Antisense display—a method for functional gene screening: evaluation in a cell-free system and isolation of angiogenesis-related genes

Hideto Yonekura, Hideyuki Migita, Shigeru Sakurai, Hua Wang, Shin-ichi Harada[1], Md. Joynal Abedin, Sho-ichi Yamagishi and Hiroshi Yamamoto*

Department of Biochemistry and [1]Center for Biomedical Research and Education, Kanazawa University School of Medicine, Kanazawa 920-8640, Japan

ABSTRACT

Presented here is an antisense-oriented method for functional gene screening, which we propose naming 'antisense display'. In principle, it consists of four steps: (i) preparation of phosphorothioate antisense repertoires that would correspond to the Kozak's consensus sequence, (ii) subgroup screening to identify active antisense molecules that could cause changes in the cellular phenotypes concerned and (iii) RT–PCR cloning of cDNA with the 5' sense complement and 3' anchor primers and sequence determination, followed by (iv) functional assays of candidate genes. Cell-free translation in rabbit reticulocyte lysate revealed that 10mer or longer antisense effectively halted protein synthesis. This required the presence of RNase H, and was achieved without prior heat-denaturation of the RNA templates. Then, subpools of the 10mer repertoire were administered to human microvascular endothelial cells in culture, and screened for anti-angiogenic activities. A single species having the sequence 5'-GGCTCATGGT-3' consistently inhibited the endothelial cell growth under hypoxia. Through RT–PCR with the corresponding sense primer, we came across three candidate cDNAs. Experiments employing longer unique antisense reproduced marked growth inhibitions in two of the three cDNAs. One encoded a mitochondrial protein and the other, which encoded a putative type-2 membrane protein containing Rab-GAP/TBC and EF-hand like domains, was a gene previously undescribed in human. The results suggest that the antisense display method is potentially useful for isolating new genes towards elucidating their functions.

INTRODUCTION

The mammalian genome contains approximately 70 000 different genes, and ~15% of them are thought to be expressed in individual cells (1). Which sets of genes are expressed will determine vital cellular processes such as differentiation, signal transduction and growth regulation. Health and diseases are also influenced by the (mal)expression of genes and (dys)function of their products. Therefore, it is of prime importance in molecular and cell biology to discover how gene expressions and functions change during various biological and pathological processes. Differential display, a method originally developed by Liang and Pardee (2), has been used to detect altered gene expression in eukaryotic cells. The key element of this method is to use arbitrary, short oligonucleotides for quantitative amplification of a diverse set of mRNAs expressed in certain cells under various conditions. Despite the wide usage and reliability of this method, determining the functions of the genes identified has usually been a difficult task. Insertional mutagenesis and rescue cloning with transgenic animals have been used to identify over a dozen genes based on their functions *per se* (3,4). Such an approach is promising because it can elucidate relationships between certain genic loci and their phenotypic accounts, with mice now being regarded as a good source for yet unidentified genes (3). However, this approach is laborious and time consuming.

In this study, we have attempted to develop a much simpler method for functional gene isolation. Experience with the differential display and antisense methodologies (5–9) led us to the notion of using short, arbitrary antisense oligonucleotides for identifying the genes responsible for various cellular phenotypes. Figure 1 depicts a flow chart of this method, tentatively named 'antisense display'. In brief, the first step is to prepare repertoires of antisense complements around the Kozak's consensus sequence (10) in order to mask almost all the mRNA species. The second step is to screen for active antisense species that can block the cellular functions concerned. The third step consists of isolation by reverse transcription–polymerase chain reaction (RT–PCR) of the corresponding cDNA, and sequence determination; with the sense counterpart

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as the 5' primer and 3' anchor-type primers, the entire open reading frame can be expected to be covered by this single step. The fourth step is to confirm the functional role of the corresponding gene with unique antisense against different regions of the same mRNA or with cDNA-expression vectors.

The present paper shows our initial attempts toward establishing the antisense display method. The first part deals with the evaluation of antisense repertoires in a cell-free translation system. The second part demonstrates its application to a cell culture system.

MATERIALS AND METHODS

DDBJ/EMBL/GenBank database accession nos AB017114 (AD 3), AB017115 (AD 2), AB017116 (AD 1) and AB024057 (full-length AD 3).

Materials

[³H]Thymidine (90 Ci/mmol) and [³⁵S]methionine (1175 Ci/mmol) were purchased from Dupont NEN (Boston, MA). Rabbit reticulocyte lysate was from Promega (Madison, WI). RNase H was from TOYOBO (Osaka, Japan). Cytolite and MTT reagents were from Packard (Groningen, Netherlands) and Chemicon International Inc. (Temecula, CA), respectively. The Marathon cDNA amplification kit and RNA blot derived from multiple human tissues were from Clontech Laboratories (Palo Alto, CA). The TA cloning kit was from InVitrogen Corp. (Carlsbad, CA).

Cells

Endothelial cells from human skin microvessels (Cascade Biologics, Inc., Portland, OR) were maintained in a Hu-Media MV2 medium supplemented with 5% fetal bovine serum (FBS), 5 ng/ml basic fibroblast growth factor, 10 µg/ml heparin, 10 ng/ml epidermal growth factor, 1 µg/ml hydrocortisone and 39.3 µg/ml dibutyryl cyclic AMP according to the supplier's instructions (Kurabo Corp., Osaka, Japan). Cells at 5–10 passages were used for the experiments. When assayed for their growth ability, cells were cultured in medium supplemented only with 5% FBS, 5 ng/ml basic fibroblast growth factor and 10 µg/ml heparin. Human lung arterial vascular smooth muscle cells (Cascade Biologics) were maintained in a Hu-Media SG2 medium supplemented with 5% FBS, 2 ng/ml basic fibroblast growth factor, 5 µg/ml insulin and 0.5 ng/ml epidermal growth factor according to the supplier's instructions (Kurabo Corp.). MRC-5 cells, a fibroblast cell line derived from human fetal lung, were cultured as described previously (11).

Synthesis of oligonucleotides

6mer to 11mer oligodeoxyribonucleotide repertoires, 10mer subpools and subgroups were synthesized as mixtures of 4 nucleotides at non-consensus positions and C or T at their 3'-termini, using an Applied Biosystems Inc. model 392 DNA synthesizer by the phosphorothioate approach with tetraethylthiuram disulfide (12). The oligodeoxyribonucleotides were purified by two cycles of reverse-phase HPLC. Longer oligodeoxyribonucleotides were synthesized at TOAGOSEI Co. (Tokyo).

Cell-free translation

500 ng of poly(A)+ RNA from bovine placenta were translated in 25 µl of rabbit reticulocyte lysate containing [³⁵S]methionine in the presence or absence of 0.1 U/µl RNase H, antisense oligonucleotides or control oligo(dA) at 30°C for 1 h. Incorporation of ³⁵S-radioactivity into translation products was determined as described previously (13). In brief, 2 µl of the mixture were spotted onto Whatman No.1 paper filter and precipitated in ice-cold 5% (w/v) trichloroacetic acid (TCA) for 15 min. Then, the filter was placed in 5% TCA at 95°C for 15 min, and washed once with ice-cold 5% TCA, once with ethanol and once with acetone. ³⁵S-radioactivity was then measured by liquid scintillation counting. Another 2 µl aliquot of the *in vitro* translation products was analyzed by SDS–polyacrylamide gel electrophoresis under reducing conditions, followed by autoradiography.

Cell proliferation assays

Human microvascular endothelial cells were seeded at a density of 2×10^3 cells per well in a 96-well plate, and incubated at 37°C under an atmosphere of 5% O_2/5% CO_2/90% N_2 in 0.1 ml of the culture medium containing or not containing the oligonucleotides. After incubation, cell proliferation was assessed with Cytolite reagents (Packard) (14), by the MTT method (15) or by [³H]thymidine incorporation (8).

RT–PCR cloning and sequencing

Poly(A)+ RNA was isolated from human microvascular endothelial cells with a Quick prep mRNA purification kit (Pharmacia), and reverse-transcribed with a 3' anchored primer ($T_{15}N$). The resultant cDNA was PCR-amplified with the 5' sense complement of the functional 10mer antisense, the 3' anchor primer and rTth DNA polymerase (Perkin Elmer). Thermal cycling parameters were as follows: 94°C (30 s), 40°C (30 s) and 68°C (4 min). The PCR products were cloned into a pCR2.1 vector (InVitrogen), and the resultant plasmids were purified using a Flexiprep plasmid isolation kit (Pharmacia). Upstream and downstream extensions of cDNA were carried out under a 5'- and 3'-rapid amplification of cDNA end (RACE) protocols (16). The 5' end of AD 3 mRNA was determined using a human microvascular endothelial cell-derived oligo-capped cDNA library (Nippon Gene, Toyama, Japan). By this cap site cDNA cloning method, unique cap sites can be reliably identified (17,18). Nucleotide sequences were determined with an Applied Biosystems Inc. model 373 DNA sequencer (Perkin Elmer). Sequence homology was searched for with the GenBank and the EMBL databases by BLAST algorisms.

Northern blot analysis

Northern blot analysis was performed as described (19). In brief, 2 µg each of poly(A)+ RNAs from human endothelial cells, smooth muscle cells and fibroblasts were electrophoresed on a 1.5% agarose gel containing 1.1 M formaldehyde, transferred to a Hybond N+ nylon membrane (Amersham), and hybridized to a cloned cDNA fragment which had been labeled with [α-³²P]dCTP by the random labeling method (20). After hybridization, the membrane was washed and autoradiographed. Northern blot derived from multiple human tissues containing 2 µg of poly(A)+ RNA per
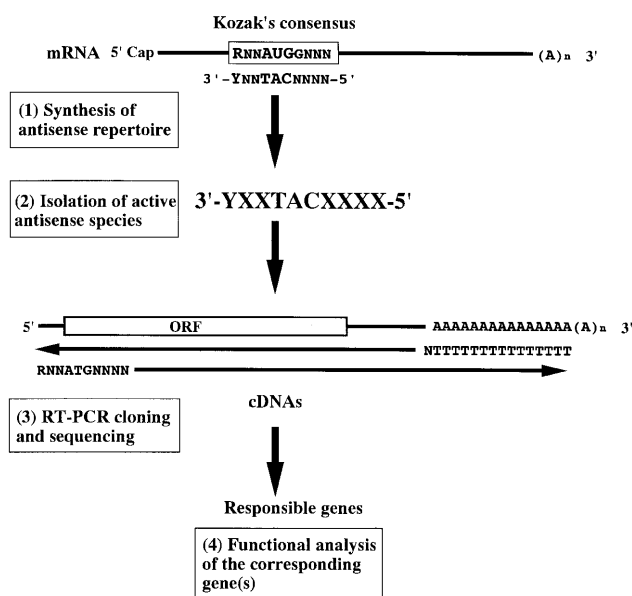
Figure 1. Flow chart of the antisense display method.

lane was probed with the ^{32}P-labeled AD 3 cDNA fragment. The blots were reprobed with a human β-actin cDNA fragment.

RESULTS

Design of antisense repertoires

As the mRNA region to be targeted by the antisense display method, we chose the Kozak's consensus sequence (10) around the initiation codon. The rationale for this was that this region is known to usually form a loop structure which would be accessible to antisense (5–9,21,22), and also because the consensus sequence would specify complementary nucleotides and thus minimize the diversity of the antisense repertoires.

The consensus sequence is composed of purine at nucleotide –3, AUG at +1 to +3, and G at +4 (Table 1), the occurrence of which in vertebrate mRNAs is reported to be 97, 100 and 46%, respectively (10). Accordingly, we selected –3 purine as the 5'

end of the target. The 3' terminal of the antisense oligodeoxy-ribonucleotides would then be T or C. At 3–5 nucleotides 5' to it, TAC corresponding to the AUG codon followed. +4 G was not considered because of its relatively infrequent occurrence. Table 1 lists the repertoires of the prepared phosphorothioate antisense. Their lengths ranged from 6mer to 11mer, the respective diversity being from 32 to 32 768 species. These arbitrary antisense oligos would thus be theoretically capable of masking the majority of mammalian mRNA species.

Evaluation in a cell-free system

The effects of the 6mer to 11mer antisense repertoires on mRNA translation were first tested in a cell-free system. Protein synthesis was conducted in reticulocyte lysate using bovine placental poly(A)[+] RNA as a template in the presence of each set of arbitrary antisense oligonucleotides. Oligo(dA)s having the same chain lengths were used as controls. As shown in Figure 2A, [^{35}S]methionine incorporation into acid-insoluble fractions was found to decrease as the length of the antisense oligos increased, while the control oligo(dA)s essentially gave no changes. The antisense inhibition required the addition of RNase H; without it, ^{35}S-radioactivity incorporation did not significantly differ either among the six antisense repertoires or between each repertoire and the respective oligo(dA) control (data not shown). When the translation products were analyzed by SDS–polyacrylamide gel electrophoresis, both the number of bands and their intensities were found to decrease as the length of the antisense increased (Fig. 2B). The repertoires longer than 10mer almost totally abolished the poly(A)[+] RNA-dependent protein synthesis (Fig. 2B), indicating that these arbitrary antisense oligos can inhibit the translation of the majority of mRNA species. Accordingly, subsequent evaluations were conducted with the 10mer repertoire.

As shown in Figure 3A, the 10mer arbitrary antisense inhibited the *in vitro* protein synthesis in a dose-dependent manner; at 1.3 μM, the ^{35}S-radioactivity incorporation began to be inhibited, then linearly decreased, and at >13.2 μM reached near the level without the template. The same concentrations of oligo(dA)$_{10}$ only slightly inhibited ^{35}S-protein synthesis.

In standard assays, template RNAs are heat-denatured prior to starting the translation (13). In viable cell systems, however, such high temperatures cannot be applied. We therefore tested whether the template activity was changed when RNA had been placed under various temperatures. As shown in

Table 1. Antisense repertoires corresponding to the Kozak's consensus sequence

		–3	–2	–1	+1	+2	+3	+4	+5	+6	+7	+8		
mRNA	5'-	R	N	N	A	U	G	G	N	N	N	N	-3'	
11mer	3'-	Y	N	N	T	A	C	N	N	N	N	N	-5'	(32 768)
10mer	3'-	Y	N	N	T	A	C	N	N	N	N	-5'		(8192)
9mer	3'-	Y	N	N	T	A	C	N	N	N	-5'			(2048)
8mer	3'-	Y	N	N	T	A	C	N	N	-5'				(512)
7mer	3'-	Y	N	N	T	A	C	N	-5'					(128)
6mer	3'-	Y	N	N	T	A	C	-5'						(32)

Numbers on the top indicate nucleotide positions in mRNA with the first letter of the initiation codon counted as +1. R, purine; N, mixture of the four bases; Y, pyrimidine. Numbers in parentheses indicate total number of antisense species in each repertoire.
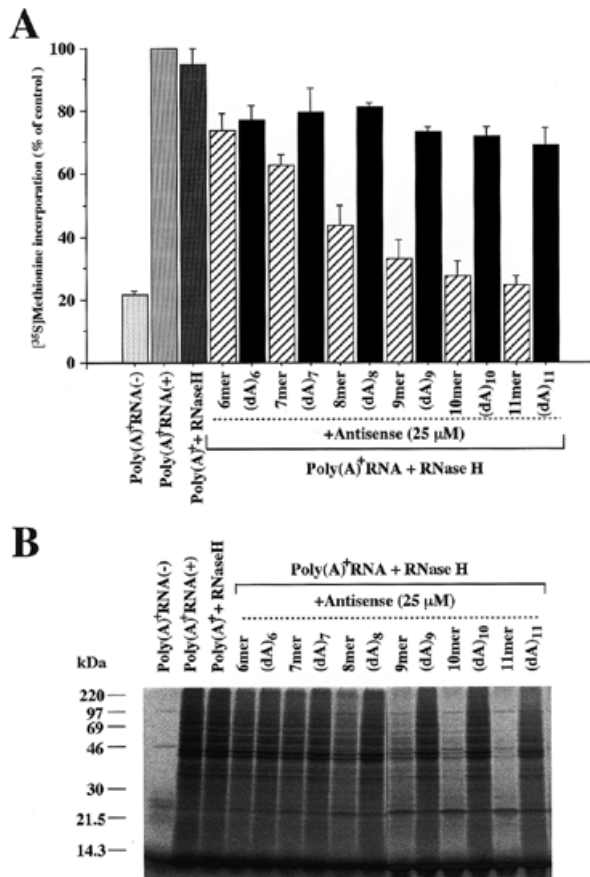
Figure 2. Effects of arbitrary antisense oligonucleotides on *in vitro* protein synthesis. (**A**) [^{35}S]Methionine incorporation. 500 ng of bovine placenta poly(A)$^+$ RNA were translated in rabbit reticulocyte lysate containing RNase H and [^{35}S]methionine in the presence of 25 μM each of 6mer to 11mer antisense repertoires or respective control oligo(dA), and the products were assayed for [^{35}S]methionine incorporation as described in Materials and Methods. Columns indicate the average values of three independent experiments. Bars indicate standard errors. (**B**) SDS–polyacrylamide gel electrophoresis of the translation products. The reaction mixture (2 μl) was electrophoresed on 12.5% SDS–polyacrylamide gels under reducing conditions. After electrophoresis, gels were dried and analyzed with BAS-1000 (Fuji Film, Hamamatsu, Japan). Positions of size markers are presented on the left.

Figure 3. (**A**) Dose-dependency in the arbitrary antisense inhibition of the *in vitro* protein synthesis. The translation reaction was performed under the indicated concentrations of the 10mer antisense mixture (closed circle) or oligo(dA)$_{10}$ (open circle), and assayed for [^{35}S]methionine incorporation. Symbols indicate the mean values of three separate experiments. Bars represent standard errors. (**B**) Antisense effects after placing template RNA at various temperatures. Previously non-heat-denatured bovine placenta poly(A)$^+$ RNAs were incubated at the indicated temperatures for 10 min with or without 25 μM 10mer oligos, and then *in vitro* translated at 30°C for 60 min. Columns indicate the average values of three independent experiments. Bars indicate standard errors.

Figure 3B, the translation with the RNA template pretreated at 37°C was efficiently inhibited by the 10mer antisense to an extent similar to that with RNA pretreated at 67°C, the condition usually employed for denaturation. This suggests that mRNAs within cells can achieve access to the arbitrary antisense oligonucleotides under the physiological temperature.

Application to a cell culture system: screen for angiogenesis-related genes

We then applied the antisense display method to cells in culture. As a model system, human microvascular endothelial cells were employed, and angiogenesis-related genes were screened for.

First, the 10mer antisense repertoire was divided into 128 subpools, each containing 64 independent sequences. They were added to culture media to a final concentration of 64 μM, and cells were incubated at 37°C for 72 h under
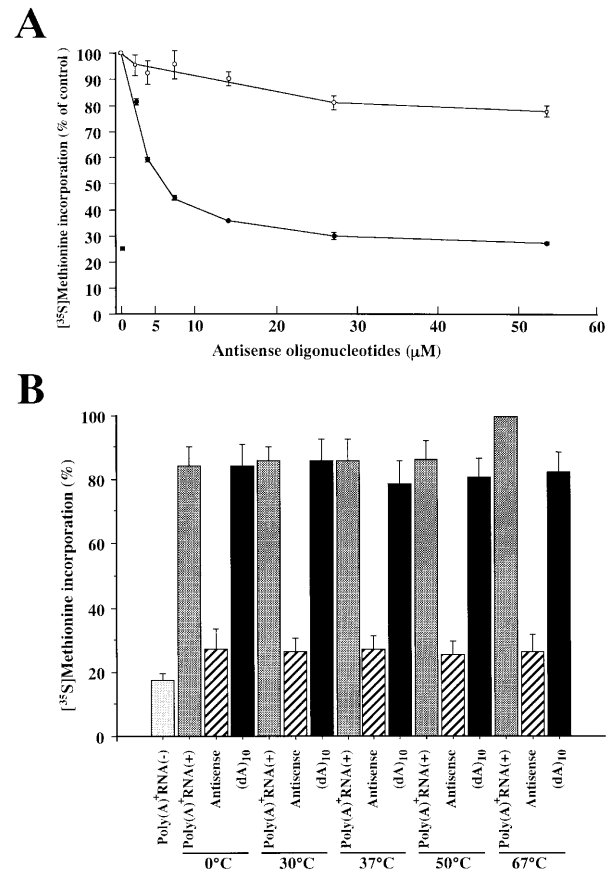
hypoxia, the principal inducer of angiogenesis (8), after which their growth was assayed. In the initial screening, several subpools were inhibitory but only one subpool designated T39 exhibited a consistent inhibition of the endothelial cell growth in several separate experiments (data not shown). The T39 subpool then underwent second and third screenings (Fig. 4). In this screening, we could not find subpools whose antisense effects were growth-stimulatory.

Then, we divided the T39 10mer subpool into 16 subgroups, each composed of four independent sequences, and assayed. As shown in Figure 4A, one subgroup designated T39l was found to be growth-inhibitory. The third screening revealed that, among the four unique 10mer sequences, one species designated T39l-3 could effectively inhibit the proliferation of endothelial cells (Fig. 4B). Its sequence was 5'-GGCTCAT-GGT-3'.
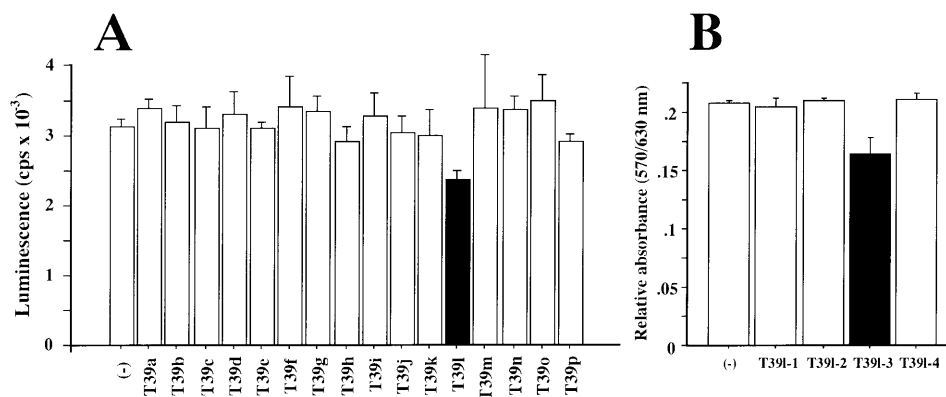
Figure 4. Screen for angiostatic antisense. (**A**) Second screening. The subpool T39 judged as positive in the first screen was divided into 16 subgroups designated T39a–T39p, each consisting of four independent sequences. The subgroups were administered to the culture of human microvascular endothelial cells at 20 μM, and cells were incubated at 37°C under 5% O_2 for 72 h. After incubation, cell proliferation was assayed with Cytolite (Packard) as during the first screening. Ordinate indicates Cytolite luminescence liberated from viable cells. Values are expressed as the mean ± SE in triplicate determinations. The subpool T39l judged as positive in the second screen (closed bar). (**B**) Third screening. Four unique antisense species in the positive subgroup T39l were added to the culture media at 10 μM, and endothelial cells were incubated at 37°C under 5% O_2 for 72 h, after which cell proliferation was assessed by the MTT method. Ordinate indicates the ratio of absorbances at 570 nm versus 630 nm. Values are expressed as the mean ± SE in triplicate determinations. The unique antisense species T39l-3 judged as positive in the third screening (closed bar).

RT–PCR identification of candidate mRNA

To identify mRNA sequences that would have been targeted by the T39l-3 antisense, poly(A)+ RNA from the endothelial cells was analyzed by RT–PCR with the corresponding 5'-sense decamer (5'-ACCATGAGCC-3') and 3'-anchored oligo(dT) primers. This approach yielded three candidate cDNA clones designated AD (antisense display) 1, 2 and 3. Nucleotide sequence analysis of the cloned cDNAs and comparison with the sequences stored in DNA databases revealed that they were derived from three different genes.

As shown in Figure 5A, AD 1 was 130 nt long and had a sequence identical to the known sequence of the mitochondrial NADH-ubiquinone oxidoreductase chain 3 cDNA (23). The human mitochondrial genome contained one 10-nt stretch that perfectly matched the T39l-3 sequence only at this locus (nucleotide no. 10283–10292). However, it corresponded to an internal AUG sequence but not to the initiator codon AUA of the oxidoreductase subunit mRNA. AD 2 was 786 nt long, had the same sequence as the cDNA for KIAA0551 protein (24) and seemed to be primed from an A-rich region of the mRNA by oligo(dT) primer (Fig. 5B). AD 3 was 691 nt long (Fig. 5C) and met no sequences that were identical to it except for several ESTs (expressed sequence tags). Since AD 3 appeared to correspond to a novel gene, we isolated and sequenced the full-length AD 3 cDNA by using cap site labeling (17,18) and RACE protocols. The full-length cDNA stretched 4404 nucleotides, not counting the poly(A) tract (DDBJ/EMBL/GenBank accession no. AB024057). The AD 3 cDNA fragment initially obtained by RT–PCR seemed to be primed from an A-rich region of the mRNA by oligo(dT) primer (Fig. 5C). Both the KIAA0551 protein cDNA and AD 3 cDNA contained only one stretch each that was homologous to the T39l-3 10mer antisense: In both cases, 9 nucleotides out of 10 were complementary to the T39l-3 sequence, but did not reside at the initiator codon regions (Fig. 5B and C).

Functional assays of the candidate mRNAs

We then investigated which candidate(s) took an active part in the regulation of the endothelial cell growth. For this, we synthesized longer antisense oligos spanning those 10-nt regions and their sense complements, each of which had an overall sequence unique to the respective candidate mRNA; they were 16–18 nt long with T_m at ~60°C. As shown in Figure 6A, the endothelial cell synthesis of DNA was markedly inhibited by the long antisense oligos against AD 1 and AD 3 (AD 3a), but not by the antisense against AD 2. The sense controls gave no changes. In addition, a long antisense against a different region of AD 3 mRNA (AD 3b) also specifically inhibited DNA synthesis by the endothelial cells (Fig. 6). Next, we conducted northern blot analyses to determine the intracellular concentrations of putative target mRNAs in cells treated with antisense or sense oligonucleotides. In northern blot analyses, antisense oligo against AD 1 as well as T39l-3 caused a decrease in AD 1 mRNA (Fig. 6B), and antisense oligos against AD 3 (AD 3a and AD 3b) as well as T39l-3 also caused decreases in AD 3 mRNA (Fig. 6D). The sense controls gave no significant changes (Fig. 6B and D). These seemed to be indications that the oligos did act on the target mRNAs and that RNase H-like activity-driven degradation of target mRNA was primarily responsible for the antisense action. Furthermore, it seemed likely that blockade of ribosomal readthrough might also have accounted in part for the antisense action in viable cell systems, because the degree of DNA synthesis inhibition was above that of mRNA. These results suggest that, among the three candidates, genes corresponding to AD 1 and 3 may have roles in the growth of endothelial cells. AD 2 antisense as well as T39l-3 caused only a slight decrease in AD 2 mRNA (Fig. 6C). This suggested that, in the RT–PCR reactions, the 10mer region was accessible to the sense primer under denatured conditions, but in the viable cell system the 10mer region in AD 2 mRNA could form a structure that was hardly accessible to the antisense. These results would thus indicate that AD 2 was not an actual target of T39l-3 in this screening.

A

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AD 1          ACCATGAGCCCTACAAACAACTAACCTGCCACTAATAGTTATGTCATCCCTCTTATTAAT    60
              ************************************************************
Mitochondria  ACCATGAGCCCTACAAACAACTAACCTGCCACTAATAGTTATGTCATCCCTCTTATTAAT  10342

AD 1          CATCATCCTAGCCCTAAGTCTGGCCTATGAGTGACTACAAAAAGGATTAGACTGAGCCGA   120
              ************************************************************
Mitochondria  CATCATCCTAGCCCTAAGTCTGGCCTATGAGTGACTACAAAAAGGATTAGACTGAGCCGA  10402

AD 1          ATAAAAAAAA   130
              **
Mitochondria  AT   10404
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B

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AD 2          ACCATGAGCCTGCCAAGGTGAAACCAGAAGAATCCAGGGACATT    44
              ********************************************
KIAA0551      GGATCACCTGTGCTTCCCCATGAGCCTGCCAAGGTGAAACCAGAAGAATCCAGGGACATT  2280

AD 2          ACCCGGCCCAGTCGACCAGCT-----------------------GATCTGACGGCATTA    80
              ********************                       **************
KIAA0551      ACCCGGCCCAGTCGACCAGCTAGCTACAAAAAAAGCTATAGATGAGGATCTGACGGCATTA  2340

AD 2          GCCAAAGAACTAAGAGAACTCCGGATTGAAGAAACAAACCGCCCAATGAAGAAGGTGACT   140
              ************************************************************
KIAA0551      GCCAAAGAACTAAGAGAACTCCGGATTGAAGAAACAAACCGCCCAATGAAGAAGGTGACT  2400

AD 2          GATTACTCCTCCTCCAGTGAGGAGTCAGAAAGTAGCGAGGAAGAGGAGGAAGATGGAGAG   200
              ************************************************************
KIAA0551      GATTACTCCTCCTCCAGTGAGGAGTCAGAAAGTAGCGAGGAAGAGGAGGAAGATGGAGAG  2461

AD 2          AGCGAGACCCATGATGGGACAGTGGCTGTCAGCGACATACCCAGACTGATACCAACAGGA   200
              ************************************************************
KIAA0551      AGCGAGACCCATGATGGGACAGTGGCTGTCAGCGACATACCCAGACTGATACCAACAGGA  2520

AD 2          GCTCCAGGCAGCAACGAGCAGTACAATGTGGGAATGGTGGGGACGCATGGGCTGGAGACC   320
              ************************************************************
KIAA0551      GCTCCAGGCAGCAACGAGCAGTACAATGTGGGAATGGTGGGGACGCATGGGCTGGAGACC  2580

AD 2          TCTCATGCGGACAGTTTCAGCGGCAGTATTTCAAGAGAAGGAACCTTGATGATTAGAGAG   380
              ************************************************************
KIAA0551      TCTCATGCGGACAGTTTCAGCGGCAGTATTTCAAGAGAAGGAACCTTGATGATTAGAGAG  2640

AD 2          ACGTCTGGAGAGAAGAAGCGATCTGGCCACAGTGACAGCAATGGCTTTGCTGGCCACATC   440
              ************************************************************
KIAA0551      ACGTCTGGAGAGAAGAAGCGATCTGGCCACAGTGACAGCAATGGCTTTGCTGGCCACATC  2700

AD 2          AACCTCCCTGACCTGGTGCAGCAGAGCCATTCTCCAGCTGGAACCCCGACTGAGGGACTG   500
              ************************************************************
KIAA0551      AACCTCCCTGACCTGGTGCAGCAGAGCCATTCTCCAGCTGGAACCCCGACTGAGGGACTG  2761

AD 2          GGGCGCGTCTCAACCCATTCCCAGGAGATGGACTCTGGGACTGAATATGGCATGGGGAGC   560
              ************************************************************
KIAA0551      GGGCGCGTCTCAACCCATTCCCAGGAGATGGACTCTGGGACTGAATATGGCATGGGGAGC  2820

AD 2          AGCACCAAAGCCTCCTTCACCCCCTTTGTGGACCCCAGAGTATACCAGACGTCTCCCACT   620
              ************************************************************
KIAA0551      AGCACCAAAGCCTCCTTCACCCCCTTTGTGGACCCCAGAGTATACCAGACGTCTCCCACT  2880

AD 2          GATGAAGATGAAGAGGATGAGGAATCATCAGCCGCAGCTCTGTTTACTAGCGAACTTCTT   680
              ************************************************************
KIAA0551      GATGAAGATGAAGAGGATGAGGAATCATCAGCCGCAGCTCTGTTTACTAGCGAACTTCTT  2940

AD 2          AGGCAAGAACAGGCCAAACTCAATGAAGCAAGAAAGATTTCGGTGGTAAATGTAAACCCA   740
              ************************************************************
KIAA0551      AGGCAAGAACAGGCCAAACTCAATGAAGCAAGAAAGATTTCGGTGGTAAATGTAAACCCA  3000

AD 2          ACCAACATTCGGCCTCATAGCGACACACCAAAAAAAAAAAAAAAAAAA   786
              ***************************  ***   *  ****  *  **
KIAA0551      ACCAACATTCGGCCTCATAGCGACACACCAGAAATCAGAAAATACAAGAAACGATTCAAC  3060
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Expression of AD 3 gene in vascular cells and various human tissues

Since AD 3 appeared to correspond to a novel gene, we conducted northern blot analysis. When poly(A)$^+$ RNAs from human skin microvascular endothelial cells, human lung arterial smooth muscle cells and MRC-5 human fibroblasts were probed with the cDNA insert of AD 3, a major hybridizing band was clearly marked at 5 kb (Fig. 7A). The level of the mRNA in endothelial cells was 7- and 14-fold higher than those in smooth muscle cells and fibroblasts, respectively. The results thus indicated the presence of AD 3 mRNA in vascular cells. We also analyzed the expression of AD 3 mRNA in various human tissues. Northern analysis of human tissue poly(A)$^+$ RNAs showed that AD 3 mRNA is present in nearly all human tissues examined (Fig. 7A). AD 3 was highly expressed in the heart, skeletal muscle and pancreas, barely detectable in the

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RT-PCR       1  ACCATGAGCCCTACCCTGTGACTGATATTTCGGACCTGATC       41
                **** ********************************
AD3 cDNA     CATGCCTTTTTCTCCGACGACCAGGAGCCCTACCCTGTGACTGATATTTCGGACCTGATC   2680


RT-PCR       CGGGATTCCTATGAGAAATTTGGAGACCAGTCTGTGGAGCAGATCGAGCACCTACGTTAC   101
             ************************************************************
AD3 cDNA     CGGGATTCCTATGAGAAATTTGGAGACCAGTCTGTGGAGCAGATCGAGCACCTACGTTAC   2740


RT-PCR       AAGCACAGGATCAGGGTCCTCCAAGGCCACGAGGACACCACAAAGCAGAACGTGCTTCGA   161
             ************************************************************
AD3 cDNA     AAGCACAGGATCAGGGTCCTCCAAGGCCACGAGGACACCACAAAGCAGAACGTGCTTCGA   2800


RT-PCR       GTCGTTATCCCGGAAGTCTCAATTCTTCCTGAAGACCTAGAGGAGCTCTACGACTTATTC   221
             ************************************************************
AD3 cDNA     GTCGTTATCCCGGAAGTCTCAATTCTTCCTGAAGACCTAGAGGAGCTCTACGACTTATTC   2860


RT-PCR       AAGAGAGAACATATGATGAGCTGTTACTGGGAGCAGCCCAGGCCCATGGCCTCACGCCAC   281
             ************************************************************
AD3 cDNA     AAGAGAGAACATATGATGAGCTGTTACTGGGAGCAGCCCAGGCCCATGGCCTCACGCCAC   2920


RT-PCR       GACCCCAGCCGGCCCTATGCTGAGCAGTACCGCATAGACGCCCGGCAGTTTGCACACCTG   341
             ************************************************************
AD3 cDNA     GACCCCAGCCGGCCCTATGCTGAGCAGTACCGCATAGACGCCCGGCAGTTTGCACACCTG   2980


RT-PCR       TTTCAGCTAGTCTCGCCCTGGACCTGCGGGGCCCACACGGAGATCCTCGCCGAAAGGACG   401
             ************************************************************
AD3 cDNA     TTTCAGCTAGTCTCGCCCTGGACCTGCGGGGCCCACACGGAGATCCTCGCCGAAAGGACG   3040


RT-PCR       TTCAGGCTCTTGGATGACAACATGGACCAGCTCATCGAGTTCAAAGCGTTTGTGAGCTGC   461
             ************************************************************
AD3 cDNA     TTCAGGCTCTTGGATGACAACATGGACCAGCTCATCGAGTTCAAAGCGTTTGTGAGCTGC   3100


RT-PCR       CTCGATATTATGTATAATGGAGAAATGAATGAGAAGATTAAACTATTATACAGGCTTCAT   521
             ************************************************************
AD3 cDNA     CTCGATATTATGTATAATGGAGAAATGAATGAGAAGATTAAACTATTATACAGGCTTCAT   3160


RT-PCR       ATCCCTCCAGCACTCACTGAAAATGACCGAGACAGCCAGTCGCCGTTGAGGAATCCTCTG   581
             ************************************************************
AD3          ATCCCTCCAGCACTCACTGAAAATGACCGAGACAGCCAGTCGCCGTTGAGGAATCCTCTG   3220


RT-PCR       TTGTCAACATCGAGACCCCTGGTTTTCGGGAAACCCAATGGTGATGCAGTTGATTATCAG   641
             ************************************************************
AD3 cDNA     TTGTCAACATCGAGACCCCTGGTTTTCGGGAAACCCAATGGTGATGCAGTTGATTATCAG   3280


RT-PCR       AAACAGCTGAAGCAGATGATTAAGGATTTAGCCAAAAAAAAAAAAAAAAAAA          691
             ***********************************  *****  * ****
AD3 cDNA     AAACAGCTGAAGCAGATGATTAAGGATTTAGCCAAAGAAAAAGATAAAACTGAGAAAGAA   3340
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Figure 5. (Opposite and above) Nucleotide sequences of cloned cDNAs (AD 1, AD 2 and AD 3). (**A**) Upper sequence represents the nucleotide sequence of AD 1 isolated by the RT–PCR cloning. The lower one represents the mitochondrial sequence for NADH-ubiquinone oxidoreductase chain 3 (23) aligned to maximize homology. Asterisks indicate identical nucleotides. Double underlines indicate nucleotides matched to the 10mer sense primer used in the RT–PCR cloning. Underlines indicate nucleotides corresponding to the longer antisense oligos (AD 1) used in the functional assay (Fig. 6). (**B**) Upper sequence represents the nucleotide sequence of AD 2. The lower one represents the sequence of cDNA for KIAA0551 protein (24) aligned to maximize homology. Nine nucleotides out of 10 were complementary to the T39l-3 sequence, and did not reside at the initiator codon regions. Double underlines indicate nucleotides matched to the 10mer sense primer used in the RT–PCR cloning. Underlines indicate nucleotides corresponding to the longer antisense oligos (AD 2) used in the functional assay. (**C**) Upper sequence represents the nucleotide sequence of AD 3 isolated by the RT–PCR cloning. The lower one represents the sequence of the full-length AD 3 cDNA (DDBJ/EMBL/ GenBank accession no. AB024057) which was obtained by cap site cDNA and RACE approaches. Nine nucleotides out of 10 were complementary to the T39l-3 sequence, and did not reside at the initiator codon regions. Double underlines indicate nucleotides matched to the 10mer sense primer used in RT–PCR. Underlines indicate nucleotides corresponding to the longer antisense oligos (AD 3a) used in the functional assay.

brain, placenta and lung, and at intermediate levels in the liver and kidney. The major AD 3 transcript was 5 kb, while trace amounts of an ~7 kb form were also detected.

DISCUSSION

In this study, we have attempted to develop an antisense-oriented method for functional gene screening, which we propose naming 'antisense display' (Fig. 1). Studies in many laboratories, including our own, have shown that antisense oligos against the region around the translation initiation codon work better than those against other regions of mRNA (5–9,21,22), and it has been suggested that an 11mer oligonucleotide, or perhaps smaller, could bind to a unique sequence if the non-random nature of the mammalian RNA sequence is taken into account (21,22). Accordingly, we synthesized 6mer to 11mer antisense phosphorothioate oligonucleotide repertoires corresponding to Kozak's sequon (Table 1), and evaluated first their effects on cell-free protein synthesis. The *in vitro* evaluation demonstrated that antisense oligonucleotides longer than 10mer specifically and efficiently inhibited protein synthesis in a manner dependent on RNase H (Figs 2 and 3), the degradation by which has been regarded as the major mode of action of phosphorothioate antisense oligodeoxyribonucleotides (25).
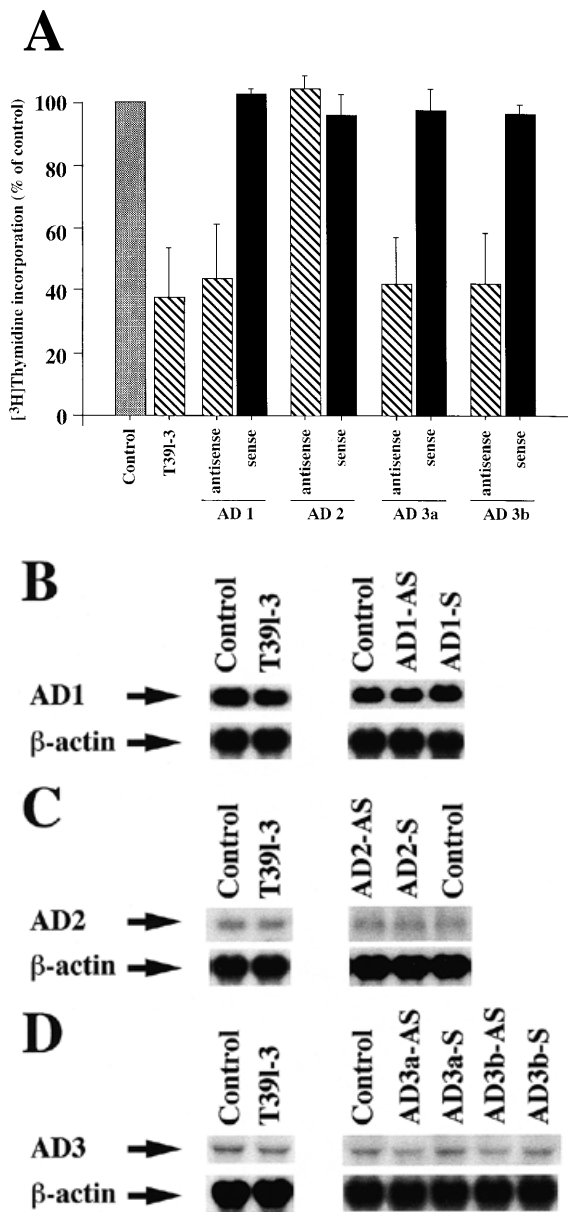
A

B

C

D

Figure 6. (**A**) Confirmation of the function of candidate mRNAs. Effects of longer oligodeoxyribonucleotide complements of regions spanning the 10mer sequence (AD 1, AD 2 and AD 3a, Fig. 5) and of a different region of AD 3 mRNA (AD 3b) on endothelial cell growth. Antisense sequences corresponding to AD 1, AD 2, AD 3a and AD 3b were 5'-GTTTGTAGGGCTCATGGT-3', 5'-TTGGCAGGCTCATGGG-3', 5'-GGGTAGGGCTCCTGGT-3' and 5'-GCTCGTGTCCTCCATCTT-3' (the last one corresponding to nucleotides 1439–1456 of the full-length AD 3 cDNA), respectively. These antisense oligos and their sense controls were added to culture media to a final concentration of 10 μM, and cells were incubated at 37°C for 24 h under 5% O_2, after which cells were labeled with [^{3}H]thymidine for 4 h. Columns indicate the mean values of three independent experiments. Bars, standard errors. Effects of antisense oligos on endothelial cell contents of AD 1 (**B**), AD 2 (**C**) and AD 3 (**D**) mRNAs. The human microvascular endothelial cells were incubated in medium containing 10 μM antisense or sense oligos for 48 h, after which total RNAs were isolated as described previously (19). Ten μg of the RNA were electrophoresed and analyzed by northern blotting using AD 1, AD 2 or AD 3 cDNA fragments as probes. AD 1, AD 2 and AD 3 mRNAs were detected at ~0.4, ~10 and ~5 kb, respectively (see also Fig. 7 for AD 3 mRNA). Hybridization to 1.9-kb β-actin transcript is shown in the bottom panel as a loading control.
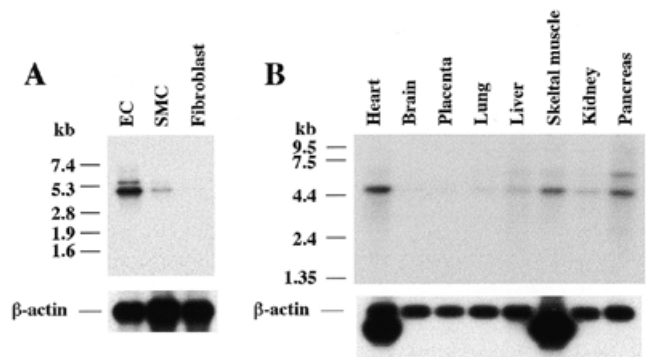

Figure 7. (**A**) Probing poly(A)$^+$ RNA from vascular cells with AD 3 cDNA. Two μg each of poly(A)$^+$ RNAs isolated from human skin microvascular endothelial cells (EC), human lung arterial smooth muscle cells (SMC) and MRC-5 human fibroblasts (Fibroblast) were electrophoresed on 1.5% agarose gel, transferred to a nylon membrane and hybridized to ^{32}P-labeled AD 3 cDNA fragment. Positions of size markers are presented on the left. The same filter hybridized to the ^{32}P-labeled β-actin probe. (**B**) AD 3 mRNA levels in human tissues. A filter containing 2 μg each of poly(A)$^+$ RNA from indicated human tissues was hybridized to the ^{32}P-labeled AD 3 cDNA fragment.

Based on the results, we chose a 10mer antisense oligonucleotide repertoire for application to cellular systems.

We applied the antisense display method to human microvascular endothelial cells in culture as a model system, and screened for angiogenesis-related genes. Angiogenesis, the formation of new capillary networks, is physiologically essential for embryogenesis, reproduction and wound healing, and is also related to various pathological states, including tumor growth and metastasis, diabetic retinopathy and rheumatoid arthritis (26,27). 128 subpools and 16 subgroups of the 10mer antisense repertoire were assayed for their abilities to affect the growth of endothelial cells, and this screening led to an identification of one species, designated T39l-3, that was consistently growth-inhibitory (Fig. 4). Subsequent RT–PCR with the corresponding sense primer and assays with longer unique antisense yielded two candidate cDNA clones, AD 1 and 3. AD 1 mRNA encoded mitochondrial NADH-ubiquinone oxidoreductase chain 3. Since it is involved in the electron transfer system in mitochondria, the gene may be related to endothelial cell growth as a housekeeping gene. To the authors' knowledge, this may be the first report that antisense oligonucleotides can target mitochondrial mRNA. Mitochondrial uptake of phosphorothioate oligos has recently been suggested (Dr Akira Murakami, Kyoto Institute of Technology, personal communication). AD 3 mRNA would seem to be derived from a previously undescribed human gene. It was expressed abundantly in endothelial cells but at much lower levels in arterial smooth muscle cells and fibroblasts (Fig. 7), suggesting that AD 3 has some endothelial cell-related functions. The AD3 cDNA had one large open reading frame of an 897-amino acid protein (Fig. 8A). Comparison of the amino acid sequence of AD 3 with the sequences stored in databases revealed that AD 3 protein exhibits a high degree of homology (90%) with a protein named mouse BUB-2 like protein (28), which has appeared in the database quite recently. AD 3 protein also has significant homology with human KIAA0676 protein (29), *Caenorhabditis elegans* Y45F10A.6 protein (30) and yeast

A

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MEQLADVTLR RLLDNEVFDL DPDLQEPSQI TKRDLEARAQ NEFFRAFFRL PRKEKLHAVV  60
DCSLWTPFSR CHTAGRMFAS DSYICFASRE DGCCKIILPL REVVSIEKME DTSLLPHPII 120
VSIRSKVAFQ FIELRDRDSL VEALLARLKQ VHANHPVHYD TSADDDMASL VFHSTSMCSD 180
HRFGDLEMMS SQNSEESEKE KSPLMHPDAL VTAFQQSGSQ SPDSRMSREQ IKISLWNDHF 240
VEYGRTVCMF RTEKIRKLVA MGIPESLRGR LWLLFSDAVT DLASHPGYYG NLVEESLGKC 300
CLVTEEIERD LHRSLPEHPA FQNETGIAAL RRVLTAYAHR NPKIGYCQSM NILTSVLLLY 360
TKEEEAFWLL VAVCERMLPD YFNHRVIGAQ VDQSVFEELI KGHLPELAEH MNDLSALASV 420
SLSWFLTLFL SIMPLESAVN VVDCFFYDGI KAIFQLGLAV LEANAEDLCS SKDDGQALMI 480
LSRFLDHIKN EDSPGPPVGS HHAFFSDDQE PYPVTDISDL IRDSYEKFGD QSVEQIEHLR 540
YKHRIRVLQG HEDTTKQNVL RVVIPEVSIL PEDLEELYDL FKREHMMSCY WEQPRPMASR 600
HDPSRPYAEQ YRIDARQFAH LFQLVSPWTC GAHTEILAER TFRLLDDNMD QLIEFKAFVS 660
CLDIMYNGEM NEKIKLLYRL HIPPALTEND RDSQSPLRNP LLSTSRPLVF GKPNGDAVDY 720
QKQLKQMIKD LAKEKDKTEK ELPKMSQREF IQFCKTLYSM FHEDPEENDL YQAIATVTTL 780
LLQIGEVGQR GSSSGSCSQE CGEELRASAP SPEDSVFADT GKTPQDSQAL PEAAERDWTV 840
SLEHILASLL TEQSLVNFFE KPLDMKSKLE NAKINQYNLK TFEMSHQSQS ELKLSNL     897
```
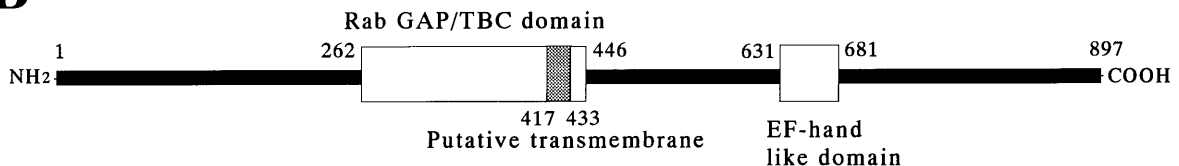
B

Rab GAP/TBC domain

```
        1                262           446      631    681           897
NH2 ┣━━━━━━━━━━━━━━━━━━━━━━[          ▓]━━━━━━━━━━[    ]━━━━━━━━━━━┫ COOH
                                    417 433
                              Putative transmembrane    EF-hand
                                                        like domain
```

Figure 8. (**A**) Amino acid sequence deduced from the full-length AD 3 cDNA sequence. The cDNA has only one large open reading frame encoding an 897-amino acid protein, which we named VRP (vascular Rab-GAP/TBC domain-containing protein). Underlines indicate TBC/Rab-GAP homology and EF-hand like domains. Double underline indicates a putative transmembrane domain. (**B**) Schematic representation of VRP.

MIC 1 protein (31). As shown in Figure 8, AD 3 protein as well as these proteins contains a conserved domain named Rab-GAP/TBC domain (32,33). Furthermore, the AD 3 protein has a putative transmembrane domain, and the C-terminal portion of AD 3 protein showed a significant homology to EF-hand-containing calcium-binding proteins (34) (Fig. 8). We thus propose naming this gene product VRP (vascular Rab-GAP/TBC domain containing protein). The physiological roles of the AD 3 gene in endothelial cell growth remain to be established.

The results obtained in this study thus suggest that the antisense display method is potentially useful for the isolation of novel functional genes. This method would have the following advantages: (i) it uses short oligonucleotides which can be easily prepared and would ensure reproducibility; (ii) it would enable the isolation of new genes based on their functions *per se*; (iii) it is applicable to a wide range of cells over various phenotypes. It should be noted here that sequence-dependent, non-antisense mechanisms like the aptamer mechanism may also work; the antisense repertoire screening has the potential to encounter active oligonucleotide molecules that can act through interactions with proteins. However, this antisense display method would seem to require several improvements. The first point concerns specificity. In the present experiments, a single 10mer antisense yielded plural candidate clones, and the targeted site of each mRNA did not correspond to the Kozak's consensus sequence. This issue may be resolved by

increasing the chain-length of the antisense oligos. The second point concerns the sensitivity. In this study, we screened subpools each of which contained 64 sequences, and were able to identify only one active antisense species. Because of their toxicity at high concentrations, phosphorothioate oligos should not be added to the culture at concentrations >100 μM. This perhaps results in incomplete inhibition, especially that of abundant mRNA species. This may explain why only one inhibitory subpool or no stimulatory subpool was detected and why two mRNAs were identified from the single subpool; the latter case could be regarded as an indication that a certain functional synergism of plural mRNA species in the inhibition of endothelial cell growth might contribute to positive detection. This point may be improved by decreasing the number of antisense species per subpool or by using appropriate carriers for intracellular delivery such as cationic liposomes (35) or streptolysin O (36); a higher concentration of each antisense species can then be achieved. Thirdly, in searching for genes the antisense inhibition of which would cause a loss of function, the blockade of housekeeping genes might give false-positive results. We thus think that this method would work better when searching for genes the antisense inhibition of which would cause a gain of function, i.e., suppresser genes like those for angiostatic factors or antioncogenes.

Our results presented here show that antisense oligos will play an important role in functional genomics, and that genes

once identified by this method and their products may become good targets for a strategy of inhibition by specific drugs or antisense oligos.

REFERENCES

1. Gabor Miklos,G.L. and Rubin,G.M. (1996) *Cell*, **86**, 521–529.
2. Liang,P. and Pardee,A.B. (1992) *Science*, **257**, 967–971.
3. Rijkers,T., Peetz,A. and Rüther,U. (1994) *Transgenic Res.*, **3**, 203–215.
4. Kuro-o,M., Matsumura,Y., Aizawa,H., Kawaguchi,H., Suga,T., Utsugi,T., Ohyama,Y., Kurabayashi,M., Kaname,T., Kume,E. *et al.* (1997) *Nature*, **390**, 45–51.
5. Inoue,C., Shiga,K., Takasawa,S., Kitagawa,M., Yamamoto,H. and Okamoto,H. (1987) *Proc. Natl Acad. Sci. USA*, **84**, 6659–6662.
6. Yamagishi,S., Hsu,C.-C., Taniguchi,M., Harada,S., Yamamoto,Y., Ohsawa,K., Kobayashi,K. and Yamamoto,H. (1995) *Biochem. Biophys. Res. Commun.*, **213**, 681–687.
7. Nakashima,E., Matsushita,R., Negishi,H., Nomura,M., Harada,S., Yamamoto,H., Miyamoto,K. and Ichimura,F. (1995) *J. Pharm. Sci.*, **84**, 1205–1209.
8. Nomura,M., Yamagishi,S., Harada,S., Hayashi,Y., Yamashima,T., Yamashita,J. and Yamamoto,H. (1995) *J. Biol. Chem.*, **270**, 28316–28324.
9. Kaji,M., Yonemura,Y., Harada,S., Liu,X., Terada,I. and Yamamoto,H. (1996) *Cancer Gene Ther.*, **3**, 393–404.
10. Kozak,M. (1991) *J. Cell Biol.*, **115**, 887–903.
11. Yamagishi,S., Kobayashi,K. and Yamamoto,H. (1993) *Biochem. Biophys. Res. Commun.*, **190**, 418–425.
12. Thomas,L.L. and Price,C.M. (1992) In Beserga,R. and Denhardt,D.T. (eds), *Antisense Strategies*. New York Academy of Sciences, New York, pp. 328–330.
13. Perbal,B. (1988) *A Practical Guide to Molecular Cloning*, 2nd Ed. John Wiley & Sons, Inc., New York.
14. Kolb,A.J. (1994) *Laboratory Information Management*, **26**, 107–113.
15. Mosmann,T. (1983) *J. Immunol. Methods*, **65**, 55–63.
16. Frohman,M.A., Dush,M.K. and Martin,G.R. (1988) *Proc. Natl Acad. Sci. USA*, **85**, 8998–9002.
17. Maruyama,K. and Sugano,S. (1994) *Gene*, **138**, 171–174.
18. Yamabe,Y., Shimamoto,A., Goto,M., Yokota,J., Sugawara,M. and Furuichi,Y. (1998) *Mol. Cell. Biol.*, **18**, 6191–6200.
19. Watanabe,T., Yonekura,H., Terazono,K., Yamamoto,H. and Okamoto,H. (1990) *J. Biol. Chem.*, **265**, 7432–7439.
20. Feinberg,A.P. and Vogelstein,B. (1983) *Anal. Biochem.*, **132**, 6–13.
21. Crooke,S.T. (1995) *Medical Intelligence Unit-Therapeutic Applications of Oligonucleotides*. R. G. Landes Company, Austin, TX.
22. Helene,C. and Toulme,J.J. (1990) *Biochim. Biophys. Acta*, **1049**, 99–125.
23. Anderson,S., Bankier,A.T., Barrell,B.G., de Bruijn,M.H.L., Coulson,A.R., Drouin,J., Eperon,I.C., Nierlich,D.P., Roe,B.A., Sanger,F. *et al.* (1981) *Nature*, **290**, 457–465.
24. Nagase,T., Ishikawa,K., Miyajima,N., Tanaka,A., Kotani,H., Nomura,N. and Ohara,O. (1998) DDBJ/EMBL/GenBank nucleotide sequence accession no. AB011123.
25. Dash,P., Lotan,I., Knapp,M., Kandel,E.R. and Goelet,P. (1987) *Proc. Natl Acad. Sci. USA*, **84**, 7896–7900.
26. Hanahan,D. and Folkman,J. (1996) *Cell*, **86**, 353–364.
27. Risau,W. (1997) *Nature*, **386**, 671–674.
28. Guimaraes,M.J. and Bazan,J.F. (1999) DDBJ/EMBL/GenBank nucleotide sequence accession no. U88873.
29. Ishikawa,K., Nagase,T., Suyama,M., Miyajima,N., Tanaka,A., Kotani,H., Nomura,N. and Ohara,O. (1998) DDBJ/EMBL/GenBank nucleotide sequence accession no. AB014576.
30. Wilson,R., Ainscough,R., Anderson,K., Baynes,C., Berks,M., Bonfield,J., Burton,J., Connell,M., Copsey,T., Cooper,J. *et al.* (1998) DDBJ/EMBL/GenBank nucleotide sequence accession no. AL021483.
31. Serpe,M. and Kosman,D.J. (1996) DDBJ/EMBL/GenBank nucleotide sequence accession no. U52667.
32. Richardson,P.M. and Zon,L.I. (1995) *Oncogene*, **11**, 1139–1148.
33. Neuwald,A.F. (1997) *Trends Biol. Sci.*, **22**, 243–244.
34. Moncrief,N.D., Kretsinger,R.H. and Goodman,M. (1990) *J. Mol. Evol.*, **30**, 522–562.
35. Duzgunes,N. and Felgner,P.L. (1993) *Methods Enzymol.*, **221**, 303–306.
36. Giles,R.V., Spiller,D.G., Grzybowski,J., Clark,R.E., Nicklin,P. and Tidd,D.M. (1998) *Nucleic Acids Res.*, **26**, 1567–1575.