BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **227,** 70–76 (1996)
ARTICLE NO. 1469

The Mouse Genes for the EP_1 Prostanoid Receptor and the PKN Protein Kinase Overlap[1]

Björn Båtshake[2] and Johan Sundelin

Division of Molecular Neurobiology, Wallenberg Neurocentre, Lund University, Sölvegatan 17, S-223 62 Lund, Sweden

Received August 23, 1996

PKN is a newly discovered protein kinase that has been shown to mediate GTPase Rho dependent intracellular signalling. We show in this report that the mouse PKN gene is situated at the mouse EP_1 prostanoid receptor gene locus and that the two genes are overlapping in a tail-to-tail manner. An ''exon trap'' strategy was used to identify the overlap phenomenon. By using RT-PCR and 3′ RACE we have identified two major PKN transcripts that are produced by alternative polyadenylation. The 3′ end of the short PKN transcript overlaps the 3′ untranslated region of the EP_1 gene with ~280 bp, while the long PKN transcript overlaps the whole EP_1 gene. Remarkably, none of the three transcripts originating from this locus display the consensus AAUAAA polyadenylation signal. The last seven exons of the PKN gene, corresponding to the last third of the PKN cDNA, have been recognised in 7.2 kb of continuous genomic sequence that we have collected from the EP_1/PKN genetic locus. The 3′ part of the PKN gene is highly fragmented and its intron/exon organisation is reminiscent of that of the *Drosophila* protein kinase C gene. The possibility of a natural antisense regulation of these genes is discussed.

The prostanoids (prostaglandins and thromboxanes) are arachidonic acid metabolites and act as paracrine and autocrine mediators in a large number of physiological systems. Their receptors constitute a defined family within the super-family of G protein-coupled receptors (1). The EP_1 prostanoid receptor is one of four identified subtypes of the prostaglandin E_2 receptor (2).

Earlier we reported the cloning and the exon-intron organisation of the mouse gene encoding the EP_1 receptor (2). The EP_1 gene (*ep1*) consists of three exons and is predominantly expressed in the kidney and in the hypothalamus of the brain. In subsequent studies we used reverse transcription PCR (RT-PCR), with primers located in different exons, to analyse in greater detail the expression of *ep1* transcripts, and look for possible splice variants. Apart from amplified fragments of the expected size, we also obtained fragments with a size corresponding to the genomic unprocessed sequence. Further analysis showed that genomic contamination of the cDNA preparations could not explain the results. A second possible explanation, amplification of unspliced *ep1* transcript, was also disregarded because the longer amplified fragments were abundant in some tissues that completely lacked the normal *ep1* transcript. These circumstances led us to suspect the presence of an additional transcript, in the antisense orientation, from this locus. We show in this report that such a transcript indeed is produced in many tissues, and we have identified it as a transcript from the PKN gene (*pkn*).

PKN is a newly discovered protein kinase, related to the protein kinase C family of kinases (3). PKN was cloned from a human hippocampus cDNA library, and subsequently from a rat lung library (3) and a *Xenopus laevis* kidney library (4). It has also been isolated under the

[1] The sequence data of the mouse genomic DNA containing the EP_1 and PKN genes have been deposited in the EMBL Nucleotide Sequence Database under Accession No. Y07611.

[2] To whom correspondence should be addressed. Fax: +46-46-2220568. E-mail: bjorn.batshake@mphy.lu.se.

0006-291X/96 $18.00

name PRK1 from a human fetal brain library (5). PKN is activated by the small GTPase Rho (an intracellular signal transducing protein belonging to the Ras superfamily of small GTPases) and is thought to be a mediator of Rho-dependent signalling (6, 7).

MATERIALS AND METHODS

Oligonucleotides and enzymes. All oligonucleotides [L100 (5′-TAGGATCCTTAGCTTCTGGGCACATTCAG-3′), L104 (5′-CGCACACGATGTGGAAATGG-3′), L143 (5′-GACAACAGGGGAGTCATCC-3′), L155 (5′-CTTGAC-CTTTCAAATGTGCCC-3′), L156 (5′-GGACCCCGAGAACTTGTCC-3′), L160 (5′-GAGGCAAGGCAGACT-CTTGG-3′), L161 (5′-GGATAAACAGGCCTTAGAGATG-3′), L165 (5′-CTGAGCGCGATGCAGAAGATGTG-3′), L174 (5′-TGGTTCCCCACTCACCCAGG-3′)] were purchased from the BioMolecular Resource Facility (Lund University). Enzymes and other reagents were obtained from GibcoBRL or Boehringer Mannheim, unless otherwise stated.

Preparation of RNA and cDNA synthesis. Total RNA was isolated from mouse tissues after extraction with guanidinium isothiocyanate and acid phenol (8). Poly(A)$^+$ RNA was selected on oligo(dT) cellulose, and treated with DNase I to remove any contaminating DNA. After phenol-extraction, 1 μg of poly(A)$^+$ RNA was converted to first strand cDNA using random hexamer primers and Moloney murine leukemia virus reverse transcriptase (Superscript, GibcoBRL). Control reactions, without reverse transcriptase, were carried out in parallel. After removal of RNA by alkaline hydrolysis, the cDNA was precipitated from 0.3 M NaAc with ethanol, and finally dissolved in TE-buffer.

Exon trap experiment. A 10-kb EcoRI mouse (strain 129) genomic DNA fragment containing the whole EP_1 receptor gene (2) was cloned in the vector pET01 (Mo Bi Tec) with *ep1* in the reverse orientation. The construct was used to transfect COS-7 cells by the DEAE dextrane method (CellPhect Transfection Kit, Pharmacia). After 48 h total RNA was extracted (9) and 10 μg used for cDNA synthesis as described above, but instead of random primers a specific primer (L104) from the *ep1* gene was used. The cDNA obtained was subjected to PCR amplification using Taq polymerase and Taq Extender PCR additive (Stratagene). The primer L155, derived from the *ep1* gene, and a primer from the pET01 vector were used.

PCR-amplification and subcloning. Amplification of fragments less than 1000 bp was carried out with a standard program of 30 cycles (1 min each at 94, 55, and 72° C) using 0.2 μM of each primer and 1 unit of Taq polymerase (AmpliTaq, Perkin Elmer). For amplification of longer fragments, Taq Extender (Stratagene) was included in the reaction mixture and the elongation time at 72° C was increased. Selected PCR-fragments were cloned with a TA-cloning kit (Invitrogen).

Rapid amplification of 3′ cDNA ends (3′ RACE). Amplification of the 3′ part of cDNAs was performed with a 3′-Amplifinder RACE Kit from Clontech. Mouse heart poly(A)$^+$ RNA (2 μg) was reverse transcribed using a 3′ RACE oligo(dT) primer. Two sets of nested PCRs were then performed using the specific primers L165 and L160 for the short *pkn* transcript. For the long transcript primers L143 and L156 were used. PCR fragments were cloned with a TA-cloning kit from Invitrogen. Four TA-clones from each RACE experiment were randomly picked and sequenced.

DNA sequencing. Sequencing of double stranded plasmid DNA was done with an Applied Biosystems Inc 373A sequencer and the Taq DyeDeoxy Terminator Cycle Sequencing Kit (ABI). Vector-primers as well as specific primers were used. Sequences were analysed using the Wisconsin package, Genetics Computer Group. The nucleotide sequence submitted to the EMBL database was determined from both strands of genomic mouse DNA clones.

RESULTS

When analysing the expression of *ep1* in various mouse tissues by RT-PCR we observed, besides the expected product, a fragment with a size corresponding to an unspliced *ep1* transcript. To exclude the possibility of accidental amplification of contaminating genomic or plasmid DNA, the RT-PCR experiment was repeated under more stringent conditions. RNA was isolated from mouse kidney and heart, and treated with DNase I before reverse transcription. Control reactions without reverse transcriptase were also performed before amplification with primers located in different exons of *ep1*. Even with these precautions, as shown in Figure 1, a fragment corresponding in length to the genomic distance between the primers (780 bp) was amplified both from kidney and heart cDNA. As expected the 360 bp *ep1* cDNA fragment could be amplified from kidney cDNA, but not from heart cDNA, suggesting that the 780 bp fragment was not derived from an unspliced *ep1* transcript. These results therefore indicate the presence of a transcript originating from the strand opposite the strand encoding the EP_1 receptor.

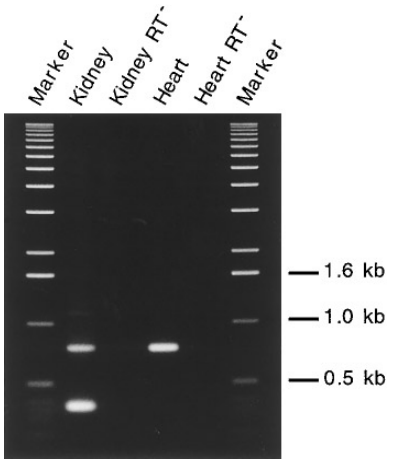

FIG. 1. Reverse-transcriptase PCR with EP_1 primers on mouse kidney and heart cDNA. PCR fragments obtained using two primers (L104 and L100) located in the second and third exon, respectively, of the EP_1 receptor gene with cDNA templates from mouse kidney and heart were visualised by ethidium bromide staining after agarose gel electrophoresis. Besides the expected 0.36 kb EP_1 cDNA fragment from kidney (lane 2), a fragment corresponding in length to the genomic distance (0.78 kb), is seen from both kidney and heart (lane 2 and 4). The cDNA was prepared from DNase treated RNA, and mock-synthesised cDNA without reverse transcriptase (RT^-), was used in control PCRs (lane 3 and 5). The sizes, in kb, of selected DNA markers (lane 1 and 6) are shown.

To address this possibility, an exon trap experiment was performed (10). A 10 kb mouse genomic fragment, containing the *ep1* gene, was cloned into the vector pET01 with *ep1* in reverse orientation (Fig. 2). The eukaryotic expression vector pET01 has functional donor and acceptor splice sites separated by an intron which harbours a cloning linker, providing the opportunity to trap exons from genomic fragments as long as they are cloned in the correct orientation. COS-7 cells were transfected with the pET01 construct, RNA was prepared and reverse transcribed with a primer (L104) located in the second exon of *ep1*. PCR amplification with a primer in the third exon of *ep1* (L155) together with a vector specific primer yielded a single 1.1 kb fragment which was subcloned (Fig. 2).

Sequence analysis of the cloned cDNA revealed a 849 bp open reading frame with a deduced amino acid sequence 98 % identical to the carboxyterminal part of the rat protein kinase PKN

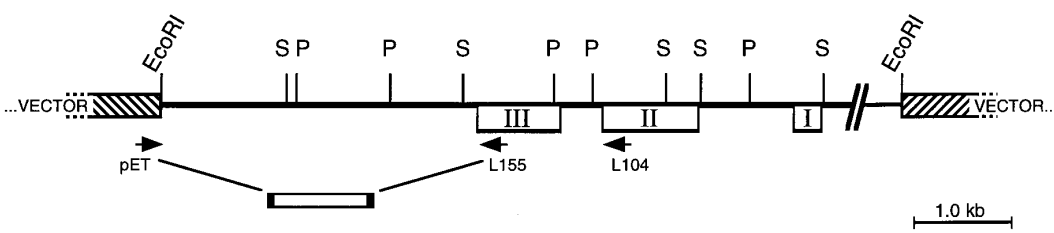

FIG. 2. Organisation of the *ep1* gene, and trapping of additional exons from the *ep1* locus. A 10 kb EcoRI genomic DNA fragment containing the three exons of *ep1* on the antisense strand (marked with roman numerals) was cloned in the vector pET01 (*striped* boxes). After transfection into COS-7 cells, RNA was isolated and converted to cDNA using the primer L104. The cDNA was used as template in a PCR, using a vector-primer (pET-primer) and a primer (L155) located in the third exon of the *ep1* gene, to amplify a 1.1 kb fragment (shown below the genomic fragment). The filled boxes at the ends of the amplified fragment depict the two primers. PstI (P) and SmaI (S) restriction enzyme recognition sites are indicated.

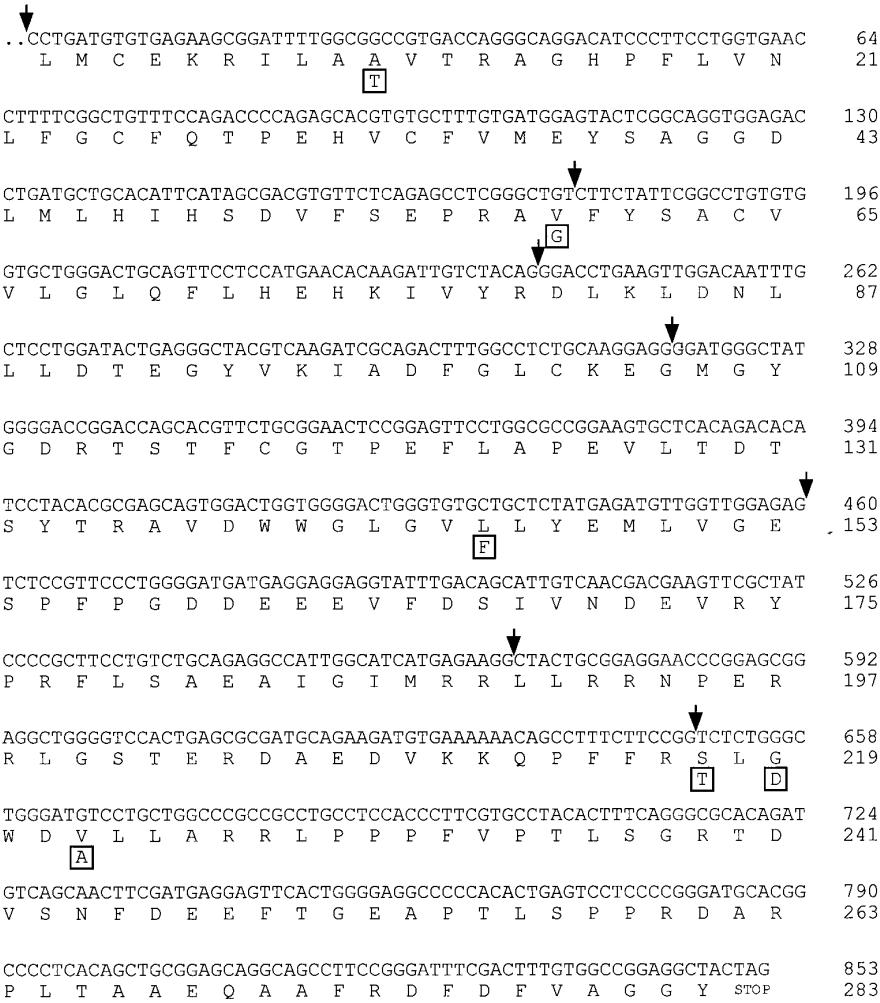

FIG. 3. Partial nucleotide and amino acid sequence of the mouse PKN and comparison with the rat PKN sequence. The 283 most carboxyterminal amino acids of the mouse PKN were deduced from a cDNA obtained from exon-trap and from genomic nucleotide sequence. Amino acid residues are shown in one-letter code below the first base of each codon. Rat amino acid residues that differ from the mouse sequence are shown in boxes below the mouse sequence. The arrows indicate the positions of introns in the mouse PKN gene.

(3) (Fig. 3). We conclude that the cloned cDNA encodes the mouse PKN, and that the PKN gene is located at the *ep1* locus and overlaps with *ep1* in a tail-to-tail manner.

Analysis of *pkn* transcripts in rat tissues by Northern blot showed two transcripts: one 3.1 kb and one 6.8 kb (3). Should a similarly long *pkn* transcript exist in the mouse, such a transcript would completely overlap *ep1*. In order to test this possibility, the extension of the *pkn* gene in the 3′ direction was mapped. Starting at the PKN stop codon and continuing in the 3′ direction, successive and overlapping PKN cDNAs were amplified with RT-PCR. To avoid interference from *ep1* or genomic DNA we used cDNA template prepared from DNase treated mouse heart RNA (which does not contain detectable levels of *ep1* transcripts; see figure 1). Several overlapping RT-PCR fragments, corresponding in length to genomic fragments, could be generated (data not shown). The last primer pair that resulted in an amplification of a PKN cDNA fragment was L143 + L161 (located in the 5′ flanking region of *ep1*). The

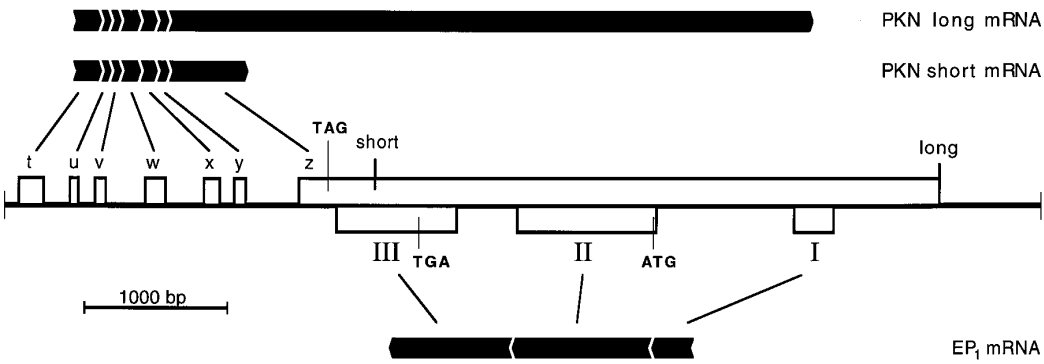

FIG. 4. Gene organisation at the mouse *ep1*/PKN locus and transcripts produced. The centre line represents the 7.2 kb of sequenced genomic DNA (deposited in the database). Open boxes represent exons. Above the line are the seven last exons of the PKN gene, denoted by letters **t** to **z.** The *ep1* exons are numbered I to III and are shown under the line. ''ATG'' and ''TGA'' are the start and stop codons of the *ep1* gene, while ''TAG'' indicates the PKN stop codon. The 3′-ends of the two identified *pkn* transcripts (short and long) are shown in the last PKN exon. Solid bars illustrate the three mRNAs produced from this locus.

next primer pair, L156 + L174, failed to produce a fragment from the heart cDNA used, but did so readily from genomic DNA. Assuming that the failure of L156 and L174 to amplify heart cDNA reflects the end of *pkn* transcript, we concluded that the *pkn* gene extends at least 4256 bp (primer L161) but no further than 4956 bp (primer L174) downstream of its stop codon. This implies that the whole *ep1* gene is contained within the 3′ untranslated region of a long *pkn* transcript.

To recognise and identify PKN polyadenylation sites the 3′ RACE method was employed. Template cDNA was prepared from heart RNA using a modified oligo(dT) primer. Two different RACE experiments were carried out; one for each of the two presumed PKN polyadenylation sites. For the short transcript the nested primers L165 and L160 were used, and for the long *pkn* transcript, primers L143 and L156. Each experiment produced, after nested PCR amplification, a single band on agarose gels. The two different PKN fragments generated were then ligated into a PCR cloning vector. From each experiment four RACE clones were randomly picked. Sequence analysis of the clones corresponding to a short *pkn* transcript showed that they all end within a 11 bp region situated ~330 nucleotides after the PKN stop codon (i.e. in the 3′ untranslated region of *ep1*). A consensus polyadenylation site (AAUAAA) could, however, not be found before this polyadenylation site. The second set of 3′ RACE clones ends at a point as far as 4273 bp after the PKN stop codon. The termination point of these RACE clones are also colinear with a genomic poly(dA) stretch suggesting that they may be products of internal priming rather than products of the actual poly(A) tail of the transcript. No AAUAAA signal was found in the proximity of this termination site either.

By comparing the PKN nucleotide sequences obtained from the 3′ RACE and exon trap experiments with 7.2 kb of continuos genomic sequence from the *ep1/pkn* locus, the organisation of the 3′ part of the *pkn* gene could be determined (Fig. 4). The *pkn* gene is highly fragmented: the 3′ part is split into seven exons (arbitrarily denoted by the letters **t** - **z**). The PKN exons identified are relatively short (the smallest is only 63 bp long) and are all flanked by consensus splice sites.

The organisation of the mouse *pkn* gene is reminiscent of the *Drosophila melanogaster* protein kinase C (PKC) gene (11) which is also highly fragmented and is split in at least 14 exons. The positions of two of the introns in *pkn* (introns **t** and **x**) are exactly matched by intron positions in the *Drosophila* PKC gene (introns 10 and 12).

DISCUSSION

As shown here the mouse prostanoid receptor EP_1 gene and the PKN protein kinase gene overlap and are transcribed in opposite directions. Three transcripts have been identified from the *ep1/pkn* gene locus; the previously identified EP_1 transcript (2), and two *pkn* transcripts that partially and totally, respectively, overlap the *ep1* transcript. The short *pkn* transcript overlaps the 3′ untranslated region of the identified EP_1 transcript by approximately 280 nucleotides, and apparently corresponds to the sequenced rat and human PKN/PRK1 cDNAs (3, 5). According to RT-PCR mapping, the long *pkn* transcript ends somewhere between 4256 and 4956 bp after the PKN stop codon. By 3′ RACE the polyadenylation site of the long *pkn* transcript was more accurately determined to be 4273 bp after the stop codon. But since this RACE result may be due to internal priming of the oligo(dT) RACE primer to an adenosine rich genomic stretch, it is possible that the actual polyadenylation site is to be found further down stream: i.e. in the interval 4273-4956 bp after the stop codon. The size difference between the long and the short transcript, ~3.9 kb, corresponds approximately to the difference between the two rat PKN transcripts of 3.1 kb and 6.8 kb identified by Northern blotting (3).

An unusual feature of the *pkn* transcripts identified from this locus is that neither they, nor the *ep1* transcript (2), contain the polyadenylation signal AAUAAA. The sequenced rat and human PKN cDNAs also apparently lack this highly conserved signal (3, 5). In the region in which polyadenylation of the long *pkn* transcript could possibly occur, no AAUAAA consensus polyadenylation signal can be found. Although the polyadenylation signal, which often is followed by a downstream U-rich element (12), is highly conserved, some mRNAs do not contain a recognisable AAUAAA signal, see (13). It is not clear how the 3′ processing of these mRNAs occurs, but it may involve premature termination of transcription (see (14) and references therein), and appears to be associated with alternative polyadenylation (13).

Overlapping, bidirectionally transcribed genes like the mouse *ep1* and *pkn* are uncommon in eukaryotes, and in only a few cases do the overlapping transcripts appear to encode proteins. Examples of this phenomenon include members of the rat thyroid/steroid hormone receptors c-*erb* family (15), an exon of the c-*myb* proto-oncogene that is encoded on the antisense strand of the SC35 splicing factor gene (16), a locus for signal-transducing components of the T-cell receptor that, on the opposite strand, encodes the transcription factor Oct-1 (17), and an antisense transcript at the BCMA gene locus that encodes a protein with unknown function (18).

What is the functional significance of an antisense transcript? This question has been addressed only in a limited number of studies, but the available information indicates that antisense transcripts often have a regulatory function. Besides interference at the transcriptional level, several different control mechanisms are possible. RNA processing of transcripts from the c-*erbA* gene (15), and the N-*myc* gene (19), is influenced by naturally occurring antisense transcripts. The stability and translation of mRNAs may also be regulated by antisense transcripts. For example, the expression of a protein encoded by the *Dictyostelium* gene EB4 (20) and the basic fibroblast growth factor (21, 22) is apparently regulated by antisense transcripts forming RNA hybrids, that may be degraded (20) or destroyed by covalent modification (23).

Regarding the overlapping transcripts from the *ep1/pkn* locus, we presently do not know if they interfere with each other or if they are present in the same cell. PKN is widely expressed (3, 5) whereas EP_1 shows a more restricted tissue distribution. With *in situ* hybridisation we found EP_1 to be expressed mainly in the collecting ducts of the kidney and in the hypothalamus (2). Using sense probes as control we obtained a less distinct hybridisation to sections of kidney, which was interpreted as a non-specific background signal. Since the sense probes now are known to hybridise to the long *pkn* transcript, it is likely that this interpretation was wrong, and that the signal instead reflects the long *pkn* transcript which is present in mouse

kidney (see Fig. 1). Additional studies are needed to determine if indeed *ep1* and *pkn* are transcribed in the same cells. If they are, it seems probable that their transcripts would interfere with each other. Finally, it is also possible, although highly speculative, that the EP_1 receptor and PKN are functionally coupled. Activation of EP_1 by prostaglandin E_2 evokes an increase in the intracellular concentration of Ca^{2+} (2), a known activator of phospholipase A_2 (24). Activation of phospholipase A_2 in turn leads to an increased generation of arachidonic acid which is an activator of PKN (25, 26).

ACKNOWLEDGMENTS

This work was supported by grants from the Swedish Medical Research Council (13X-09467) and by Pharmacia and the Medical Faculty, Lund University.

REFERENCES

1. Pierce, K. L., Gil, D. W., Woodward, D. F., and Regan, J. W. (1995) *Trends Pharmacol. Sci.* **16,** 253–256.
2. Båtshake, B., Nilsson, C., and Sundelin, J. (1995) *Eur. J. Biochem.* **231,** 809–814.
3. Mukai, H., and Ono, Y. (1994) *Biochem. Biophys. Res. Commun.* **199,** 897–904.
4. Mukai, H., Mori, K., Takanaga, H., Kitagawa, M., Shibata, H., Shimakawa, M., Miyahara, M., and Ono, Y. (1995) *Biochem. Biophys. Acta* **1261,** 296–300.
5. Palmer, R. H, Ridden, J., and Parker, P. J. (1995) *Eur. J. Biochem.* **227,** 344–351.
6. Watanabe, G., Saito, Y., Madaule, P., Ishizaki, T., Fujisawa, K., Morii, N., Mukai, H., Ono, Y., Kakizuka, A., and Narumiya, S. (1996) *Science* **271,** 645–648.
7. Amano, M., Mukai, H., Ono, Y., Chihara, K., Matsui, T., Hamajima, Y., Okawa, K., Iwamatsu, A., and Kaibuchi, K. (1996) *Science* **271,** 648–650.
8. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual (Ford, N., Nolan, C., and Ferguson, M., Eds.), 2nd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
9. Xie, W., and Rothblum, L. I. (1991) *Biotechniques* **11,** 324–327.
10. Auch, D., and Reth, M. (1990) *Nucleic Acids Res.* **18,** 6743–6744.
11. Rosenthal, A., Rhee, L., Yadegari, R., Paro, R., Ullrich, A., and Goeddel, D. V. (1987) *EMBO J.* **6,** 433–441.
12. Chou, Z.-F., Chen, F., and Wilusz, J. (1994) *Nucleic Acids Res.* **22,** 2525–2531.
13. Wahle, E., and Keller, W. (1992) *Annu. Rev. Biochem.* **61,** 419–440.
14. Rabbitts, K. G., and Morgan, G. T. (1992) *Nucleic Acids Res.* **20,** 2947–2953.
15. Lazar, M. A., Hodin, R. A., Darling, D. S., and Chin, W. W. (1989) *J. Mol. Cell. Biol.* **9,** 1128–1136.
16. Fu, X.-D., and Maniatis, T. (1992) *Science* **256,** 535–538.
17. Lerner, A., D'Adamio, L., Diener, A. C., Clayton, L. K., and Reinherz, E. L. (1993) *J. Immunol.* **151,** 3152–3162.
18. Laabi, Y., Gras, M., Brouet, J., Berger, R., Larsen, C., and Tsapis, A. (1994) *Nucleic Acids Res.* **22,** 1147–1154.
19. Krystal, G. W., Armstrong, B. C., and Battey, J. F. (1990) *Mol. Cell. Biol.* **10,** 4180–4191.
20. Hildebrandt, M., and Nellen, W. (1992) *Cell* **69,** 197–204.
21. Volk, R., Köster, M., Pöting, A., Hartmann, L., and Knöchel, W. (1989) *EMBO J.* **8,** 2983–2988.
22. Knee, R. S., Pitcher, S. E., and Murphy, P. R. (1994) *Biochem. Biophys. Res. Commun.* **205,** 577–583.
23. Kimelman, D., and Kirschner, M. W. (1989) *Cell* **59,** 687–696.
24. Clark, J. D., Lin, L. L., Kriz, R. W., Ramesha, C. S., Sultzman, L. A., Lin, A. Y., Milona, N., and Knopf, J. L. (1991) *Cell* **65,** 1043–1051.
25. Mukai, H., Kitagawa, M., Shibata, H., Takanaga, H., Mori, K., Shimakawa, M., Miyahara, M., Hirao, K., and Ono, Y. (1994) *Biochem. Biophys. Res. Commun.* **204,** 348–356.
26. Kitagawa, M., Mukai, H., Shibata, H., and Ono, Y. (1995) *Biochem. J.* **310,** 657–664.