *e*lement (ABE). ABE binds angiogenin specifically and exhibits angiogenin-dependent promoter activity in a luciferase reporter system. CT repeats, or inverted GA box, which are abundantly distributed in the eukaryotic genome and are often located in the 5′-flanking region, have been implicated in regulating gene expression. We have previously shown that angiogenin stimulates rRNA synthesis. The present results suggest that the nuclear function of angiogenin may not only be related to rRNA production but also play a role in regulating expression of genes containing CT repeats.

Angiogenin was one of the earliest angiogenic proteins to be identified (*1*). It is also the only angiogenic protein that is a ribonuclease and is the only member of the ribonuclease superfamily that is angiogenic (*2*). The ribonucleolytic activity of angiogenin is about $\sim 10^6$-fold less than that of pancreatic ribonuclease (*3*). However, this seemingly weak but characteristic ribonucleolytic activity is necessary for angiogenin to induce angiogenesis (*4*, *5*). Besides an absolute requirement of enzymatic activity, angiogenin must also interact with endothelial and smooth muscle cells to induce a wide range of cellular responses including a second messenger response (*6*), mitogen-activated protein kinase (MAPK)[1] activation (*7*, *8*), invasion (*9*), and tube formation (*10*). Furthermore, angiogenin must undergo nuclear translocation to be active as an angiogenic factor (*11*, *12*).

Nuclear translocation of exogenous angiogenin occurs very rapidly in sparse endothelial cell cultures. Angiogenin enters the nucleus within 2 min after addition to the culture medium, reaches saturation in 15 min, and remains nuclearly associated for at least 24 h (*13*). Although the mechanistic details of nuclear translocation remain unknown, it is apparently mediated by endocytosis (*12*) and is independent of the microtubule system (*14*). More importantly, nuclear translocation is essential for angiogenin to induce angiogenesis. Inhibition of nuclear translocation of angiogenin by neomycin (*15*) or through mutagenesis (*12*) abolishes its angiogenic activity. Neomycin does not interfere with the ribonucleolytic activity of angiogenin (*15*) and does not inhibit the phosphorylation of Erk 1/2 in endothelial cells (*7*) or that of SAPK/JNK in smooth muscle cells (*8*) that are induced by angiogenin. Therefore, a direct nuclear function of angiogenin seems likely.

Recently, we showed that angiogenin stimulates the synthesis of ribosomal RNA both in intact endothelial cells and in isolated endothelial nuclei (*16*). Because rRNA transcription is the rate-limiting step in ribosome biogenesis, it is essential for protein translation and cell growth. Therefore, angiogenin-stimulated rRNA synthesis might be a key step in the process by which angiogenin induces endothelial cell proliferation and new blood vessel formation. Nuclear run-on experiments indicated that angiogenin-stimulated rRNA synthesis in isolated nuclei is independent of cell type or the proliferation status under which the nuclei were isolated, suggesting that it is the nuclear translocation process that controls and regulates the overall angiogenin activity. It is known that angiogenin does not undergo nuclear

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[1] Abbreviations: ABE, angiogenin-binding element; bFGF, basic fibroblast growth factor; CT-1, angiogenin antisense oligonucleotide; CT-2, control oligonucleotide; EGF, epidermal growth factor; FBS, fetal bovine serum; HuASM, human umbilical artery smooth muscle; HUVE, human umbilical vein endothelial; MAPK, mitogen-activated protein kinase; NSB, nuclei storage buffer; PDGF, platelet-derived growth factor.

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translocation in confluent endothelial and smooth muscle cells nor in normal non-blood vessel cells such as fibroblasts. Because angiogenin binds to DNA in the nucleolus and stimulates rRNA transcription, we hypothesized that angiogenin may bind to the intergenic spacer where various regulatory elements for rRNA transcription are located. In the present study, an angiogenin-binding DNA sequence has been identified from the nontranscribed region of rRNA gene and characterized as possessing angiogenin-dependent promoter activity that directs the expression of a luciferase reporter gene.

EXPERIMENTAL PROCEDURES

Cell Culture. Human umbilical vein endothelial (HUVE) and arterial smooth muscle (HuASM) cells were purchased from Cell Systems Corp. (Kirkland, WA). HeLa and U-937 cells were from ATCC. HUVE cells were cultured in human endothelial serum-free medium (Invitrogen) plus 5% fetal bovine serum (FBS) and 5 ng/mL basic fibroblast growth factor (bFGF), HuASM and HeLa cells were cultured in DMEM plus 10% FBS, and U-937 cells were cultured in RPMI plus 10% FBS, at 37 °C under 5% humidified CO_2. Medium was changed every 2 days. Cells were subcultured in a 1:3 split ratio.

Antisense Treatment. The so-called second-generation antisense oligonucleotides, phosphorothioate-2′-*O*-methyl RNA chimera, were used (Oligo Etc.). CT-1 (5′-CAACAAAACGCCCAGGCC-3′) hybridizes with nucleotides 122−139 of the angiogenin mRNA. CT-2 (5′-CCGGACCCGCAAAACAAC-3′) is a control oligonucleotide that has the same base composition as CT-1 but in the reverse sequence orientation and thus does not hybridize with angiogenin mRNA. CT-1 and CT-2 were transfected into HeLa cells in the presence of 5 μL/mL Effectene (Qiagene).

Cloning of the Angiogenin-Binding Sequence. A 3.0 kb fragment was obtained by *Dpn*I digestion of a 4.6 kb insert of the nontranscribed region of rDNA that was isolated by *Pst*I cleavage of plasmid pHuPP4.6 (ATCC 61088). The 3 kb fragment (100 μg) was incubated with 180 μg of angiogenin in 0.8 mL of 40 mM Tris-HCl, pH 7.9, containing 10 mM NaCl, 6 mM $MgCl_2$, and 10 mM $CaCl_2$ at room temperature for 10 min. DNase I (5 units) was added, and the mixture was incubated at room temperature for 5 min to digest the unprotected DNA. Angiogenin-binding oligonucleotides were separated by Centricon-10, extracted with phenol, cloned into the pPCR-Script Amp vector (Stratagene), and sequenced according to the manufacturer's instructions.

Gel Retardation Assay. Angiogenin (10 pmol) was incubated with 0.1 pmol of ^{32}P-labeled, double-stranded ABE in 10 μL of 10 mM Tris-HCl, pH 7.5, containing 50 mM NaCl, 1 mM $MgCl_2$, 0.5 mM EDTA, 0.5 mM DTT, and 4% glycerol at room temperature for 20 min. The reaction mixtures were subjected to 8% nondenaturing acrylamide gel electrophoresis and autoradiography.

Angiogenin−Sepharose Affinity Chromatograpy. An angiogenin affinity column was prepared by coupling 5 mg of angiogenin to a 1 mL column of NHS-activated Sepharose and equilibrated in 50 mM Tris-HCl, pH 7.5. Both double- and single-stranded ABE (150 pmol) were injected, and elution was achieved by a linear gradient from 0.15 to 1 M NaCl.

Filter Binding Assay. A fixed concentration of ABE (0.2 nM) was mixed with varying concentrations of angiogenin ranging from 5 nM to 2.5 μM in 20 mM Tris-HCl, pH 7.5, containing 50 mM KCl, 0.1 mM DTT, 10% glycerol, and 50 μg/mL BSA. The mixture was incubated at room temperature for 25 min and filtered through a nitrocellulose membrane. The radioactivity retained on the membrane was determined by liquid scintillation counting. The apparent K_{eq} was calculated from a Scatchard analysis of the data.

Luciferase Expression of Various Constructs. ABE was inserted at different sites of luciferase expression vectors (pGL3B, pGL3P, pGL3E, and pGL3C), and the plasmids were transfected into cells with Effectene (Qiagen). For a typical transfection, 0.2 μg of plasmid and 2 μL of Effectene were used for 30000 cells in 0.7 mL of DMEM + 10% FBS per well of the 24-well plates. The cells were incubated at 37 °C for 48 h and lysed with passive lysis buffer (Promega). Luciferase activity was measured by the Dual-Luciferase assay system (Promega) with pRL-TK as the internal control that was cotransfected with the testing plasmids. The angiogenin expression vectors pRM-Ang(+) and pRM-Ang(−) were prepared by inserting the angiogenin coding region at the *Sma*I site of pRmHa-3 in forward and reverse directions, respectively. The DNA sequence encoding angiogenin was obtained from a PCR reaction with pAngC (ATCC no. 65422) DNA as the template and a pair of nucleotide primers with the sequences 5′-TCTGAATTCATGCAGGATAACTCCAGGTACAC-3′ and 5′-TCTGGTCGACTGGTTACGGACGACGG-3′).

Nuclear Run-On Transcription Assay. Endothelial nuclei were isolated from HUVE cells. Cells were trypsinized, washed twice with HBSS, resuspended at 2×10^7 cells/mL in a resuspension buffer (RSB: 10 mM Tris-HCl, pH 7.5, containing 10 mM NaCl, 3 mM $MgCl_2$, and 3 mM DTT), incubated on ice for 10 min, and lysed by 0.3% NP-40. The nuclei were isolated by centrifugation at 1000*g* for 5 min, washed once each with RSB and once with the nuclei storage buffer [NSB: 50 mM HEPES, pH 7.9, containing 20% glycerol (v/v), 90 mM KCl, 5 mM $MgCl_2$, 0.2 mM DTT, and 0.2 mM EDTA], and stored in liquid nitrogen at $\geq 10^8$ nuclei/mL in NSB. Nuclear run-on transcription was initiated by adding the NTP mixture (0.3 mM each of ATP, GTP, and UTP, 0.03 mM CTP, and 10 μCi of [α-^{32}P]CTP) to 4×10^5 nuclei in 100 μL of NSB. Angiogenin was mixed with the nuclei at 4 °C immediately before NTP addition. After 30 min incubation at 30 °C, the reaction was terminated by acidic phenol extraction. RNA was precipitated by ethanol and analyzed by 6% acrylamide gel electrophoresis.

RESULTS

Identification of the Angiogenin-Binding DNA Sequences. Angiogenin enters the nucleus, binds to DNA (*13*), and induces RNA synthesis (*16*) in endothelial cells. Northern blotting results showed that rRNA is one of the products stimulated by angiogenin (*16*). We therefore examined binding of angiogenin to the regulatory region of the rRNA gene. A 4.6 kb fragment of the nontranscribed region of the human ribosomal RNA gene was digested with *Dpn*I to obtain five smaller fragments of 3.0, 1.4, 1.0, 0.8, and 0.6 kb, respectively (Figure 1A). A gel retardation assay shows that the mobility of the 3.0 and 1.4 kb fragments but not

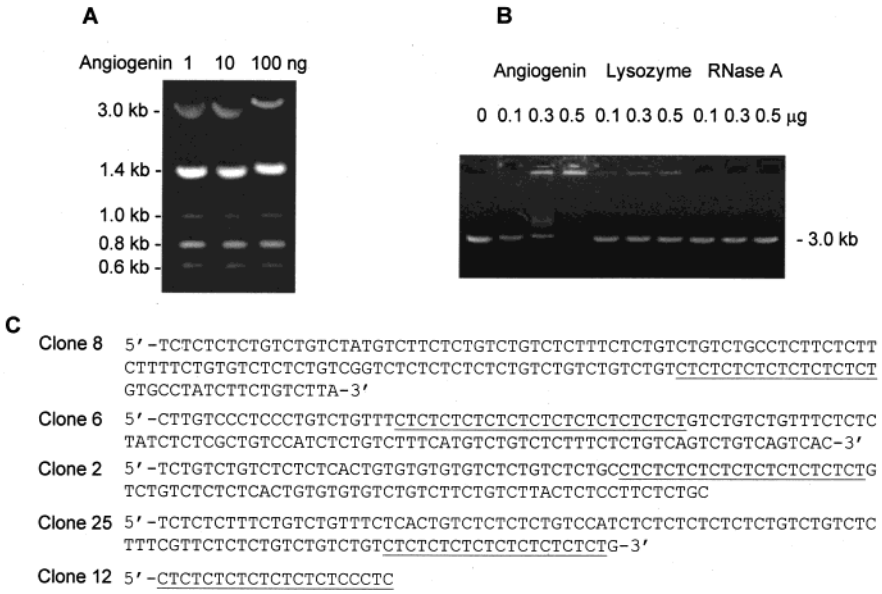


FIGURE 1: Identification and characterization of angiogenin-binding element (ABE) from rDNA. (A) Gel mobility shift assay. Plasmid DNA pHuPP4.6 (7.3 kb, ATCC 61088) was isolated and cleaved by *Pst*I to obtain the 4.6 kb insert of the nontranscribed spacer of rDNA. This 4.6 kb DNA (0.6 μg) was digested with *Dpn*I, and the resultant fragments (3.0, 1.4, 1.0, 0.8, and 0.6 kb, respectively) were mixed with 1, 10, or 100 ng of angiogenin in 15 μL of 6 mM Tris-HCl, pH 7.5, containing 6 mM $MgCl_2$, 50 mM NaCl, and 1 mM DTT. After incubation at room temperature for 10 min, the mixtures were subjected to 1% agarose gel electrophoresis. (B) Specificity of binding between angiogenin and the 3.0 kb DNA fragment. The 3.0 kb fragment (150 ng) was mixed with the indicated amounts of angiogenin, lysozyme, or RNase A in 10 μL of PBS, incubated at room temperature for 10 min, and subjected to 1% agarose gel electrophoresis. (C) Sequence of the five angiogenin-binding DNA fragments obtained from the 3.0 kb fragment of the regulatory region of rDNA.

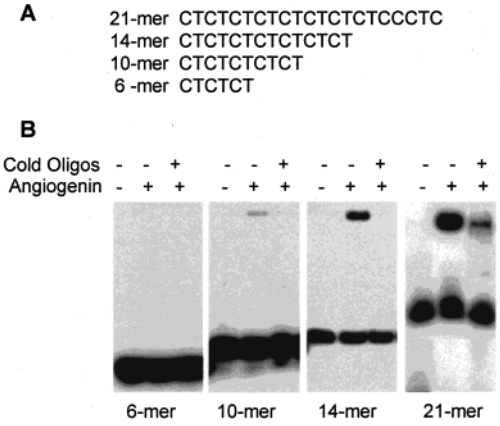
that of the 1.0, 0.8, and 0.6 kb fragments is retarded by angiogenin (Figure 1A). Since the 3.0 kb fragment showed the most significant mobility shift, it was used for further characterization of angiogenin binding. As shown in Figure 1B, the binding between angiogenin and the 3.0 kb fragment is specific. As the concentration of angiogenin increases, the intensity of the 3.0 kb DNA decreases and is completely abolished at 50 μg/mL angiogenin (0.5 μg in 10 μL). Instead, a band appears just below the loading well which may represent the angiogenin−DNA complex. Lysozyme, a basic protein whose MW and p*I* values are similar to those of angiogenin, has only marginal affinity for the DNA fragment. The homologous protein RNase A, which has 53% overall sequence similarity to angiogenin but is nonangiogenic, does not bind at all.

The complex of the 3.0 kb DNA and angiogenin was digested with DNase I, and the angiogenin-binding fragments were isolated, cloned, and sequenced. Figure 1C shows the DNA sequences of five clones. A common feature of these oligonucleotides is that they are rich in CT and contain a region of at least eight CT repeats (underlined). The shortest angiogenin-binding sequence (clone 12) identified so far, a 21-mer oligonucleotide consisting of eight CT repeats and CCCTC, has been designated *a*ngiogenin *b*inding *e*lement (ABE).

Binding of Angiogenin to ABE. To demonstrate that angiogenin indeed binds to ABE and to determine the minimum number of CT repeats necessary for angiogenin binding, four oligonucleotides with the sequences shown in Figure 2A were synthesized and their affinities for angiogenin were examined by three different methods. First, a gel retardation assay shows that angiogenin binds to the ^{32}P-labeled double-stranded 21-mer (ABE) and 14-mer, weakly to the 10-mer, but not to the 6-mer (Figure 2B). This binding is competitive with excess unlabeled oligo. Second, a filter-binding assay (*17*) shows that angiogenin binds to the double-stranded, ^{32}P-labeled 21-, 14-, and 10-mer oligos with apparent K_{eq} of 150, 220, and >1500 nM, respectively. No binding was detected for the 6-mer (Figure 2C). Third, angiogenin affinity chromatography showed that angiogenin−Sepharose binds both the double-stranded and the sense strand (CT repeats) of the 21-mer and 14-mer, which elute from the column at 0.62, 0.64, 0.58, and 0.56 M NaCl, respectively. The antisense strand (GA repeats) does not bind to angiogenin−Sepharose. No binding was detected with the 10-mer and 6-mer oligos by this method.

Assessment of the Transcription Stimulatory Activity of ABE. The transcriptional activity of ABE was examined in a luciferase reporter system transfected into cells that produce angiogenin endogenously. ABE was inserted at different sites in four different vectors as shown in Figure 3A. Insertion of ABE at the *Hin*dIII site upstream from the luciferase gene in pGL3P did not stimulate transcription significantly in HeLa cells. In contrast, its insertion at the *Hin*dIII site in the pGL3E vector increases the expression of luciferase by 68-fold (Figure 3B), comparable to that of an SV 40 promoter (pGL3C). Insertion of ABE at the *Hin*dIII site in the pGL3B vector results in transcription stimulation, but because this vector lacks an enhancer, the total luciferase activity is very low. No further enhancement was observed when ABE was inserted in pGL3C, a vector that already contains an SV 40 promoter and enhancer. The observations that ABE has no activity in pGL3P, a vector suitable for testing enhancers, but has significant activity in pGL3E, a vector suitable for testing promoters, suggest that ABE has promoter-like activity in this reporter system.

The transcriptional activity of ABE also depends on the position of insertion in pGL3E. When the insertion site is farther upstream at *Sac*I or *Sma*I sites or downstream at the *Bam*HI site, no significant activity was observed either in



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Olig	Eluting NaCl (M)	K_{eq} (nM)
21-mer		
Double-stranded	0.62	150
Sense strand	0.64	nd
Antisense strand	No binding	nd
14-mer		
Double-stranded	0.58	220
Sense strand	0.56	nd
Antisense strand	No binding	nd
10-mer		
Double-stranded	No binding	>1000
Sense strand	No binding	nd
Antisense strand	No binding	nd
6-mer		
Double-stranded	No binding	No binding
Sense strand	No binding	nd
Antisense strand	No binding	nd

FIGURE 2: Binding of angiogenin to synthetic oligonucleotides. (A) Sequences of the four oligonucleotides. The oligonucleotides (10 pmol) were radiolabeled by use of [γ-^{32}P]ATP and T_4 polynucleotide kinase. Equal amounts of the complementary strand (GA repeats) were added to make the double-stranded oligos. (B) Gel mobility shift assay. In the competition experiments, a 10-fold molar excess of the unlabeled oligos was present. (C) Angiogenin−Sepharose affinity chromatograpy and filter-binding assays. nd, not determined.

HeLa or in HuASM cells (Figure 3C,D). ABE inserted in the reverse orientation at any of these sites has diminished activity. These results are consistent with ABE being a promoter. Furthermore, a 21-mer oligonucleotide with the sequence of 5′-TCCTCCTCCTCCTCTCCTTTC-3′ did not show appreciable activity when inserted at the *Hin*dIII site of pGL3E (data not shown).

The transcription stimulatory activity of oligonucleotides with various numbers of CT repeats has also been examined (Table 1). CT repeats of various lengths were inserted in the *Hin*dIII site of the pGL3E vector in both forward and reverse orientations. Luciferase expression from these constructs was determined in HeLa cells. Table 1 shows that the promoter activity of CT repeats increases with their length. While the activity of $(CT)_5$ merely doubles, that of $(CT)_6$ and $(CT)_7$ increases 16- and 35-fold, respectively, over the pGL3E control. The activation is saturated when the repeat number reaches eight. Thus, insertion of $(CT)_8$, $(CT)_9$, and $(CT)_{10}$ at the *Hin*dIII site results in 53-, 56-, and 58-fold increases of luciferase expression, respectively. In this set of experiments, insertion of ABE gives 72-fold activation, consistent with the results in Figure 3B. No increase of luciferase expression was detected with poly$(G)_{21}$ insertion or when the CT repeats were inserted in the reverse orientation (Table 1). In combination with the results in Figure 2 where we showed that angiogenin binds marginally to $(CT)_5$ but significantly to $(CT)_7$, the minimum number of CT repeats required for angiogenin binding is about six.

Table 1: Promoter Activity of Oligonucleotides with Different Numbers of CT Repeats[a]

oligonucleotide	insert orientation	activity[b]	oligonucleotide	insert orientation	activity[b]
poly$(G)_{21}$	forward	0.4 ± 0.1	$(CT)_7$	forward	35 ± 3
ABE	forward	72 ± 4		reverse	0.8 ± 0.1
	reverse	0.8 ± 0.2	$(CT)_8$	forward	53 ± 4
$(CT)_5$	forward	1.9 ± 0.3		reverse	1.1 ± 0.1
	reverse	0.4 ± 0.1	$(CT)_9$	forward	56 ± 10
$(CT)_6$	forward	16 ± 1		reverse	0.7 ± 0.2
	reverse	0.6 ± 0.1	$(CT)_{10}$	forward	58 ± 8
				reverse	0.7 ± 0.1

[a] Oligonucleotides were inserted in the *Hin*dIII site of the pGL3E vector. The orientation of the insert was determined by nucleotide sequencing. [b] Activity is defined as the fold increase over pGL3E.

The Transcription Stimulatory Activity of ABE Depends on the Expression Level of Cellular Angiogenin. Insertion of ABE at the *Hin*dIII site of pGL3E results in 68-, 31-, and 11-fold increases of luciferase expression in HeLa, HuASM, and U-937 cells, respectively (Figure 4A). HeLa and smooth muscle cells secrete 1.5 and 0.7 ng of angiogenin per day per 10^6 cells, respectively, whereas U-937 cells do not secrete any detectable amount of angiogenin (*18*). This correlation between the ABE activity and the cellular angiogenin level suggests that the transcription stimulatory activity of ABE is angiogenin-dependent.

To confirm this result, HuASM and U-937 cells were transfected with an angiogenin expression vector pRM-Ang-(+) (*19*) and a vector containing the angiogenin gene in the reverse orientation pRM-Ang(−) to increase and decrease cellular angiogenin expression, respectively. The increase of ABE activity in HuASM cells cotransfected with pRM-Ang-(+) was only moderate (41- and 31-fold activation with and without cotransfection, respectively), probably due to the relatively high amount of angiogenin already expressed in this type of cells (Figure 4B). However, there was a significant increase in the ABE activity in U-937 cells after cotransfection with pRM-Ang(+) (78- and 8-fold activation with and without cotransfection, respectively). In both cell types, the activity of ABE was abolished when the cells were cotransfected with pRM-Ang(−) (Figure 4B).

Angiogenin antisense oligonucleotides were also used to confirm that the transcription stimulatory activity of ABE is angiogenin-dependent. Cells treated with a specific angiogenin antisense oligonucleotide CT-1 to decrease the expression of endogenous angiogenin exhibit a corresponding decrease in ABE activity (Figure 4C). Treatment with a control oligonucleotide, CT-2, has no effect on ABE activity. Treatment with CT-1 or CT-2 does not alter the expression of luciferase from the vectors lacking the ABE insert, indicating that the effect of angiogenin antisense DNA is specific to ABE. Figure 4D shows that both angiogenin mRNA and protein levels are indeed decreased after CT-1 transfection but unchanged with CT-2 treatment.

Exogenous angiogenin is able to reverse the inhibitory effect of CT-1. Incubation with 1 μg/mL angiogenin for 40 h efficiently restores the transcription stimulatory activity of ABE that has been inhibited by CT-1 (Figure 4C). It does

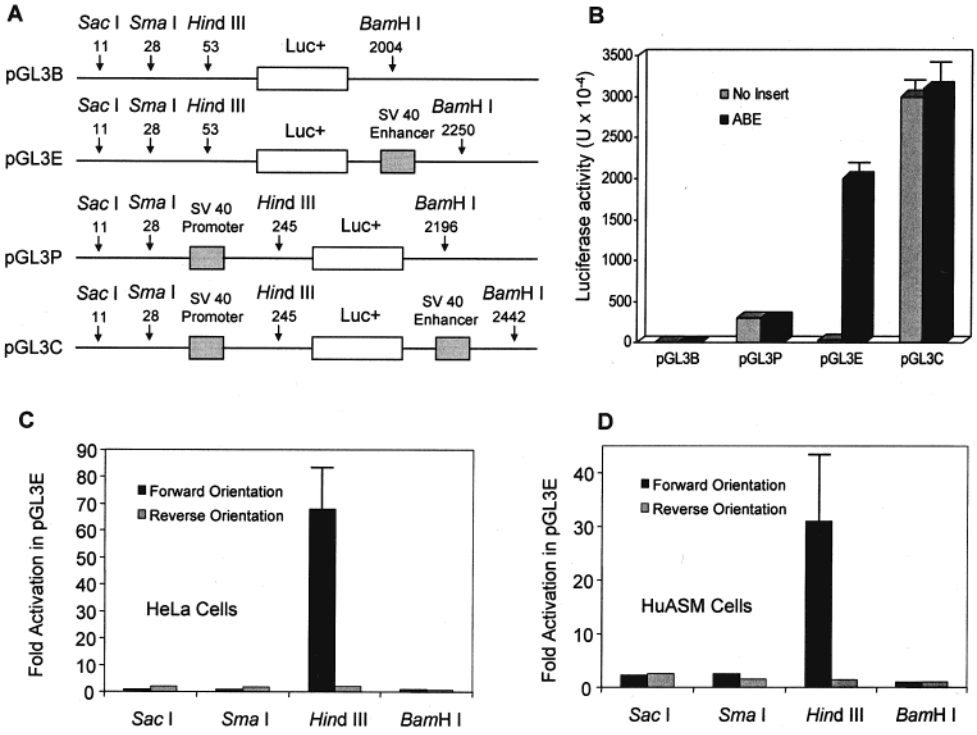

FIGURE 3: Transcription stimulatory activity of ABE. (A) Schematic diagram of the four luciferase reporter vectors (Promega). pGL3B is a basic vector containing no foreign promoters and enhancers; pGL3E is an enhancer vector containing an SV 40 enhancer downstream of the luciferase gene, suitable for testing potential promoters; pGL3P is a promoter vector containing an SV 40 promoter upstream of the luciferase gene, suitable for testing potential enhancers; pGL3C is the positive control of the luciferase reporter gene that contains both a promoter and an enhancer from SV 40. (B) Luciferase expression of various constructs after transfection in HeLa cells. ABE was inserted in the *Hin*dIII site of pGL3B, pGL3P, and pGL3E, respectively. Endotoxin-free plasmids (0.2 μg) were prepared by use of an Endofree Maxi plasmid kit from Qiagen and transfected into HeLa cells with use of 2 μL of Effectene reagent (Qiagen). The cells were incubated at 37 °C for 48 h, and the expression of luciferase was measured as described in Experimental Procedures. (C and D) The transcription stimulatory activity of ABE in the pGL3E vector depends on the insertion site and orientation. ABE was constructed both in forward [$(CT)_7$CCCTC] and in reverse [GAGGG$(AG)_7$] orientations into pGL3E at the *Sac*I, *Sma*I, *Hin*dIII, and *Bam*HI sites of pGL3E. Expression of the luciferase gene from these constructs in HeLa (C) and HuASM (D) cells was examined as described in Experimental Procedures. The data shown are the fold increases of luciferase activity over that of pGL3E.

not alter the ABE activity in untreated or in CT-2-treated cells probably because the endogenous angiogenin is adequate to activate the ABE fully.

Angiogenin Stimulates the Synthesis of both rRNA and mRNA in Nuclear Run-On Transcription Assays. The above results demonstrate that binding of angiogenin to CT repeats stimulates transcription of the luciferase gene in the pGL3E reporter systems that are usually transcribed by RNA polymerase II. We have previously shown that angiogenin stimulates rRNA synthesis in both live endothelial cells and isolated endothelial nuclei (*16*). Searching for an angiogenin-binding DNA sequence from the promoter region of rDNA was the initial purpose of this study. Indeed, we have identified an ABE from the promoter region of rDNA and proved that it has angiogenin-dependent promoter activity in the luciferase reporter system. However, the present data suggest that angiogenin may also play a role in mRNA transcription since CT repeats are abundantly distributed in eukaryotic genomes and many CT repeats are located in the 5′-flanking regions of genes (*20*). Therefore, we performed a nuclear run-on transcription assay in the presence of α-amanitin, a specific inhibitor of RNA polymerase II. Figure 5 shows that angiogenin stimulates total RNA transcription in isolated nuclei from HUVE cells, consistent with our previous results (*16*). At 1 and 10 μg/mL α-amanitin, the effective concentrations required to inhibit RNA polymerases II and III, respectively, the overall transcription is decreased significantly, but the ratio of angiogenin-stimulated synthesis remains unchanged. These results indicate that the products of the nuclear run-on assay are a mixture of mRNA and rRNA. rRNA is among the products because angiogenin is still able to increase the synthesis of nascent RNA in the presence of 10 μg/mL α-amanitin when the synthesis of mRNA and tRNA was inhibited. On the other hand, because the percentage increase of run-on transcription induced by angiogenin did not increase in the presence of α-amanitin, angiogenin must have also stimulated the synthesis of mRNA in the absence of α-amanitin. Otherwise, if only rRNA were stimulated by angiogenin, a much higher increase would have occurred when mRNA synthesis was inhibited by α-amanitin.

DISCUSSION

Our previous results that angiogenin binds to DNA in the nucleolus (*13*) and stimulates rRNA synthesis in both intact endothelial cells and isolated nuclei (*16*) prompted us to hypothesize that angiogenin may bind to the promoter region of the ribosomal RNA gene. In the present study, we have identified an angiogenin-binding sequence from the non-transcribed region of rDNA and shown that it binds to angiogenin specifically and has angiogenin-dependent promoter activity in a luciferase reporter system. Although it remains to be determined whether ABE is responsible for angiogenin-driven transcription of rRNA in vivo, the present data clearly demonstrate that the binding between angiogenin

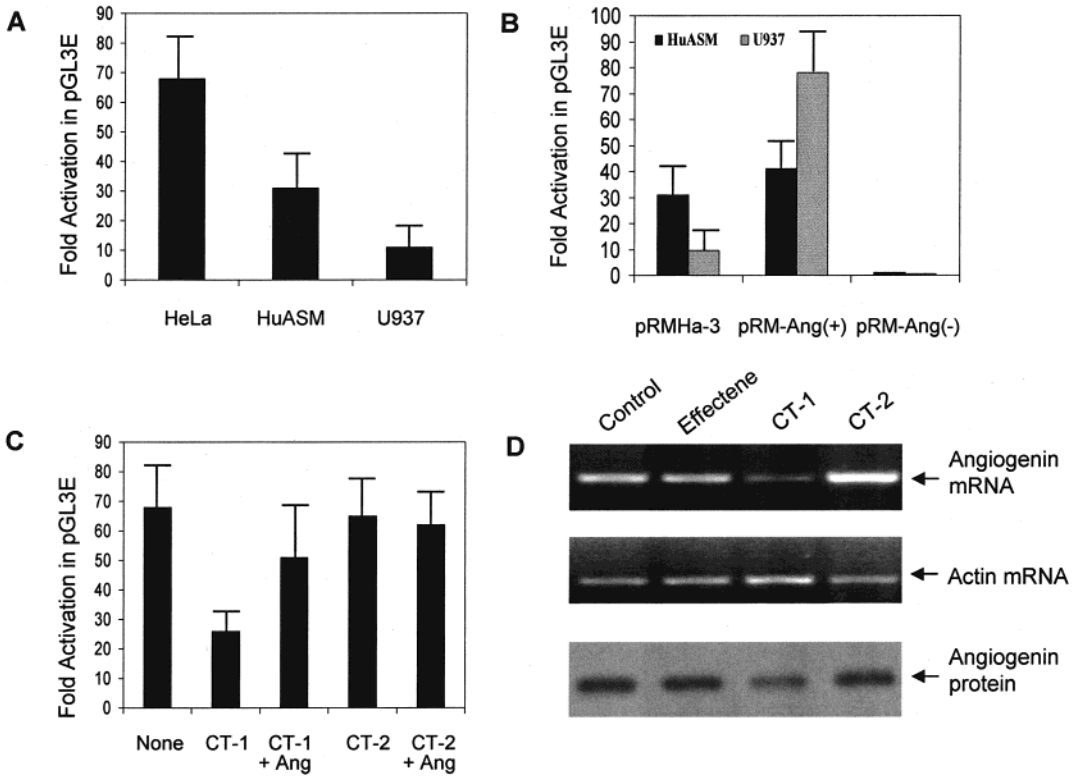

FIGURE 4: Angiogenin dependency of the ABE activity. (A) Transcription stimulatory activity of ABE in cells of different endogenous angiogenin contents. Expression of pGL3E and pGL3E-ABE (inserted at *Hin*dIII site) was examined in HeLa, HuASM, and U-937 cells that secrete 1.5, 0.7, and <0.1 ng of angiogenin per 10^6 cells per day, respectively. Data shown are the fold increases of luciferase activity from pGL3E-ABE over that from pGL3E in each cell type. (B) Transcription stimulatory activity of ABE in HuASM and U-937 cells cotransfected with angiogenin expression vectors pRM-Ang(+) and pRM-Ang(−), representing a forward- and reverse-orientated angiogenin coding DNA, respectively. pRMHa-3 is the vector control. Data shown are the fold increases of luciferase activity over that from cells transfected with pGL3E alone. (C) Effect of an angiogenin antisense oligonucleotide on the transcription stimulatory activity of ABE in HeLa cells. CT-1 and CT-2, 1.4 μg (final concentration of 0.4 μM), were cotransfected with 0.2 μg of pGL3E-ABE in the presence of 5 μL of Effectene. When exogenous angiogenin was added to rescue cells from CT-1 inhibition, it was added to the cells at a final concentration of 1 μg/mL 40 h prior to cell lysis. Data shown are the fold increases of luciferase activity over that from cells transfected by pGL3E alone. (D) RT-PCR and Western blotting analyses. HeLa cells were transfected with 0.4 μM CT-1 or CT-2 for 24 h. Total cellular RNA was extracted, and the RT-PCR reaction was carried out with the use of a ProStar RT-PCR kit (Stratgene). The primers used in this experiment were 5′-TCTGAATTCATGCAGGATAACTCCAGGTACAC-3′ and 5′-TCTGGTCGACTGGTTACGGACGACGG-3′ for angiogenin (upper panel) and 5′-ACAATGAGCTGCGTGTGGCT-3′ and 5′-TCTCCTTAATGTCACGCACGA-3′ for actin (middle panel), respectively. Conditioned medium was collected, and total proteins were precipitated with 5% TCA and subjected to SDS−PAGE and Western blotting analysis with an anti-angiogenin monoclonal antibody 26-2F.

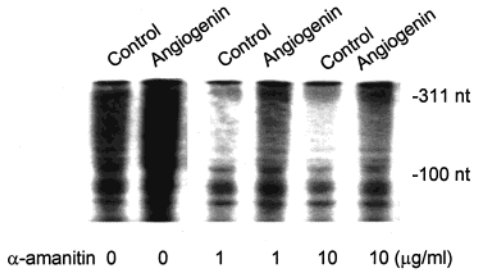

FIGURE 5: Effects of α-amanitin on angiogenin-stimulated transcription in the nuclear run-on experiments. α-Amanitin was mixed with nuclei at a final concentration of 1 and 10 μg/mL to inhibit RNA polymerases II and III, respectively. When angiogenin was present, it was added at a final concentration of 1 μg/mL immediately after α-amanitin was added to the nuclei. An NTP mixture was added to initiate the nuclear run-on reaction. The newly synthesized RNA (from 1×10^6 nuclei of HUVE cells) was visualized by autoradiography after 6% acrylamide sequencing gel electrophoresis.

and ABE is specific and productive in directing transcription of the luciferase reporter gene.

CT repeats are abundant in the eukaryotic genome (*21*) and have been implicated in a number of biological events including DNA replication (*22*), recombination (*23*), and nucleosomal organization (*24*). A search of the GenBank database using $(CT)_6$ as a query sequence generated more than 5000 hits. Many of them are located in introns with a considerable number in the 5′- and 3′-flanking regions of genes. Among them, 83 are located in the nontranscribed region of the ribosomal RNA gene.

Each human cell contains ~400 copies of the rRNA gene arranged in head-to-tail tandem repeats on five chromosomes (*25*). Within each of the repeats (44 kb) there is a transcription unit (13.3 kb) for the synthesis of a 45S primary rRNA transcript and a nontranscribed spacer (30.7 kb) where all the known transcription regulatory elements, such as the upstream control element, the core promoter, enhancers, spacer promoters, and the proximal transcription termination sequence, are located (*26*, *27*). We found that the nontranscribed region of rDNA contains three ABEs located at 16957, 22248, and 28089 bp upstream of the transcription initiation site, respectively. These ABEs are flanked with 53, 10, and 16 sequences, respectively, that have at least six CT repeats, the minimum requirement for angiogenin binding. It is not yet known how many angiogenin molecules

will actually bind to the nontranscribed region of rDNA. But with a total of 83 occurrences of $(CT)_6$, the maximum number of binding sites would be ~33200. We have previously shown that each HUVE cell cultured under sparse density can take up a maximum of $\sim 1.3 \times 10^6$ angiogenin molecules (*13*). Binding to the regulatory region of rDNA would therefore not account for all of the angiogenin molecules present in the nucleus. It is conceivable that nuclear angiogenin may also bind to the CT repeats of other genes and to other DNA sequences or nuclear proteins that remain to be identified. This is consistent with the observation that angiogenin also stimulates mRNA synthesis in the nuclear run-on assay (Figure 5).

Our results are also consistent with previous reports that short CT repeats may act as initiator elements that are able to direct transcription initiation (*28*). It is known that, in promoters that lack a TATA box, initiators alone are sufficient to direct gene expression and many initiator elements are pyrimidine-rich and contain CT repeats (*29*). It is of particular interest to note that CT-rich initiator elements have been identified in genes encoding ribosomal proteins (*30*). If the nuclear function of angiogenin is related to ribosome biogenesis, it would be pointless for angiogenin to stimulate rRNA synthesis without simultaneously stimulating the synthesis of ribosomal proteins and 5S rRNA.

Inverted CT repeats, or GAGA boxes, have been hypothesized to play a role in the transcriptional regulation of a variety of mammalian genes (*31*, *32*). GAGA-binding proteins have been identified (*33*−*36*). For example, in human proximal tubule cells, an 18 kDa nuclear protein has been detected to bind to the GAGA box of the promoter region of human angiotensin II type I receptor gene (*37*). Treatment of these cells with other growth factors such as EGF, PDGF, and insulin increase the nuclear localization of this GAGA-binding protein, indicating that the GAGA box may function as a more common cis-acting element for growth factor-induced transcription. At present, it is unknown whether angiogenin may also serve as one of the GAGA-binding proteins and stimulate gene transcriptions that are controlled by GAGA boxes. In this regard, it is interesting to note that treatment of human umbilical artery smooth muscle cells with PDGF induces nuclear localization of cytosolic angiogenin (G.-f. Hu, unpublished data).

Structurally, CT repeats are dynamic and form non-B-DNA structures such as H-DNA (*38*). The unusual DNA structures adopted by CT repeats have been studied extensively as models for non-B-DNA conformations formed by polypyrimidine tracts (*39*, *40*). We showed that angiogenin binds to both single-stranded $(CT)_n$ and double-stranded $(CT)_n(GA)_n$ but does not bind to single-stranded $(GA)_n$. Angiogenin does not bind to 5′-TCCTCCTCCTCCTCTC-CTTTC-3′, indicating that a simple non-B-DNA structure is inadequate for angiogenin to bind. Accordingly, both this sequence and $poly(dC)_{21}$ or $oligo(dT)_{21}$ do not drive the expression of the luciferase reporter gene. Moreover, insertion of ABE in reversed orientation at the *Hin*dIII site of the pGL3E vector does not have angiogenin-dependent promoter activity.

Four different types of luciferase reporting vectors were used to determine whether and how ABE stimulates transcription. pGL3B is a basic vector that does not contain foreign promoters and enhancers. Expression of luciferase activity in cells transfected with this plasmid depends on proper insertion and orientation of a functional promoter upstream from the luciferase gene. Insertion of ABE at the *Hin*dIII site upstream from the luciferase gene resulted in an increase of luciferase activity. More stable and significant stimulation was obtained when ABE was inserted in the *Hin*dIII site of pGL3E, a vector that has no promoter but contains an SV 40 enhancer downstream from the luciferase gene and the poly(A) signal. The presence of an enhancer results in higher levels of transcription of the luciferase gene, and the stimulation from ABE insertion reached 68-fold in HeLa cells. This degree of stimulation from ABE in a promoter-less vector was totally unexpected.

We do not know at present if RNA polymerase I or II transcribes from pGL3E-ABE. Luciferase reporter systems are usually used to assess both cis- and trans-acting factors for RNA polymerase II. However, because pGL3E is a promoter-less vector, we believe that it is the foreign promoter, ABE, which controls the recruitment of RNA polymerase. It is known that some genes lack TATA elements and their transcription depends heavily on initiators (*28*, *41*, *42*). ABE may serve as an initiator to drive the expression of the luciferase reporter gene.

It is of particular interest to note that the promoters of mammalian ribosomal protein genes do not contain TATA boxes (*43*−*48*) and their transcriptions are driven by polypyrimidine initiators rich in CT repeats (*30*, *49*). Besides the established role angiogenin plays in stimulating rRNA transcription (*16*), angiogenin may also bind to the CT repeats located on the promoter region of ribosomal protein genes and stimulate their transcription, which are also required for synthesizing new ribosomes. Experiments are underway to determine the effect of angiogenin on gene expression profiles.

In any event, the transcription stimulatory activity of ABE in the pGL3E vector has been confirmed in four different cell types (HeLa, HUVE, HuASM, and U-937) under various conditions. Because the transcription stimulatory activity of ABE depends on angiogenin binding, one possible explanation is that binding of angiogenin to ABE directly recruits RNA polymerase I or II onto the initiation site. That would also provide a rationale for the nuclear run-on results. Under normal circumstances, very little initiation occurs in nuclear run-on assays. However, our previous data clearly showed that angiogenin enhances the formation of RNA products containing the initiation site sequence of 45S rRNA (*16*), suggesting that reinitiation indeed takes place in the presence of angiogenin. Studies are underway to determine the possible interaction of angiogenin with RNA polymerases.

ABE has no transcription-stimulatory activity when inserted in either pGL3P or pGL3C regardless of the insertion site and orientation, indicating that ABE does not serve as an enhancer and does not stimulate transcription when a promoter is already in place.

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