Vol. 201, No. 1, 1994 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
May 30, 1994 Pages 379-387

CLONING AND CHARACTERIZATION OF THE HUMAN THROMBOXANE SYNTHASE GENE PROMOTER

Kuan-Der Lee[1], Seung Joon Baek[1], and Rong-Fong Shen[1,2*]

[1]Division of Human Genetics, Department of Obstetrics/Gynecology, University of Maryland School of Medicine, Baltimore, MD 21201

[2]Medical Biotechnology Center, University of Maryland Biotechnology Institute, Baltimore, MD 21201

Received March 28, 1994

SUMMARY: A genomic phage clone hybridized to the 5′ end of human thromboxane synthase (TS) cDNA was isolated. Sequencing analysis of a 1.7 kb subfragment revealed that it contained the entire 5′ untranslated region and 46 bp of the coding sequence of TS cDNA, an upstream canonical TATA box (TATAAA), and several binding sites for transcription factors (AP1, PEA-3, PU.1, and GR), indicative of a promoter/first exon region of the TS gene. RNase protection assay mapped the transcription start site of the human TS gene to the nucleotide A 30 bp downstream from the TATA box. The authenticity of the promoter was further confirmed by its ability to direct expression of a CAT reporter gene in transfected HL60 cells.

Thromboxane synthase (TS) controls the biosynthesis of thromboxane A_2 (TxA_2), a potent autacoid that induces vasoconstriction and platelet aggregation (1). The ability of TxA_2 to modulate platelet function and vascular activity has led to extensive investigation of TS for its involvement in pathophysiological states such as cardiovascular diseases, pulmonary hypertension, and glomerulonephritis (2,3,4). However, a rigorous proof of the etiological relationship of TS to various diseases has yet to be established. A few studies demonstrated that a deficiency in platelet TS activity resulted in bleeding disorders (5,6), but the underlying molecular mechanisms remain to be elucidated. TS has been purified from human platelets (7) and porcine lung (8) and its tissue distribution determined by direct enzyme quantitation (9) and immunocytochemical staining (10). The enzyme exhibits interesting features in that it catalyzes the formation of both TxA_2 and HHT and undergoes self-inactivation during catalysis (8,11).

* To whom correspondence should be addressed. Fax: (410)706-6105.

The abbreviations used are: h, hour(s); HHT, 12-hydroxy-heptadecatrienoic acid; nt, nucleotide(s); PCR, polymerase chain reaction; PGH_2, prostaglandin endoperoxide H_2; TS, thromboxane synthase; TxA_2, thromboxane A_2.

0006-291X/94 $5.00

The suicide inactivation was shown to result from a covalent modification of the enzyme by its substrate PGH_2 (11), which could be protected against by competitive inhibitors (8,11). TS cDNA has recently been cloned from three mammalian species (12-16). We demonstrated that TS protein sequences, as deduced from human, porcine and murine TS cDNAs, shared greater than 75% identity and that the TS gene was expressed primarily in the lung and kidney of all three species (15,16). The conservation of enzyme structures and the similarity in the spectra of gene expression suggest that TS probably exerts similar physiological function in these mammalian species. The selective, multi-tissue expression of the TS gene also implies a delicate control at the level of transcription. Catalytic inactivation of TS presumably serves as a refractory mechanism to avoid over-production of the potent autacoid. It is likely that regeneration of a functional enzyme requires transcriptional activation of the gene. To better address these important questions, we decided to clone the promoter of the human TS gene. Here we report the isolation, sequencing, and characterization of a genomic DNA fragment containing the human TS promoter/first exon.

MATERIALS AND METHODS

Isolation of Genomic Phage Clones Containing the Human TS Gene: A human genomic DNA library constructed in λ FIX II (Stratagene) was screened with human TS cDNA probe according to the method described previously (16). Phage plaques were transferred to Hybond membranes (Amersham) and hybridized at 42°C for 12 h in 50% formamide, 5X SSPE, 10X Denhardt's solution, 0.1 mg per ml salmon sperm DNA, and 0.1% SDS. After hybridization, membranes were washed at 55°C for 1 h with 0.1 X SSC containing 0.1% SDS. Positive clones were purified to a single plaque and the phage DNA was extracted by standard methods (17). DNA was digested with NotI to release the insert, followed by partial EcoRI digestion. The digestion mixture was subcloned into the pBluscript SK (+) vector and screened with an oligonucleotide probe (5′ agatcagcctcctgtctcat 3′, nt -55 to -36 in human TS cDNA [12,13]).

DNA Sequence Analysis: Double-stranded DNA sequencing was performed by the dideoxy chain termination method (18) using [α-^{35}S]dATP and the Sequenase 2.0 kit (U.S. Biochemicals).

Cell Culture and RNA Isolation: HL60 cells were maintained in RPMI 1640 medium supplemented with fetal bovine serum (10%), L-glutamine (2 mM), penicillin (100 U/ml), and streptomycin (100 μg/ml). Total RNA was isolated according to the acid guanidinium thiocyanate procedures (19). Cells were collected by centrifugation and lysed with a solution containing 4M guanidine thiocyanate, 25 mM sodium citrate (pH 7.0), 0.5% *N*-laurylsarcosine, and 0.1 M 2-mercaptoethanol. Sodium acetate (pH 4.0) and phenol were then added to the lysate to a final concentration of 0.1 M and 50% (v/v), respectively. The mixture was homogenized with a Polytron to shear chromosomal DNA. Following the addition of 1/10 volume of chloroform and vortexing, the aqueous phase was separated by centrifugation and RNA was precipitated with isopropanol. Poly $(A)^+$ RNA was purified by oligo (dT)-cellulose column chromatography according to the described procedures (20).

Mapping of the Transcription Start Site by RNase Protection : *In vitro* transcription and RNase protection mapping were carried out according to the protocols described by Calzone *et al.* (21). RNA probe was prepared from a plasmid containing a PstI-XmnI fragment (nt 1346-1628, Fig. 2) cloned in the pBluescript SK(+) vector. The plasmid was digested with HindIII,

extracted once with phenol/chloroform, ethanol precipitated, and dissolved in 10 mM Tris (pH 8.0) buffer containing 1 mM EDTA. The DNA (1 μg) was used as the template for *in vitro* transcription using the T7-polymerase (Ambion) in the presence of 1 mM each of the three nucleotides (ATP, CTP, and GTP) and 50 μCi of [^{32}P]UTP. The synthesized probe (10^6cpm) was annealed to 2 μg of poly(A)$^+$ RNA for 12 h at 45°C in a buffer containing 80% (v/v) formamide, 100 mM sodium citrate (pH 6.4), 300 mM sodium acetate (pH6.4) and 1 mM EDTA. The hybrids were digested with RNase A (5 μg/ml) and RNase T1 (100 units/ml) at 37°C for 30 min. The reaction was terminated with an RNase inactivation mixture containing SDS and proteinase K (Ambion). The products were precipitated by guanidinium/ethanol reagent, resuspended in the loading buffer (80% formamide, 2 mM EDTA, 0.1% bromophenol blue and 0.1% xylene cyanol), and then analyzed by electrophoresis in a 6% polyacrylamide-urea sequencing gel. Autoradiography was carried out at -80 °C for 16 h using the Kodax XAR-5 film and one Cronex intensifying screen.

Rapid Amplification of 5′-cDNA Ends (5′-RACE): The 5′-RACE experiments were carried out essentially as described (22) using the 5′-AmpliFINDER RACE kit (Clontech). A first strand cDNA was generated by reverse transcription using 2 μg of poly (A)$^+$ RNA and a specific oligonucleotide (P1 primer: 5′-tagtacccacacagaggtccatac-3′, bottom strand nt 219-242 in human TS cDNA, ref. 12). After incubation for 1 h at 52 °C, a single-stranded oligonucleotide anchor was directly ligated to the 3′-end of the first-strand cDNA by T4 RNA ligase. Following anchor ligation, a portion of the cDNA was used as a template for PCR amplification, using one primer within the anchor and the other primer (P2 primer: 5′ atgagacaggaggctgatct 3′, bottom strand nt -55 to -36) in human TS cDNA. The amplified products were electrophoresed in a 3% agarose gel and DNA from the major band was isolated and cloned into pBluescript SK(+) vector for sequencing analysis.

Transfection and CAT Assay: Two genomic fragments, 0.8 kb and 1.2 kb respectively, containing the TS promoter sequence and a portion of the first exon were cloned 5′ to the CAT reporter gene, in both orientations, in the pCAT-basic vector (Promega). Plasmids for transfection were prepared by double CsCl centrifugation. HL60 cells ($5X10^6$) were transfected with 20 μg plasmid DNA by electroporation using the Bio-Rad Gene Pulser. 48 h post transfection, cell extracts were prepared for CAT activity assay according to the method described by Gorman *et al.* (23).

RESULTS AND DISCUSSION

Isolation of a 5′ Genomic Clone of the Human TS Gene: Screening of ~500,000 phage clones of a human genomic library with a full-length TS cDNA probe resulted in the isolation of ten positive clones. Southern analysis revealed that only one of these clones contained an insert hybridized to the 5′ probe of human TS cDNA (data not shown). Phage DNA from this positive clone was prepared and digested with NotI restriction enzyme to release the insert, followed by EcoRI partial digestion. The digestion mixture was shotgun cloned into a pBluescript vector previously treated with EcoRI and NotI restriction enzymes. Following identification with an oligonucleotide probe (nt -55 to -36 in human TS cDNA, ref. 12 and 13), one plasmid clone containing a 4.6 kb EcoRI/NotI fragment was isolated and its restriction map constructed for three restriction enzymes, EcoRI, PstI, and NcoI, as shown in Fig. 1. The latter two restriction sites have been found in the 5′ end of human TS cDNA.

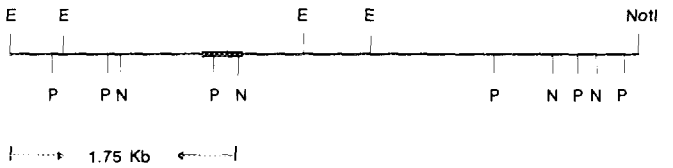

Fig. 1. **Restriction Map of the NotI/EcoRI Genomic Fragment**. The screening of a human genomic library and subcloning of a 4.6 kb fragment hybridized to a 5′ TS cDNA probe were described in "*Materials and Methods*". This NotI/EcoRI fragment was restriction mapped for EcoRI, NcoI, and PstI enzymes. The relative position of the 5′ end of human TS cDNA sequence (see Fig. 2) in this fragment is indicated by the thicker line.

Sequence Analysis of the Promoter/First Exon: To confirm that the cloned genomic DNA contained the 5′ portion of the human TS gene, a 1,753 bp subfragment (EcoRI to NcoI) was completely sequenced. As shown in Fig. 2, this fragment contained the 5′-most 173 bp sequence of human TS cDNA (1581-1753, underlined), including the entire 5′ untranslated region (127 bp) and 46 bp of the coding sequence. Within this sequence is a canonical TATA box (TATAAA, 1441-1446) 140 bp upstream from the end of the reported human platelet TS cDNA sequence (12). The rest of the sequence is very A/T rich, characteristic of a promotor region. There are several short sequence repeats in this sequence. A hexanucleotide [TAGA(T/C/G)A] sequence is found to repeat seven times (1278-1319). The heptanucleotide TTTTCTT is identified six times (at 491, 504, 560, 657, 844, and 1419), while the sequence (C/T)TTTCCT(C/T) appears seven times (at 39, 182, 231, 361, 413, 1082, and 1350). In addition to the TATA box, several potential binding sites for transcription factors were identified. There are two binding sites for PU.1 (5′ AGGAAG 3′, nt 1431-1436, and its reverse sequence 5′ CTTCCT 3′, nt 415-420) and one for the PEA3 (5′ AGGAAGT 3′, nt 1431-1437) transcription factors; the latter overlaps with the PU.1 binding site. PU.1 is an *ets*-related transcriptional activator expressed specifically in macrophages and B cells (24). PEA3 motif has been shown to mediate transcriptional activation by the c-*ets*-1 and -2 proto-oncogenes, v-src, phorbol ester, and serum components (25,26). The core TTTCCT sequence in the above mentioned (C/T)TTTCCT(C/T) repeats can be a binding site (a reverse sequence) for the PEA3 transcription factor (27). Interestingly, both PEA3 and PU.1 sites were also idenified in the promoters of murine and human prostaglandin endoperoxide synthase I (COX I) genes (28,29). Whether or not they play any role in controlling the expression of these arachidonic acid-metabolic enzymes remains to be elucidated. Two AP1 binding sites (nt 592-599 and 966-973) (30) and a glucocorticoid receptor binding site (5′ TGTTCT 3′, nt 309-314) (31) are also present in the sequence.

Determination of the Transcription Initiation Site: To verify that the isolated fragment represents the promoter/first exon of human TS gene, RNase protection assay was carried out

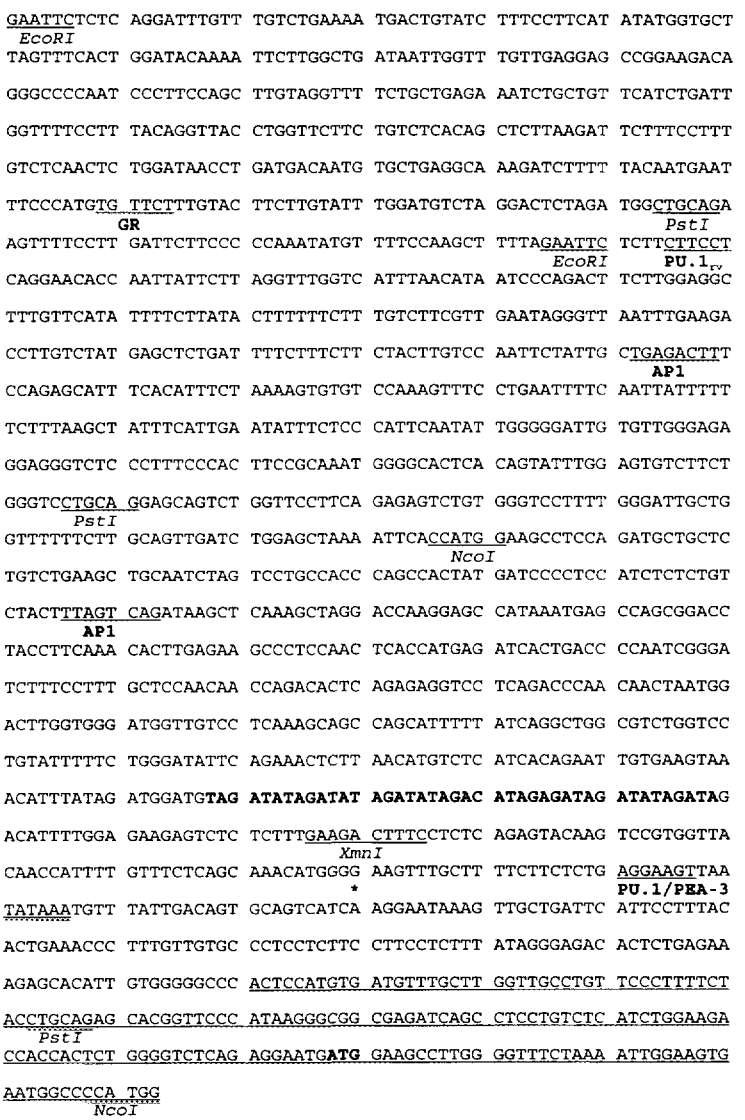

Fig. 2. **Nucleotide Sequence of the Putative Promoter/First Exon of the Human TS Gene.** The underlined sequence represents the sequence reported for the 5′ end of human TS cDNA (12). The translation start codon AUG is bold-faced. The putative TATA box is double-underlined and the hexanucleotide repeat is bold-faced. Putative binding sites for transcription factors are indicated. The star denotes the transcription start site determined by the RNase protection assay described in Fig. 3.

to localize the transcription start site(s) using mRNA of the human promyelocytic cells (HL60), which constitutively express the TS gene (32). A 340-base riboprobe, including 70 bases of the polylinker sequence, was generated by *in vitro* transcription from a plasmid containing the PstI-XmnI subfragment (Fig. 3A). Following hybridization of the riboprobe with HL60 mRNA and nuclease digestion with RNase A/T1, the products were analyzed in a sequencing gel. As shown in Fig. 3B, the longest fragment protected from RNase digestion is 152 bp, indicating that

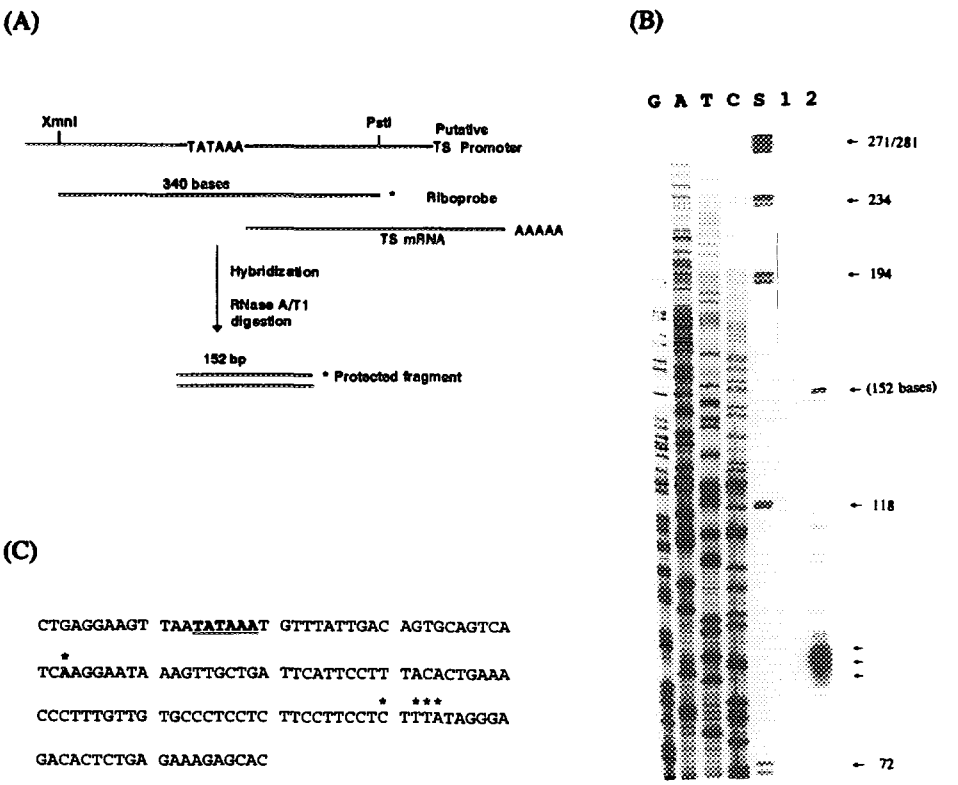

Fig. 3. **Mapping the Transcription Start Site of the Human TS gene by RNase Protection Assay and the RACE protocol.** (A): A schematic description of riboprobe preparation and RNase protection assay. An XmnI/PstI subfragment (1346-1623, Fig. 2) was used to generate a riboprobe and used for RNase protection assay, as described in "*Materials and Methods*". (B): Autoradiogram of the protected riboprobes after RNase digestion. The protected probes were precipitated, resuspended in the loading buffer, and electrophoresed in a 6% polyacrylamide-urea sequencing gel. The gel was dried and exposed to X-ray film at -80 °C for 16 h using one intensifying screen. Lane S, DNA size marker (ϕx174 HaeIII); Lane 1, control tRNA; Lane 2, HL60 mRNA. The four sequence ladders were obtained using T7 primer and a plasmid DNA containing a PstI fragment (1151-1536) of human TS cDNA. (C): Sequence showing the transcription start site of the human TS gene. The nucleotide A 30 bp from the TATA box was designated as the transcription start site, as determined by RNase protection assay. The clustered 4 nucleotides ($\sim$100 bp from the TATA box indicated by stars) were the ends of cDNA sequences determined from 4 independent plasmid clones obtained by the RACE protocol, as described in the text.

transcription starts from the nucleotide A (indicated by a star) 30 bp downstream from the TATA box (Fig. 3C). There is also a broad region containing protected probes approximately 90-base long (Fig. 3B, indicated by three arrows), representing several protected fragments whose 5′ ends are about 100 bp from the TATA box. We suspected that this may be due to either multiple alternative transcription initiation sites of the TS gene or incomplete protection of the probe caused by a secondary structure present in the 5′ end of TS mRNA. To test the latter possibility, we carried out the RACE procedure, which relies on the synthesis of the first strand cDNA by reverse transcriptase and is, therefore, more sensitive to the presence of a

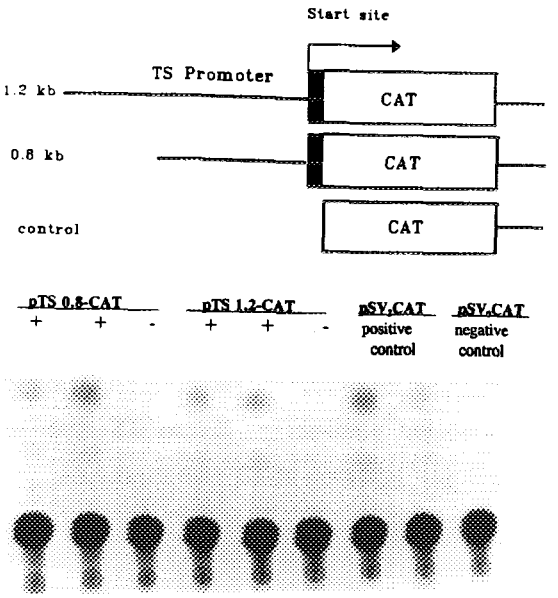

Fig. 4. **Demonstration of TS Promoter Activity by Directing CAT Expression in HL60 Cells.** HL60 cells (5×10^6) were transfected with 20 μg each of the plasmid DNA indicated by electroporation, using Bio-Rad Gene Pulser set at 300 volts and 960 μF. 48 h after transfection, cell extracts were prepared in 100 μl Tris-HCl buffer, pH 7.5. An aliquot of the extract (30 μl) was used for CAT activity assay at 37°C for 4 h using 0.5 μCi ^{14}C-chloramphenicol and 8 mM acetyl CoA. pTS1.2-CAT and pTS0.8-CAT contain 1.2 and 0.8 kb, respectively, of human TS promoter/first exon driving the CAT gene. Positive (+) and negative (-) signs indicate that the promoter fragments are cloned in the correct and reverse orientation, respectively. The two (+) samples used cell extracts prepared independently. The pSV_2CAT vector contains both the SV40 promoter and enhancer, while the pSV_0CAT vector is promoter- and enhancer-less.

secondary structure in mRNA. As described in detail in "*Material and Methods*", we used HL60 mRNA to synthesize the first strand cDNA which was then ligated to an oligonucleotide anchor. The products were then used as templates for PCR amplification, using nested primers. The PCR products were gel-purified and subcloned into a vector for sequencing analsysis. We obtained four independent plasmid clones whose 5′ ends were determined to be at C, T, T, A, respectively, about 100 bp downstream from the TATA box, as shown in Fig. 3 C. This result suggests, but did not prove, that there may indeed be a secondary structure present in the TS mRNA which prevents reverse transcription to completion. Since eukaryotic pol II transcription generally initiates at ~25-30 bp downstream from the TATA box with a CAP site motif over-represented by CA (where A is the transcription initiation site, ref. 33), we therefore assigned the nucleotide A 30 bp downstream of TATA box to be the transcription initiation site for the human TS gene.

Transient CAT Expression Controlled by the TS Promoter: We expect that if the above sequence represents the promoter/first exon of the TS gene, it would be able to direct expression

of a reporter gene in cells expressing TS. We subcloned either a ~0.8 or ~1.2 kb PstI fragment (nt 789-1626 and 357-1626, respectively, Fig.2), which included 160 bp first exon sequence, into a promoter- and enhancer-less CAT expression vector in both orientations. These plasmids were used to transfect HL60 cells by electroporation. As shown in Fig.4, constructs containing the genomic fragments in the correct orientation (pTS0.8-CAT and pTS1.2-CAT, indicated by +) and the positive control (pSV_2CAT) were able to direct CAT expression, while those containing fragments in the reverse orientation (indicated by -) and the negative control (pSV_0CAT) produced no detectable CAT activity. The result confirms that the isolated genomic fragment is indeed the promoter for the human thromboxane synthase gene. The availability of the genomic DNA containing the promoter of human TS gene should facilitate future investigation of the transcriptional regulation of TS gene expression.

ACKNOWLEDGMENTS

This work was supported by a Grant-in-Aid (to RFS) from the American Heart Association and with funds contributed in part by the AHA, Maryland Affiliate.

REFERENCES

1. Hamberg, M., Svensson, J., and Samuelsson, B. (1975). Proc. Natl. Acad. Sci. 72:994-2998.
2. Marcus, A.J., and Safier, L.B. (1993). FASEB J. 7:516-522.
3. Ogletree, M.L. (1987). Fed. Proc. 46:133-138.
4. Moncada, S., and Vane, J.R. (1982). Pharmacol. Rev. 30:293-331.
5. Mestel, F., Oetliker, O., Bech, E., Felix, R., Imbach, P. and Wafner, H-P. (1980). Lancet i, 157.
6. Weiss, H.J. and Lages, B.A. (1977). Lancet ii, 760-761.
7. Haurand, M., and Ullrich, V. (1985). J. Biol. Chem. 260:15059-15067.
8. Shen, R.-F., and Tai, H.H. (1986). J. Biol. Chem. 261:11592-11599.
9. Shen, R.-F., and Tai, H.H. (1986). J. Biol. Chem. 261:11585-11591.
10. Nüsing, R., Lesch, R., and Ullrich, V. (1990). Eicosanoids 3:53-58.
11. Jones, D.A., and Fitzpatrick, F.A. (1991). J. Biol. Chem. 34:23510-23514.
12. Yokoyama, C., Miyata, A., Ihara, H., Ullrich, V., and Tanabe, T. (1991). Biochem. Biophys. Res. Commun. 178:1479-1484.
13. Ohashi, K., Ruan, K-H., Kulmacz, R.J., Wu, K.K., and Wang, L-H. (1992). J. Biol. Chem. 267:789-793.
14. Xia, Z., Shen, R.-F., Baek, S.J., and H.H. Tai. (1993). Biochem. J. 295:457-461.
15. Zhang, L., Chase, M., and Shen, R.-F. (1993). Biochem. Biophys. Res. Commun. 194:741-748.
16. Shen, R.-F., Zhang, L., Baek, S.J., Tai, H.H., and Lee, K.D. (1994). Gene (in press).
17. Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989). Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory Press, New York.
18. Sangers, F., Nicklen, S., and Coulson, A.R. (1977). Proc. Natl. Acad. Sci. 74:5463-5467.
19. Chomczynski, P., and Sacchi, N. (1987). Anal. Biochem. 162, 156-159.
20. Aviv, H., and Leder, P. (1972). Proc. Natl. Acad. Sci. U.S.A. 69:1408-1412.

21. Calzone, F. *et al.* (1987). Methods Enzymol. 152: p611.
22. Edwards, J.B.D.M., Delort, J. and Mallet, J. (1991). Nucleic Acid Res. 19:4002.
23. Gorman, C., Moffat, L.F. and Howard, B. (1982). Mol. Cell Biol. 2:1044-1051.
24. Klemsz, M., McKercher, S. R., Celada, A., Beveren, C. V., and Maki, R.A. (1990). Cell 61:113-124.
25. Wasylyk, C., Flores, P., Gutman, A., and Wasylyk, B. (1989). EMBO J. 8:3371-3378.
26. Wasylyk, B., Wasylyk, S., Flores, P., Begue, A., Leprinc, D., and Stehelin, D. (1990). Nature 346:191-193.
27. Faisst, S. an d Meyer, S. (1990). Nucleic Acid Res. 20:3-26.
28. Kraemer, S.A., Meade, E.A., and DeWitt, D.L. (1992). Arch. Biochem. Biophys. 293:391-400.
29. Wang, L.-H., Hajibegi, A., Xu, X.M., Loose-Mitchell, D., and Wu, K.K. (1993). Biochem. Biophys. Res. Commun. 190:406-411.
30. Kim, S-J., Denhez, F., Kim K. Y., Jolt, J. T., Sporn, M. B., and Roberts, A. B. (1989) J. Biol. Chem. 264: 19373-19378.
31. Jantzen, K., Fritton, H. P., Igo-Kemenes, T., Espel, E., Janich, S., Cato, A. C. B., Mugele, K., and Beato, M. (1987). Nucleic Acid Res. 15:4535-52.
32. Goering, M., Habenicht A. J. R., Heitz, R., Zeh, W. Katus, H., Kommerell, B., Ziegler, R., and Glomset J. A. (1987). J. Clin. Invest. 79:903-911.
33. Bucher, P. and Trifonov, E. N. (1986). Nucleic Acids Res. 14:10009-10026.