0026-895X/94/030437-08$03.00/0

MOLECULAR PHARMACOLOGY, 46:437–444, 1994.

Modulation of Platelet-Derived Growth Factor-β mRNA Expression and Cell Growth in a Human Mesothelioma Cell Line by a Hammerhead Ribozyme

THAMBI DORAI, HIROYUKI KOBAYASHI,* JAMES F. HOLLAND, and TAKAO OHNUMA

Department of Medicine and the Derald H. Ruttenberg Cancer Center, Mount Sinai School of Medicine, New York, New York 10029

Received April 4, 1994; Accepted June 21, 1994

SUMMARY

Malignant mesothelioma in humans is a rare disease, but it has recently received much public attention and concern because of its strong relationship to exposure to asbestos. We have found overexpression of the gene for platelet-derived growth factor (PDGF)-β in several mesothelioma xenografts in nude mice. Because some mesothelioma cell lines such as VAMT-1 overexpress PDGF-β and PDGF-β receptors, it was considered that an autocrine loop involving PDGF-β and its receptor may contribute to the malignant phenotype of these cells. To investigate this possibility we have developed a hammerhead ribozyme against PDGF-β mRNA. This c-*sis* ribozyme was able to cleave an artificial PDGF-β RNA substrate in a cell-free system. Transduction of this ribozyme, with the aid of a constitutive expression vector, in the VAMT-1 cell line led to a decrease in the PDGF-β mRNA level. The ribozyme expressed in these cells was functional in cleaving the artificial RNA substrate *in vitro*. Ribonuclease protection assays using the ribozyme and whole PDGF-β mRNA showed that this ribozyme was capable of cleaving the whole mRNA *in vivo*. Transfectant clones containing the wild-type ribozyme showed decreased cell growth, in parallel with the decreases in PDGF-β expression. The disabled ribozyme was inactive in the cleavage reaction *in vitro* and in decreasing the cell growth rate *in vivo*. Our data indicate that in some mesothelioma cells the PDGF-β autocrine loop may be functional and transduction of the PDGF-β ribozyme leads to a significant reduction of cell growth. The c-*sis* ribozyme may be applicable in the treatment of patients with malignant mesothelioma.

Malignant mesothelioma is a relatively rare disease arising from the mesodermally derived mesothelial cells covering the surface of the pleural and peritoneal cavities (1, 2). Exposure to asbestiform fibers is the primary causal factor in the genesis of this cancer (3). Experimental studies have shown that asbestos fibers can cause cell transformation and extensive chromosomal breakage in normal human mesothelial cells (4). Among the myriad chromosomal aberrations that occur in malignant mesothelioma, certain nonrandom common elements can be discerned, especially the breakpoints and structural changes in chromosomes 1, 3, 7, 9, and 22 (5–8). However, no specific chromosomal abnormality has yet been described for this disease.

Given the complexity of the chromosomal alterations and the long latency period, it would be difficult to pinpoint a single mechanism by which the mesothelial cells are transformed. It is likely that this cancer arises in multiple stages. However, one approach to elucidating the mechanisms of asbestos carcinogenicity is to define the critical molecular alterations that occur in this cancer. Studies by Gerwin *et al.* (9) and Versnel *et al* (10) provided an important clue, i.e., that the expression of the *PDGF-β* gene is elevated in several mesothelioma cell lines. Much of the interest in PDGF in oncology stems from the observation that the v-*sis* oncogene of the simian sarcoma virus is the retroviral homologue of the cellular gene encoding the B chain of PDGF (11, 12). The *PDGF-A* chain gene is 60% homologous to the *PDGF-B* chain gene (13). Expression of a single *PDGF* gene or both genes has been found in a variety of normal and tumor cell lines (14). All possible dimeric combinations of A and B chains have been identified in normal and transformed cells (15). The tumorigenic effect of v-*sis* has been postulated to be due to its growth-stimulating activity, which requires that the cells producing c-*sis* also express the corresponding functional PDGF-β receptor (16, 17). In fact, Versnel *et al.* (18) demonstrated the expression of PDGF-β receptors but not PDGF-α receptors in a panel of 12 human mesothelioma cell lines that were previously reported to express

This study was supported in part by the Charlotte Geyer Foundation (Sarasota, FL), by the T. J. Martell Memorial Foundation for Leukemia, Cancer, and AIDS Research (New York, NY), by Hayashibara Biochemical Laboratories, Inc. (Okayama, Japan), and by the Chemotherapy Foundation (New York, NY).

* Dr. Hiruyuki Kobayashi's present address is: Dept. of Laboratory Medicine, National Defense Medical College, Tokorozawa, Saitama 359, Japan.

ABBREVIATIONS: PDGF, platelet-derived growth factor; FBS, fetal bovine serum; RT, reverse transcription; PCR, polymerase chain reaction; SDS, sodium dodecyl sulfate; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

PDGF-A and *-B* chain genes. In contrast, cultured normal mesothelial cells were found to express predominantly PDGF-α receptors. These results strongly suggested that normal and malignant mesothelial cells may be stimulated by two very different autocrine mechanisms, involving PDGF-A and PDGF-B, respectively (19, 20). This view is generally held regarding the genesis of several cancers other than mesothelioma, such as malignant fibrous histiocytoma (21) and choriocarcinoma (22). It is therefore possible that *PDGF-B* can directly or indirectly contribute to cellular transformation *in vivo*. Hence, we postulated that any method by which one can interfere with the level of expression of PDGF-β mRNA would be helpful in minimizing the oncogenic stimulus mesothelioma cells receive from their own production of PDGF-B.

To investigate this possibility we chose to use a hammerhead ribozyme specific for PDGF-β mRNA. Hammerhead ribozymes contain two functional modules, i.e., a catalytic core that cleaves the target RNA and the flanking regions that, by virtue of complementarity, direct the ribozyme core to a specific target site. By exploiting the flexibility of these two modular functions, it is possible to design a ribozyme to specifically cleave any target RNA molecule (23–25). We report here the design and synthesis of a hammerhead ribozyme against PDGF-B to address the role of the *PDGF-B* gene in the malignant phenotype of mesothelioma.

Materials and Methods

Cell lines and RNA extraction. VAMT-1, a sarcomatoid mesothelioma cell line (26), was obtained from Dr. L. M. Tibbets of the Roger Williams General Hospital (Providence, RI). This and another cell line, HEp-2, a head and neck squamous carcinoma cell line (purchased from the American Type Culture Collection, Bethesda, MD), were maintained in RPMI 1640 medium containing 10% (v/v) heat-inactivated FBS and were fed twice each week. Normal mesothelial cells transformed by transfection with a simian virus 40 large T antigen construct (Met5A), originally from John F. Lechner (National Institutes of Health, Bethesda, MD), were maintained in MCDB 105 medium supplemented with medium 199 (Sigma Chemical Co., St. Louis, MO), 13 mM sodium bicarbonate, and 10% FBS. Xenografts of human mesotheliomas, such as BG, VS, and ES, were routinely maintained in nude mice in our laboratory (27). Cellular RNA and xenograft tissue RNAs were extracted by the acid guanidinium thiocyanate-phenol-chloroform method (28). In some cases polyadenylated RNA was isolated from total RNA using oligo(dT)-cellulose columns, following standard procedures.

RT-PCR. For the RT-PCR amplification of PDGF-B transcripts from the VAMT-1 cell line, cDNA was synthesized by using Moloney murine leukemia virus reverse transcriptase (GIBCO-BRL, Gaithersburg, MD) with random hexamers (Pharmacia, Piscataway, NJ) as primer. PDGF coding exons 4–6 were then amplified from this cDNA by using a GeneAmp PCR core reagent kit (Perkin Elmer Cetus, Norwalk, CT) with oligonucleotide primers from exon 4 (5′-AACGCCAACTTCCTGGTGTG-3′) and exon 6 (5′-CCGGCGGACTCGCACCGT-3′). PGDF-β DNA sequences were adopted from published data (29). The amplification conditions used were denaturation at 94° for 1 min, annealing at 55° for 1 min, and synthesis at 72° for 1 min, for 25 cycles. The distinct 309-base pair product was identified by 1.5% agarose gel electrophoresis.

Direct cloning of PCR products and sequencing. The PCR products were ligated directly to TA vector (Invitrogen, San Diego, CA) or pT7Blue T vector (Novagen, Madison, WI). After transformation of competant *Escherichia coli*, colonies were selected and screened. Plasmid DNA was prepared by the cetyltrimethylammonium bromide precipitation method (30) and double-stranded DNA sequencing was performed directly after heat denaturation by using a Sequenase version 2 kit (United States Biochemicals, Cleveland, OH). Several clones were sequenced and analyzed for orientation. For the ribozyme studies a clone that produced the sense transcript was selected from the TA vector, and for the subsequent ribonuclease protection analyses a clone that produced the antisense transcript was chosen from the pT7Blue T vector.

***In vitro* transcription of substrate RNA.** For the generation of sense transcripts, the plasmid from the amplified RT-PCR product cloned in the TA vector was linearized at the 3′ unique *Eco*RV site. For the synthesis of antisense transcripts, the recombinant in the pT7Blue T vector was linearized at the 3′ unique *Eco*RI site. The respective linearized plasmids were transcribed using SP6 or T7 RNA polymerases (Promega, Madison, WI), using protocols recommended by the manufacturer. Briefly, the transcription reaction mixture contained 2 μg of the linearized plasmid template, 40 mM Tris·HCl, pH 7.5, 6 mM $MgCl_2$, 2 mM spermidine, 10 mM NaCl, 10 mM dithiothreitol, 0.5 mM levels each of GTP, ATP, and UTP, 50 μM CTP, 50 μCi of [α-^{32}P]CTP (specific activity, 800 Ci/mmol), 20 units of ribonuclease inhibitor RNasin (Promega), and 20 units of T7 or SP6 RNA polymerase, in a 20-μl final volume. The transciption reactions were carried out at 30° for 2 hr. The products were treated with RQ1 DNase (Promega) and extracted with phenol/chloroform, followed by ethanol precipitation.

Synthesis of c-*sis* ribozyme. To synthesize c-*sis* ribozyme using synthetic DNA templates, the method of Milligan *et al.* (31) was used. The generic T7 promoter top strand contained the sequence 5′-CATGTAATACGACTCACTATAGGG-3′. The bottom strand for the wild-type hammerhead ribozyme contained the sequence 5′-CTGCGACCTGTTTCGTCCTCACGGACTCATCAGCAGGTGAGACCCTATAGTGAGTCGTATTACATG-3′. The corresponding bottom strand for the disabled ribozyme contained 5′-CTGCGACCTGTGTCGTCCTCACGGACTCATAAGCAGGTGAGACCCTATAGTGAGTCGTATTACATG-3′. The transciption of RNA from the annealed synthetic DNA templates was carried out as described, except that all unlabeled deoxynucleoside triphosphates were used at 4 mM each, without [α-^{32}P]CTP, each synthetic single strand was used at a final concentration of 0.4 μM, $MgCl_2$ was used at 16 mM, and T7 RNA polymerase was used at 3 units/μl.

***In vitro* cleavage reaction.** The ribozyme and substrate RNA were mixed in a 10-μl final volume containing 50 mM Tris·HCl, pH 7.4, and 1 mM EDTA. The reaction mixture was heated at 100° for 2 min to denature the RNAs and quick-chilled on ice, and the reaction was initiated by addition of $MgCl_2$. The reaction mixtures were incubated at 37° for various periods of time, incubations were stopped by the addition of an equal volume of sequencing stop solution (95% deionized formamide, 20 mM EDTA, 0.05% bromphenol blue, 0.05% xylene cyanol), heated at 80° for 5 min, and analyzed in 6% polyacrylamide-7 M urea gels in 1× Tris-borate-EDTA buffer (0.09 M Tris-borate with 2 mM EDTA), and gels were dried and autoradiographed.

Expression of the hammerhead ribozyme in mammalian cells. The c-*sis* ribozyme used for the expression study was designed differently from that used in the cell-free system. Two single-stranded oligodeoxyribonucleotides were synthesized so that the 45-base ribozyme sequence contained flanking *Sal*I and *Hind*III restriction sites on both ends. The sequences synthesized were SRB-1 (5′-TCGACTCTCACCTGCTGATGAGTCCGTGAGGACGAAACAGGTCGCA-3′) and SRB-2 (5′-AGCTTGCGACCTGTTTCGTCCTCACGGACTCATCAGCAGGTGAGA-3′). For the development of the disabled ribozyme, the sequences synthesized were SRB-3 (5′-TCGACTCTCACCTGCTTATGAGTCCGTGAGGACGACACAGGTCGCA-3′) and SRB-4 (5′-AGCTTGCGACCTGTGTCGTCCTCACGGACTCATAAGCAGGTGAGA-3′). These oligonucleotides were phosphorylated with T4 polynucleotide kinase and annealed. The double-stranded ribozyme sequences were cloned into the *Sal*I and *Hind*III sites of the β-actin promoter vector pHβAPr-1-neo, which was obtained from Dr. Lawrence Kedes (University of Southern California, Los Angeles CA)

(32). The sequence and orientation of both the wild-type and mutated ribozymes were confirmed by DNA sequencing. Transfection of VAMT-1 cells with the ribozyme-expression vector construct was done according to the lipofection protocol recommended by the manufacturer (GIBCO-BRL). Two days after transfection, the antibiotic G418 was added at a final concentration of 500 μg/ml, and the cells were maintained for 4 weeks in RPMI 1640 medium containing 10% FBS and G418. Individual colonies of G418-resistant clones were picked up and expanded. To detect ribozyme expression in transfected G418-resistant clones, a set of primers was used, i.e., 5′-AGCACAGAGCCTCGCCTTT-3′(from the β-actin 5′ untranslated region) and 5′-TGGATCCCTCGAAGCTT-3′ (from the plasmid polylinker). RT reaction products, equivalent to 200 ng of total RNA from each clone, were processed through 30 cycles of PCR with denaturation at 94° for 1 min, annealing at 47° for 2 min, and synthesis at 72° for 3 min, using the PCR primers described above, in a final volume of 20 μl. Half of the reaction product was analyzed in a 2% agarose gel with a *Msp*I digest of pBR322 DNA as the marker.

Ribonuclease protection assay. To generate the antisense PDGF-B transcript, a PDGF-B RT-PCR clone in the pT7Blue T vector in which the PDGF-B sequences were in the reverse orientation was chosen. This plasmid, linearized with *Eco*RI, was transcribed with T7 RNA polymerase under the conditions described above. The 398-base RNA transcript was gel purified from a 6% polyacrylamide gel. Polyadenylated RNA was prepared from total RNA using a Microfast Track kit (Invitrogen). Unlabeled hammerhead ribozyme was synthesized according to the procedures described earlier. Two micrograms of polyadenylated RNA were mixed with 2 μg of freshly synthesized ribozyme RNA, in the presence of 10 mM Tris, pH 7.4, and 1 mM EDTA. RNAs were denatured by heating at 100° for 2 min and were quick-chilled on ice. The reaction was initiated by addition of $MgCl_2$ to a final concentration of 10 mM, in a final volume of 20 μl, and the mixture was incubated at 37°. Aliquots were obtained at various time points and EDTA was immediately added to a final concentration of 20 mM to stop the reaction. The ^{32}P-labeled antisense PDGF-B probe was added under conditions of probe excess, and the ribonuclease protection assay was performed using the "streamlined" protocol, using a RPA II kit (Ambion, Austin, TX). The various protected fragments were analyzed on 6% polyacrylamide-7 M urea gels and autoradiographed.

Analysis of PDGF-B and PDGF-B receptor protein. VAMT-1 cells propagated in RPMI 1640 medium with 10% FBS were washed with serum-free medium and incubated with serum-free medium overnight. For the measurement of PDGF-B levels, serum-starved VAMT-1 cells and various G418-resistant wild-type and mutant ribozyme-expressing clones were processed and 100,000 × *g* pellets (total membrane fractions) were prepared from each, according to the method of Leal *et al.* (33). The boiled lysates were subjected to 12% SDS-polyacrylamide gel electrophoresis under reducing conditions and blotted onto nitrocellulose. The blot was then probed with polyclonal goat anti-PDGF-B antibody (Upstate Biotechnology Incorporated, Lake Placid, NY). The immune complexes were identified with ^{125}I-labeled Protein G and visualized by autoradiography. Conditioned media from these cultures were removed and concentrated with a Centriprep concentrator (Amicon), so that 1 ml of concentrated medium was derived from approximately 1 × 10^8 cells. Protein was estimated from the various lysates and conditioned media by the Pierce bicinchoninic acid assay. For the PDGF-B receptor analysis, the cells from 25-cm^2 culture dishes were lysed in 1 ml of 10 mM sodium phosphate, pH 7.5, 100 mM NaCl, 1% Triton X-100, 0.1% SDS, 0.5% sodium deoxycholate, 0.15 unit/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, and clarified by centrifugation at 10,000 × *g* for 30 min at 4°. The xenograft tissues were minced on ice with an equal volume of buffer containing 10 mM sodium phosphate, pH 7.5, 100 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 0.15 unit/ml aprotinin, 1 mM leupeptin, 1 mM antipain, and 1 mM sodium orthovanadate. After mincing, an equal volume of 2× SDS sample buffer (100 mM Tris Hcl, pH 6.8, 200 mM dithiothreitol, 4% SDS, 0.2% bromophenol blue, and 20% glycerol) was added and the mixture was boiled at 100° for 10 min. Lysates were subjected to SDS-polyacrylamide gel electrophoresis, blotted onto nitrocellulose filters, and probed with polyclonal anti-PDGF-B receptor antibody (UBI) under conditions described by the manufacturer. Immune complexes were identified by using ^{125}I-labeled Protein A and were visualized by autoradiography.

Results

First we sought to determine whether the PDGF-B transcript is in fact overexpressed in the VAMT-1 cell line and in malignant mesothelioma xenografts being maintained in our laboratory in nude mice. As negative controls, we chose Met5A and HEp-2 cell lines. Fig. 1A shows that, at steady state, the 4.1-kilobase PDGF-B transcript was overexpressed both in the VAMT-1 cell line and in the xenografts BG and ES. The Met5A cell line also expressed the PDGF-B transcript, although at a much lower level. The HEp-2 cells did not show any detectable level of the PDGF-B transcript. Fig. 1B shows that there were no changes in GAPDH expression in any cell lines or xenograft tissues examined. The presence of PDGF-B receptors in normal mesothelial and malignant mesothelioma cell lines was demonstrated by Western blotting, as shown in Fig. 1C. The majority contained detectable levels of PDGF-B receptor. It is significant that the normal mesothelial cell line Met5A also expressed a very low but detectable level of the receptor. Both the precursor (165-kDa) and mature (180-kDa) forms of the B-type receptor could be visualized with the specific antibody. The control HEp-2 cell line, in contrast, did not show any detectable level of expression of the B-type receptor. These

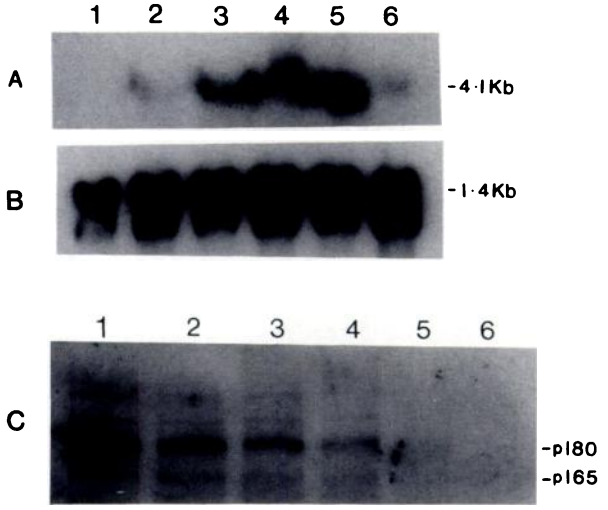

Fig. 1. A, Expression of PDGF-B mRNA in various cell lines and mesothelioma xenograft tissues from nude mice. Two micrograms of polyadenylated RNA were processed for each lane. The blot was probed with a 2.0-kilobase *Bam*HI-*Bam*HI fragment of PDGF-B cDNA containing the entire coding region, obtained from the plasmid pCDV1 (American Type Culture Collection). *Lane 1*, HEp-2 cells; *lane 2*, Met5A cells; *lane 3*, VAMT-1 cells; *lanes 4*, *5*, and *6*, nude mouse xenografts BG, ES, and VS, respectively. B, Expression of an internal standard, GAPDH, in the cell lines and tissues described for A. The GAPDH probe used was a 1.1-kilobase cDNA fragment obtained from Clontech (Palo Alto, CA). C, Expression of the PDGF-B receptor in the various cell lines and tissues analyzed. One hundred micrograms of total protein were loaded in each lane. *Lane 1*, VAMT-1 cells; *lanes 2*, *3*, and *4*, mesothelioma xenografts BG, ES, and VS, respectively; *lane 5*, Met5A cells; *lane 6*, HEp-2 cells.

data are consistent with the conclusion that malignant mesothelial cells overexpress both PDGF-B and its cognate receptor.

In the present work, a hammerhead ribozyme was designed to target codon 151 in exon 4 of the PDGF mRNA, based on the model proposed by Haseloff and Gerlach (34). The sequences of the target site and the hammerhead ribozyme are shown in Fig. 2. A disabled ribozyme was also synthesized by mutating position 3 (guanine to uracil) and position 22 (adenine to cytosine) of the central core of the ribozyme, based on the stringent sequence requirements for cleavage by hammerhead ribozymes, as described by Ruffner *et al* (35). Oligonucleotides encoding the catalytic core of the flanking sequences complementary to the target sequence and a T7 RNA polymerase promoter were synthesized. Using a generic oligonucleotide containing only the top strand of the T7 promoter sequence, a partially double-stranded synthetic substrate was made and transcribed with T7 RNA polymerase to generate a 45-base ribozyme, according to published procedures (31). To create a synthetic PDGF-B substrate RNA, a fairly large region encompassing the target site (position 1475) (29), which could thus mimic the secondary structures in the native mRNA, was selected between codons 115 and 217. Sequence analysis of the cloned PCR product indicated that there were no mutations in the PDGF-B mRNA in the region amplified, which included the target site (data not shown). Transcribing the sense RNA construct yielded a 406-base labeled RNA substrate. This substrate and the ribozyme were used to determine the cleavage reaction conditions in a cell-free system. As shown in Fig. 3, the hammerhead ribozyme cleaved the 406-base PDGF-B substrate in a time-, pH-, molar ratio-, and magnesium concentration-dependent manner. The cleaved fragments, labeled 5′F and 3′F, are the correct sizes predicted from the location of the cleavage site of the ribozyme, as shown in Fig. 2. Fig. 3A shows that the cleavage reaction was time dependent; however, there was considerable degradation of both the substrate and products after 6 hr of incubation. Fig. 3B shows that the ribozyme could work at physiological pH values. Hence, cleavage reactions at pH values of <6.5 or >8.5 were not attempted. Fig. 3C shows that the ribozyme reaction could take place at Mg^{2+} concentrations as low as 0.5 mM, which is roughly equivalent to the physiological Mg^{2+} concentration inside the cell (36). It is possible that, although the intracellular Mg^{2+} concentration is suboptimal, other divalent catalysts, e.g., Mn^{2+} and Zn^{2+}, may participate in the ribozyme reaction *in vivo*. Fig. 3D shows that incubation of the PDGF-B substrate with increasing amounts of ribozyme resulted in increased cleavage, although complete cleavage of the artificial substrate could not be achieved under the experimental conditions used. Fig. 3E shows that the disabled ribozyme prepared was completely inactive in the *in vitro* cleavage reaction. Thus, the optimized conditions for RNA cleavage by the PDGF-B ribozyme *in vitro* were determined to be pH 7.4–8.5, with 10 mM $MgCl_2$, for 1–3 hr at 37°, which is consistent with the characterization of the pH and Mg^{2+} dependence of hammerhead ribozymes (37, 38). Subsequent studies showed that this *in vitro* cleavage reaction could be observed after incubation for as little as 5 min (data not shown).

After introduction of c-*sis* ribozyme-containing plasmid pHβAPr-1-neo into VAMT-1 cells by lipofection, 12 G418-resistant clones were screened for the stable integration of the c-*sis* ribozyme plasmid by PCR analysis of their DNA (data not shown). Expression of the ribozyme was demonstrated in total cellular RNA, isolated from individual wild-type and disabled ribozyme clones, by RT-PCR analysis using the β-actin 5′ untranslated region primer and a plasmid polylinker primer to amplify a 116-base mature (spliced) form of the ribozyme RNA product, which can be seen with the corresponding unspliced version in Fig. 4. Fig. 4 also shows that the wild-type and mutant clones expressed the ribozyme at equivalent levels. To demonstrate that the ribozyme transcribed in the VAMT-1 cells retained its cleavage ability, polyadenylated RNAs from both the mutated and wild-type clones were analyzed in the cell-free assay system described above. In this analysis, only the RNA from the wild-type ribozyme-expressing clone possessed cleavage activity, as shown in Fig. 5.

We next sought to determine whether the wild-type c-*sis* ribozyme expressed *in vitro* had the ability to recognize and cleave the whole PDGF-B mRNA. For this analysis, we designed a sensitive ribonuclease protection assay to trap the cleaved RNA fragments with the use of an antisense PDGF-B probe, as described in Materials and Methods. The results are shown in Fig. 6. When the 398-base antisense PDGF substrate encompassing the target site on both sides was used, the fully protected 318-base fragment was clearly visible. The cleaved RNA fragments of 198 (3′F) and 120 (5′F) bases were visible (after a long exposure to X-ray film). However, there were no

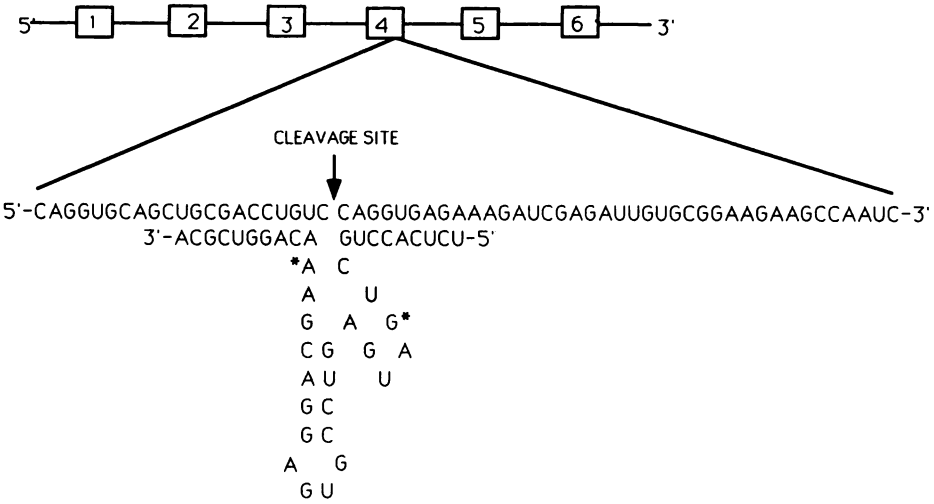

Fig. 2. PDGF-B exons shown diagrammatically with the sequence in and around the ribozyme cleavage site. Also shown is the structure of the PDGF-B ribozyme with the complementary flanking sequence and the conserved core hammerhead sequence. *, Mutations generated to derive the disabled ribozyme; they are guanine to uracil and adenine to cytosine.

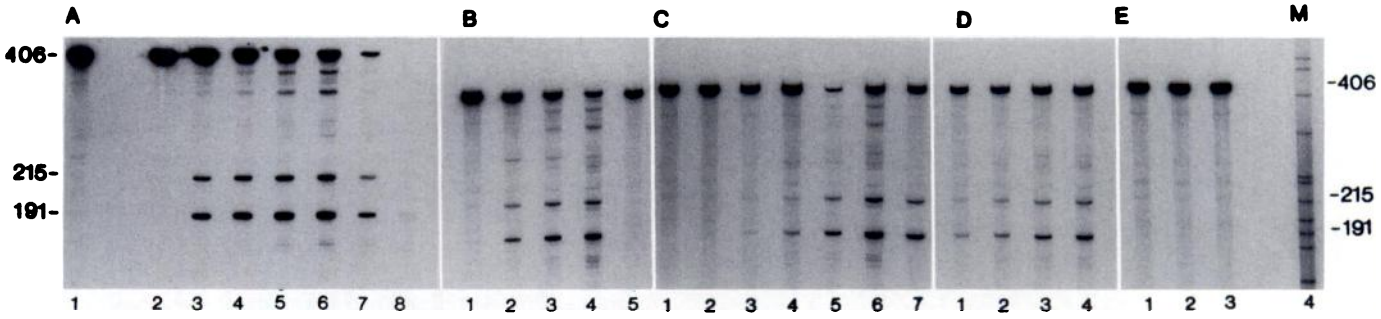

Fig. 3. Cleavage of the PDGF-B RNA substrate (406 bases) into two cleavage products by the hammerhead ribozyme *in vitro*. The ribozyme produced a 191-base 5′ fragment (5′F) and a 215-base 3′ fragment (3′F) in A-E. Only the substrate was labeled with [α-^{32}P]CTP (specific activity, 800 Ci/mmol; DuPont/NEN, Boston, MA). A, Reaction time versus activity profile for the PDGF-B ribozyme. PDGF-B RNA substrate and one third the molar amount of the ribozyme were incubated as described in Materials and Methods. The $MgCl_2$ concentration was 10 mM. *Lane 1*, substrate only; *lane 2*, time 0; *lane 3*, 30 min; *lane 4*, 1 hr; *lane 5*, 2 hr; *lane 6*, 3 hr; *lane 7*, 6 hr; *lane 8*, 12 hr. B, Effect of pH on the cleavage reaction. *Lane 1*, pH 6.5; *lane 2*, pH 7.4; *lane 3*, pH 8.0; *lane 4*, pH 8.5; *lane 5*, ^{32}P-labeled substrate only. Incubation was for 1 hr at 37°. The $MgCl_2$ concentration was 10 mM and the ribozyme/substrate ratio was 1:1. C, Effect of $MgCl_2$ concentration on the cleavage activity. *Lanes 1-7*, reaction mixtures contained 0, 0.1, 0.5, 1.0, 5, 10, and 20 mM $MgCl_2$, respectively. Incubation was for 1 hr at 37° and the ribozyme/substrate ratio was 1:1. D, Cleavage reaction with increasing proportions of the c-*sis* ribozyme. *Lanes 1-4*, ^{32}P-labeled substrate/ribozyme, on a molar basis, was 1:0.25, 1:0.5, 1:1, and 1:2, respectively. Incubation was for 1 hr was at 37°. The $MgCl_2$ concentration was 10 mM. E, Cleavage reactivity of the disabled ribozyme. Incubation conditions were the same as for the wild-type ribozyme. *Lanes 1* and *2*, reaction mixtures incubated for 1 and 2 hr, respectively; *lane 3*, ^{32}P-labeled substrate only. *Lane M*, ^{32}P-labeled *Msp*I digest of pBR322 plasmid DNA, as the molecular weight standard.

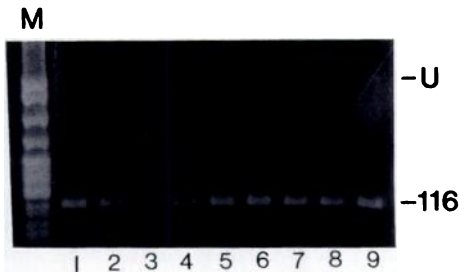

Fig. 4. Expression of wild-type and disabled ribozyme in total cellular RNA isolates from individual G418-resistant clones of VAMT-1 cells. *116*, the mature 116-base product of the expressed ribozyme. *U*, PCR product arising from the same set of primers but from the unspliced version of the RNA. *Lane M*, *Msp*I digest of pBR322, as the molecular weight standard. *Lanes 1-6*, RT-PCR products from individual clones expressing the wild-type ribozyme; *lanes 7-9*, RT-PCR products arising from individual clones expressing the disabled ribozyme.

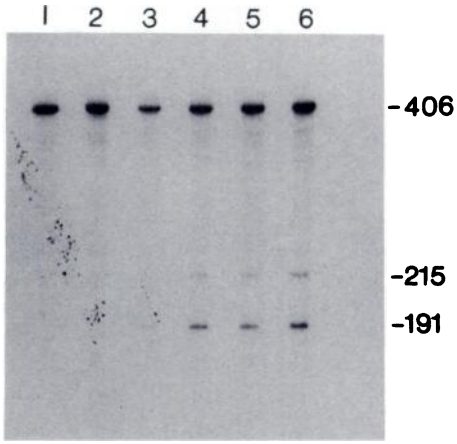

Fig. 5. Cleavage reaction with polyadenylated RNA from G418-resistant VAMT-1 clones. Polyadenylated RNA from both wild-type and disabled ribozyme-expressing, G418-resistant, VAMT-1 clones was examined in the cell-free cleavage assay, as described for Fig. 3. *Lane 1*, labeled PDGF-B substrate only; *lane 2*, cleavage reaction with 2 μg of polyadenylated RNA from a disabled ribozyme-expressing clone; *lanes 3-6*, cleavage reaction with 0.5, 1, 2, and 3 μg, respectively, of polyadenylated RNA from wild-type ribozyme-expressing clone 12. The incubation time was 1 hr at 37°, the $MgCl_2$ concentration was 10 mM, and the ribozyme/substrate ratio was 1:1.

corresponding increases in the intensity of the cleaved fragments as time progressed. This could possibly be due to the limitations of this assay, because the signals seen are the net effect of RNA fragment trapping and degradation. Although these studies demonstrate that the transcribed ribozyme is active, the data cannot be extrapolated to predict what might happen *in vivo*, because the *in vitro* annealing conditions are never encountered in cells. Nevertheless, these studies strongly suggest that the ribozyme expressed is functional *in vitro* and, hence, very likely also functional *in vivo*. Subsequent studies by Northern gel analyses showed that the level of PDGF mRNA was decreased in wild-type ribozyme-containing clones (Fig. 7A, *lanes 6-9*), compared with the parent VAMT-1 cell line (Fig. 7A, *lane 5*). Mutant clones analyzed in parallel had the same level of expression as did the wild-type VAMT-1 cell line (Fig. 7A, *lanes 1-3*). Fig. 7A, *lane 4*, represents the level of expression of PDGF-β mRNA from Met5A cells, used as a negative control. The most obvious decreases in the PDGF message levels occurred in clones 8 and 12 (Fig. 7A, *lanes 8* and *9*), after calculation of the PDGF:GAPDH message ratios by densitometry (see the legend to Fig. 7). There was no appreciable change in the level of expression of GAPDH mRNA in any of the cell lines examined (Fig. 7B).

To investigate whether the difference in the levels of expression of PDGF-mRNA is actually reflected at the protein level, we prepared enriched membrane extracts from various clones, subjected them to Western blotting, and probed the blots with a polyclonal goat antiserum specific for the PDGF-BB dimer. The results are shown in Fig. 8. The culture medium conditioned by the VAMT-1 cell line did not contain detectable PDGF-B protein (Fig. 8, *lane 1*). When whole-cell lysates were prepared from serum-starved VAMT-1 cells by freeze-thawing and analyzed for PDGF-B protein expression, the cell-associated p24 PDGF-B protein was not detected (Fig. 8, *lanes 2* and *3*). However, the membrane-associated p24 PDGF-B protein was easily detectable when enriched cell membrane

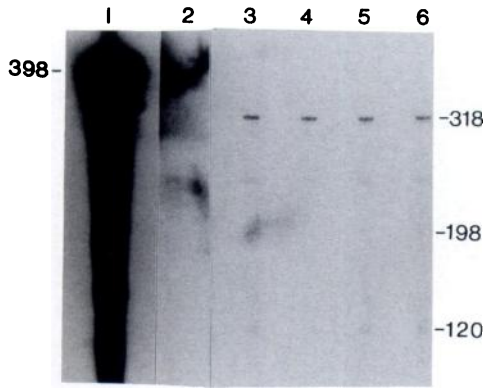

Fig. 6. Ribonuclease protection assay to screen for the cleavage of whole PDGF-B mRNA by the wild-type ribozyme expressed in VAMT-1 cells. *Lane 1*, probe hybridized with 10 μg of yeast tRNA, without ribonucleases A and T_1 (only 20% of the sample was loaded); *lane 2*, probe hybridized with 10 μg of yeast tRNA, digested with ribonucleases A and T_1; *lanes 3-6*, protected fragments from aliquots obtained for time points 15, 30, 45, and 60 min, respectively, digested with ribonucleases A and T_1. The 198-base band is visible after 72 hr of exposure of the X-ray film, whereas the 120-base fragment is clearly visible in the figure.

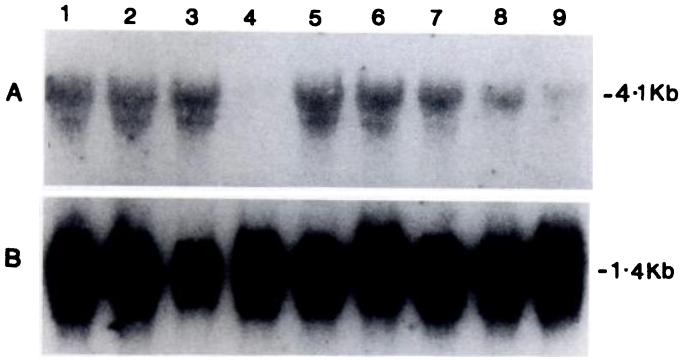

Fig. 7. A, Levels of expression of the PDGF-B mRNA in various G418-resistant clones expressing disabled and wild-type c-*sis* ribozyme. One microgram of polyadenylated RNA from selected clones of VAMT-1 cells was processed on a Northern gel as described for Fig. 1A. *Lanes 1*, *2*, and *3*, VAMT-1 cells expressing three individual clones of the mutant ribozyme; *lane 4*, Met5A cells as a negative control; *lane 5*, VAMT-1 parent cells; *lanes 6*, *7*, *8*, and *9*, VAMT-1 cells expressing the wild-type ribozyme clones 6, 7, 8, and 12, respectively. The exposure time for this film was 48 hr. B, Levels of expression of the GAPDH mRNA as the internal standard, as described for Fig. 1B. The exposure time for this film was 30 min. The most obvious decreases in PDGF-β expression are seen in clones 8 and 12 (*lanes 8* and *9*, respectively), based on the subsequent densitometric scanning analysis. The PDGF:GAPDH message ratios were found to be 0.23, 0.20, 0.22, 0.02, 0.24, 0.19, 0.15, 0.11, and 0.07 for *lanes 1* through *9*, respectively.

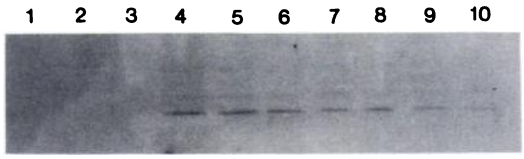

Fig. 8. Expression of PDGF-B in the various G418 clones and in conditioned media. *Lane 1*, conditioned medium from VAMT-1 cells (50 μg); *lanes 2* and *3*, whole-cell lysate protein from VAMT-1 cells (50 μg and 100 μg, respectively); *lane 4*, enriched cell membrane fraction from wild-type VAMT-1 cells; *lanes 5* and *6*, enriched membrane fraction from two independent disabled ribozyme-expressing clones; *lanes 7-10*, enriched membrane fractions from wild-type ribozyme-expressing clones 6, 7, 8, and 12, respectively (50 μg of protein applied for *lanes 4-10*).

fractions from the wild-type VAMT-1 cell line (Fig. 8, *lane 4*), the mutant ribozyme-expressing clones (Fig. 8, *lanes 5* and *6*), and the wild-type ribozyme-expressing clones (Fig. 8, *lanes 7-10*) were analyzed. As Fig. 8 illustrates, the decrease in the level of PDGF-B protein expression parallels the decreases in PDGF-B mRNA expression observed in Fig. 7. The effect of PDGF-B wild-type ribozyme on VAMT-1 cell growth roughly correlates with the reduction in PDGF-B mRNA expression, with the clone producing the lowest level of PDGF-B mRNA (clone 12) inhibiting cell growth by approximately 40%. The mutant cell line examined had a growth curve close to that of the wild-type parent cell line (Fig. 9).

Discussion

In the present study we found that three of four human mesothelioma cell lines tested had increased expression of PGDF-B and PDGF-B receptors and that a specifically designed c-*sis* ribozyme could cleave the mRNA sequence for PDGF-B chain in a cell-free system. After transfection into a mesothelioma cell line the expressed ribozyme reduced the levels of both PDGF-B mRNA and protein (p24). Subclones of cells transduced with wild-type c-*sis* ribozyme had significant reduction in cell growth, compared with those with the disabled ribozyme. Thus, our results strongly implicate the pathogenic role of this oncogene in the development of malignant mesothelioma. In addition, c-*sis* ribozymes may have therapeutic potential in the treatment of patients with mesothelioma, leading to a specific reduction in cell growth without affecting normal mesothelial cells. Incorporating this ribozyme in suitable expression vectors and direct *in vivo* gene transfer into the tumor tissue might hold promise for the treatment of this cancer.

It is of note that van der Meeren *et al.* (39) reported that VAMT-1 cells did not produce detectable PDGF-B protein. When whole-cell lysates of VAMT-1 cells were used we also failed to detect PDGF-B protein (see Fig. 8). In fact, the inability to detect cell-associated p24 PDGF-B protein when whole-cell extracts were used was reported when Igarashi *et al.* (40) analyzed EJ or A172 cells. When enriched membrane components were analyzed, the PDGF-B product was readily detectable in their study. We believe that the failure of van der Meeren *et al.* to detect PDGF-B protein in VAMT-1 cells is mainly due to the method they used.

Because ribozymes are antisense RNA sequences to begin with, it is theoretically possible that they function in an antisense fashion, rather than as a catalytic RNA endonuclease, in transfected cells. The lack of biochemical and biological effects on mesothelial cells transfected with the disabled ribozyme, possessing its full complement of antisense flanking sequences, indicated that the effects of the ribozymes on RNA expression levels and cell growth are negligible, if present at all. We conclude that the reduction in the expression of PDGF-B mRNA is due to the catalytic effect of the ribozyme, rather than its antisense effect, because the wild-type and disabled riboyzmes were expressed in VAMT-1 cells at virtually identical levels.

In the present study it was not possible to cleave the RNA substrate completely either *in vitro* or *in vivo*. The reasons for this difficulty are unclear. This could be due to the three-dimensional structures of the PGDF-B mRNA substrate and the interacting ribozyme, resulting in limited access of the

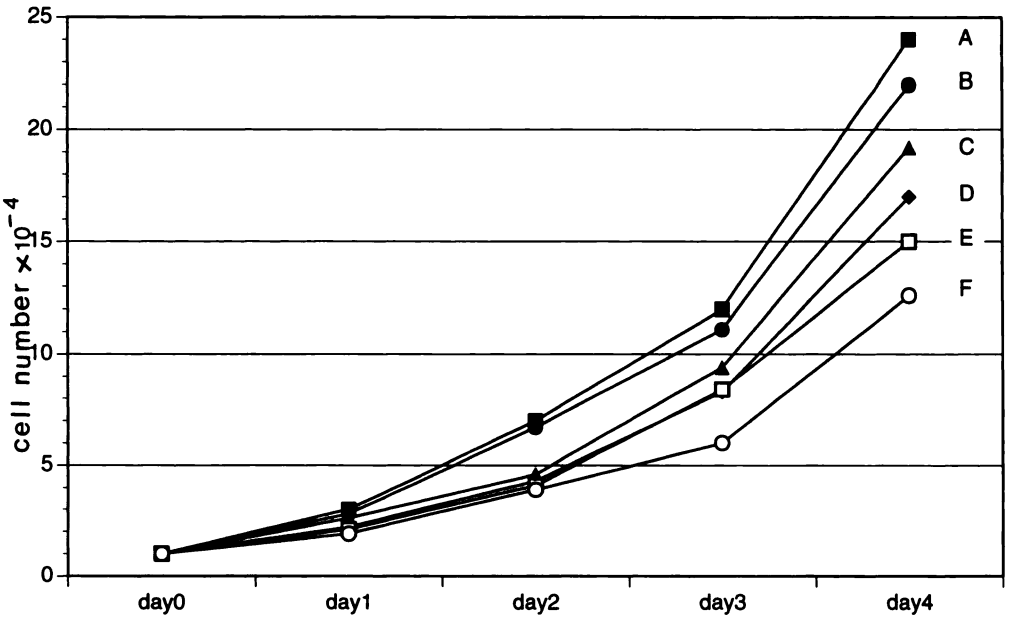

Fig. 9. Growth curves of parent VAMT-1 cells and VAMT-1 cells transfected with various ribozyme constructs expressing the c-*sis* ribozyme. Cells (1×10^4) from each clone were seeded on day 0 in serum-free RPMI 1640 medium and were grown in Falcon six-well tissue culture plates (no. 3046; Becton Dickinson) as described in Materials and Methods. Cells were counted on days 1 through 4. Each *point* represents the mean of triplicate determinations in a single experiment. Standard deviations were within 5% of the mean. Similar results were obtained in two other separate experiments. *A*, Parent VAMT-1 cells; *B*, a typical clone expressing the disabled ribozyme; *C*, *D*, *E*, and *F*, wild-type ribozyme-expressing clones 6, 7, 8, and 12, respectively.

ribozyme to the target site (41). Although the ribozymes are expressed at similar levels in various G418-resistant clones, the clones had varying degrees of inhibition of PDGF mRNA expression. This could be partly due to the high level of expression of the ribozyme transcripts by the powerful β-actin promoter, which is highly active in all nonmuscle cells, making it hard to see the relative differences in their expression in various clones. Ribozymes should be catalytic in the cleavage reaction, but this feature may not be operating to the fullest extent inside the cells. The efficacy of ribozymes is dependent on the ratio of ribozyme to substrate, the availability of the ribozyme in the same subcellular compartment as the substrate, the stability of the expressed ribozyme inside the cell, inhibition of ribozyme action by cellular factors, and the intracellular Mg^{2+} concentration, to name a few. Thus, there are many factors that contribute to the maximum inhibition of PDGF mRNA expression, which was only approximately 50% with the present ribozyme. Studies are in progress to synthesize and evaluate c-*sis* ribozymes affecting GUC sites other than the presently studied codon 151.

Finally, the treatment of VAMT-1 mesothelioma cells with suramin, an agent known to interfere with the interaction of PDGF-B with its cognate receptor and hence interrupt the autocrine loop (19, 42), inhibited cell growth by only approximately 50% (data not shown). This result strongly suggests that PDGF-B may not be the only stimulating factor in VAMT-1 cell proliferation. There is accumulating evidence that neoplastic progression involves a series of stepwise genetic alterations leading to growth factor-independent, frankly malignant cells (43, 44). With a panel of tumor cell lines, Fleming *et al.* (19) showed that, in the tumor cell lines they tested, it was not possible to impair proliferation by using suramin or anti-PDGF antibody, despite interference with the PDGF autocrine pathway. Thus, it is entirely possible that the number of genetic steps associated with the acquisition of the malignant phenotype in such tumors was sufficient to permit their sustained proliferative drive despite effective blocking of PDGF stimulation. We believe that a similar situation might exist in malignant mesothelioma, considering its long latency period and numerous structural alterations in the chromosomes. Data reported by Metcalf *et al.* (45) strongly suggest that neither *p53* alteration nor k-*ras* activation constitutes a critical step in the development of human mesothelioma. It is also of interest to note, from recent studies by Heintz *et al.* (46), that persistent induction of AP-1 transcription factors by astestos suggests a model of asbestos-induced carcinogenesis involving chronic stimulation of cell proliferation through activation of the early-response gene pathway that includes c-*jun* and/or c-*fos*. Thus, by a process of elimination and selection, we can arrive at a panel of multiple possible molecular lesions the mesothlioma cells could have acquired in their genesis, namely the overexpression of *PDGF-B*, *PDGF-A*, c-*jun*, and c-*fos*. We are currently approaching this problem with a molecular construct containing multiple ribozymes, each one directed against the specific mRNA of the aforementioned oncogenes.

Acknowledgments

The authors wish to thank Lenina Szrajer and Feng Li Li for their expert technical assistance throughout the course of this study. We also wish to thank Lawrence Brennan for his assistance in preparing the manuscript.

References

1. Antman, K. H., H. I. Pass, and A. Recht. Benign and malignant mesothelioma, in *Cancer: Principles and Practice* (V. T. Devita, S. Hellman, and S. A. Rosenberg, eds.), Ed. 3. J. B. Lippincott, Philadelphia, 1399–1417 (1989).
2. Craighead, J. E. The epidemiology and pathogenesis of malignant mesothelioma. *Chest* **96**:935–949 (1989).
3. Chahinian, A. P., T. G. Pajak, and J. F. Holland. Diffuse malignant mesothelioma: prospective evaluation of 69 patients. *Ann. Int. Med.* **96**:746–755 (1982).

4. Lechner, J. F., T. Tokiwa, M. LaVeck, W. F. Benedict, S. Banks-Schlegel, H. Yeager, A. Banerjee, and C. C. Harris. Asbestos-associated chromosomal changes in human mesothelial cells. *Proc. Natl. Acad. Sci. USA* **82**:3884–3888 (1985).
5. Sincock, A., and M. Seabright. Induction of chromosome changes in Chinese hamster cells by exposure to asbestos fibers. *Nature (Lond.)* **254**:56–58 (1975).
6. Tiainen, M., L. Tammilehto, K. Mattson, and S. Knuutila. Nonrandom chromosomal abnormalities in malignant pleural mesothelioma. *Cancer Genet. Cytogenet.* **33**:251–274 (1988).
7. Hagemeijer, A., M. A. Versnel, E. van Drunen, M. Moret, M. J. Bouts, T. H. van der Kwast, and H. C. Hoogsteden. Cytogenetic analysis of malignant mesothelioma. *Cancer Genet. Cytogenet.* **47**:1–28 (1990).
8. Popescu, N. C., A. P. Chahinian, and J. A. DiPaolo. Nonrandom chromosomal alterations in human malignant mesothelioma. *Cancer Res.* **48**:142–147 (1988).
9. Gerwin, B. I., J. F. Lechner, R. R. Reddel, A. B. Roberts, K. C. Robbins, E. W. Gabrielson, and C. C. Harris. Comparison of production of transforming growth factor-β and platelet-derived growth factor by normal human mesothelial cells and mesothelioma cell lines. *Cancer Res.* **47**:6180–6184 (1987).
10. Versnel, M. A., A. Hagemeijer, M. J. Bouts, T. H. van der Kwast, and H. C. Hoogsteden. Expression of c-*sis* (PDGF-B chain) and PDGF-A chain genes in ten human malignant mesothlioma cell lines derived from primary and metastatic tumors. *Oncogene* **2**:601–605 (1988).
11. Doolittle, R. F., M. W. Hunkapillar, L. E. Hood, S. G. Devare, K. C. Robbins, S. A. Aaronson, and H. N. Antoniades. Simian sarcoma virus oncogene, v-*sis*, is derived from the gene (or genes) encoding platelet-derived growth factor. *Science (Washington D. C.)* **221**:275–277 (1983).
12. Waterfield, M. D., G. T. Scrace, N. Whittle, P. Stroobant, A. Johnsson, A. Wasteson, B. Westermark, C.-H. Heldin, J. S. Huang, and T. F. Deuel. Platelet-derived growth factor is structurally related to the putative transforming protein p28^{sis} of simian sarcoma virus. *Nature (Lond.)* **304**:35–39 (1983).
13. Betsholtz, C., A. Johnsson, C.-H. Heldin, B. Westermark, P. Lind, M. S. Urdea, R. Eddy, T. B. Shows, K. Philpott, A. L. Mellor, T. J. Knott, and J. Scott. cDNA sequence and chromosomal localization of human platelet-derived growth factor A chain and its expression in tumor cell lines. *Nature (Lond.)* **320**:695–699 (1986).
14. Raines, E. W., D. F. Bowen-Pope, and R. Ross. *Handb. Exp. Pharmacol.* **95**:173–262 (1990).
15. Hammacher, A., U. Hellman, A. Johnsson, A. Ostman, K. Gunnarsson, B. Westermark, A. Wasteson, and C.-H. Heldin. A major part of platelet-derived growth factor purified from human platelets is a heterodimer of one A and one B chain. *J. Biol. Chem.* **263**:16493–16498 (1988).
16. Sporn, M. B., and G. J. Todaro. Autocrine secretion and malignant transformation of cells. *N. Engl. J. Med.* **303**:878–880 (1980).
17. Huang, J. S., S. S. Huang, and T. F. Deuel. Transforming protein of simian sarcoma virus stimulated autocrine growth of SSV transformed cells through PDGF cell-surface receptors. *Cell* **39**:79–87 (1984).
18. Versnel, M. A., L. Claesson-Welsh, A. Hammacher, M. J. Bouts, T. H. van der Kwast, A. Eriksson, R. Willemsen, S. M. Weima, H. C. Hoogsteden, A. Hagemeijer, and C.-H. Heldin. Human malignant mesothelioma cell lines express PDGF-β receptors whereas cultured normal mesothelial cells express predominantly PDGF-α receptors. *Oncogene* **6**:2005–2011 (1991).
19. Fleming, T. P., T. Matsui, C. J. Molloy, K. C. Robbins, and S. A. Aaronson. Autocrine mechanism for v-*sis* transformation requires cell surface localization of internally activated growth factor receptors. *Proc. Natl. Acad. Sci. USA* **86**:8063–8067 (1989).
20. Fleming, T. P., T. Matsui, M. A. Heidaran, C. J. Molloy, J. Artrip, and S. A. Aaronson. Demonstration of an activated platelet-derived growth factor autocrine pathway and its role in human tumor cell proliferation *in vitro*. *Oncogene* **7**:1355–1359 (1992).
21. Smits, A., K. Funa, F. S. Vassboyn, M. Beausang-Linder, F. Afekenstam, C.-H. Heldin, B. Westermark, and M. Nister. Expression of platelet-derived growth factor and its receptors in proliferative disorders of mesenchymal origin. *Am. J. Pathol.* **140**:639–648 (1992).
22. Holmgen, L., F. Flam, E. Larsson, and R. Ohlsson. Successive activation of the platelet-derived growth factor β receptor and platelet-derived growth factor B genes correlates with the genesis of human choriocarcinoma. *Cancer Res.* **53**:2927–2931 (1993).
23. Cech., T. R., and B. Bass. Biological catalysis by RNA. *Annu. Rev. Biochem.* **55**:599–629 (1986).
24. Rossi, J. J., E. M. Cantin, N. Sarver, and P. F. Chang. The potential use of catalytic RNAs in therapy of HIV infection and other diseases. *Pharmacol. Ther.* **50**:245–254 (1991).
25. Snyder, D. S., Y. Wu, J. L. Wang, J. J. Rossi, P. Swiderski, B. E. Kaplan, and S. J. Forman. Ribozyme mediated inhibition of *bcr-abl* gene expression in a Philadelphia chromosome-positive cell line. *Blood* **82**:600–605 (1993).
26. Tibbets, L. M. Loss and restoration of tumorigenicity in a human mesothelioma cell line. *Prog. Am. Assoc. Cancer Res.* **25**:45 (1984).
27. Ohnuma, T., L. Szrajer, J. F. Holland, M. Kurimoto, and J. Minowada. Effects of natural interferon-α, natural tumor necrosis factor-α and their combination on human mesothelioma xenografts in nude mice. *Cancer Immunol. Immunother.* **36**:31–36 (1993).
28. Chomczynski, P., and N. Sacchi. Single step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* **162**:156–159 (1987).
29. Rao, C. D., H. Igarashi, M. W. Pech, K. C. Robbins, and S. A. Aaronson. Oncogenic potential of the human platelet-derived growth factor transcription unit. *Cold Spring Harbor Symp. Quant. Biol.* **51**:959–966 (1986).
30. Sal, G. D., G. Manfioletti, and C. Schneider. The CTAB-DNA precipitation method: a common miniscale preparation of template DNA from phagemids, phages or plasmids suitable for sequencing. *Biotechniques* **7**:514–519 (1989).
31. Milligan, J. F., D. R. Groebe, G. W. Witherell, and O. C. Uhlenbeck. Oligoribonucleotide synthesis using T7 RNA polymerase and synthetic DNA templates. *Nucleic Acids Res.* **15**:8783–8798 (1987).
32. Gunning, P., J. Leavitt, G. Muscat, S.-Y. Ng, and L. Kedes. A human β-actin expression vector system directs high level accumulation of antisense transcripts. *Proc. Natl. Acad. Sci. USA* **84**:4831–4835 (1987).
33. Leal, F., L. T. Williams, K. C. Robbins, and S. A. Aaronson. Evidence that v-*sis* gene product transforms by interaction with the receptor for platelet-derived growth factor. *Science (Washington D. C.)* **239**:327–330 (1985).
34. Haseloff, J., and W. J. Gerlach. Simple RNA enzymes with new and highly specific endoribonuclease activities. *Nature (Lond.)* **334**:585–591 (1988).
35. Ruffner, D. E., G. D. Stormo, and O. C. Uhlenbeck. Sequence requirements of the hammerhead RNA self-cleavage reaction. *Biochemistry* **29**:10695–10702 (1990).
36. Alberts, B., D. Bray, J. Lewis, M. Raff, K. Roberts, and J. D. Watson. *Molecular Biology of the Cell*, Ed. 2. Garland Publishing Inc., New York, 301 (1989).
37. Uhlenbeck, O. C. A small catalytic oligoribonucleotide. *Nature (Lond.)* **328**:596–600 (1987).
38. Koizumi, M., and E. Ohtsuka. Effects of phosphorothioate and 2-amino groups in hammerhead ribozymes on cleavage rates and Mg^{2+} binding. *Biochemistry* **30**:5134–5150 (1991).
39. van der Meeren, A., M. B. Seddon, C. A. Betsholtz, J. F. Lechner, and B. I. Gerwin. Tumorigenic conversion of human mesothelial cells as a consequence of platelet-derived growth factor-A chain overexpression. *Am. J. Respir. Cell. Mol. Biol.* **8**:214–221 (1993).
40. Igarashi, H., C. D. Rao, M. Siroff, F. Leal, K. C. Robbins, and S. A. Aaronson. Detection of PDGF-2 homodimers in human tumor cells. *Oncogene* **1**:79–85 (1987).
41. Koizumi, M., Y. Hayase, S. Iwai, H. Kamiya, H. Inoue, and E. Ohtsuka. Design of RNA enzymes distinguishing a single base mutation in RNA. *Nucleic Acids Res.* **17**:7059–7071 (1989).
42. Williams, L. T., P. M. Tremble, M. F. Lavin, and M. E. Sunday. Platelet-derived growth factor receptors form a high affinity state in membrane preparation. *J. Biol. Chem.* **259**:5287–5294 (1984).
43. Vogelstein, B., E. R. Fearon, S. R. Hamilton, S. E. Kern, A. C. Preisinger, M. Leppert, Y. Nakamura, R. White, A. M. M. Smits, and J. L. Boss. Genetic alterations during colorectal tumor development. *N. Engl. J. Med.* **319**:525–532 (1988).
44. Fearon, E. R., and B. Vogelstein. A genetic model for colorectal tumorigenesis. *Cell* **61**:759–767 (1990).
45. Metcalf, R. A., J. A. Welsh, W. P. Bennett, M. B. Seddon, T. A. Lehman, K. Pelin, K. Linnainmaa, L. Tammilehto, K. Mattson, B. I. Gerwin, and C. C. Harris. *p53* and Kirsten-*ras* mutations in human mesothelioma cell lines. *Cancer Res.* **52**:2610–2615 (1992).
46. Heintz, N. H., Y. M. Janssen, and B. T. Mossman. Persistent induction of c-*fos* and c-*jun* expression by asbestos. *Proc. Natl. Acad. Sci. USA* **90**:3299–3303 (1993).

Send reprint requests to: Thambi Dorai, Derald H. Ruttenberg Cancer Center, Box 1128, Mount Sinai School of Medicine, One Gustave L. Levy Place, New York, NY 10029-6574.