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# Expression of Plasma Prekallikrein mRNA in Human Nonhepatic Tissues and Cell Lineages Suggests Special Local Functions of the Enzyme

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At present it is generally accepted that plasma prekallikrein (PPK) is synthesized in the liver and secreted into the bloodstream. Surprisingly, it has recently been shown that PPK mRNA is present also in RNA from the kidney, adrenal gland and placenta. In spite of its novelty and possible important physiological implications this finding has been neglected. Here we report that PPK mRNA is expressed also in the human brain, heart, lung, trachea, endothelial cells and leukocytes as well as in a variety of fibroblast and epithelial cell lines. Expression of PPK mRNA in fibroblasts, endothelial cells and leukocytes suggests that PPK mRNA detected in RNA preparations from whole tissue may originate solely from these ubiquitously occurring cells. However, PPK mRNA expression in various epithelial cell lines demonstrates that tissue-specific cells also transcribe the PPK gene. The presence of PPK mRNA in nonhepatic tissues and cells indicates that they have the capacity to synthesize the protein. The physiological role of PPK synthesized in extrahepatic tissue is unknown. It may participate in local actions within tissues as well as contributing to the PPK pool in blood plasma. Cultured cells will provide a valuable model for exploring the physiological significance of extrahepatic PPK expression.

*Key words:* Contact activation / Kallikrein-kinin system / Plasma kallikrein / Reverse transcription PCR / RT-PCR.

# Introduction

Plasma prekallikrein (PPK), the zymogen of the serine proteinase plasma kallikrein (PK; EC 3.4.21.34) is synthesized in hepatocytes and secreted into the blood stream (Bhoola *et al.*, 1992). Its concentration in plasma is about 50 mg/l of which seventy five percent is complexed with high molecular weight kininogen (HK). The active enzyme is generated in the contact system, the early phase of the intrinsic pathway of blood coagulation. The contact system is activated when plasma is exposed to negatively charged surfaces: the PPK-HK complex is bound via cationic regions of domain 5 of HK to the anionic surface (Mandle *et al.*, 1976), and activated factor XII (FXIIa) generates plasma kallikrein which in turn produces FXIIa (Mandle and Kaplan, 1977). Thus, by a positive feedback reaction, a local increase in the concentration of both PK and FXIIa is achieved.

The two proteinases, PK and FXIIa, generated during contact activation can be involved in a number of subsequent processes. Both enzymes can activate granulocytes (Schapira *et al.*, 1982, 1983; van der Graaf *et al.*, 1982; Wachtfogel *et al.*, 1983, 1986) and the complement system (Ghebrehiwet *et al.*, 1981, 1983; DiScipio, 1982). FXIIa activates factor XI and in this manner triggers the intrinsic blood coagulation cascade. PK is an efficient activator of urokinase-type plasminogen activator precursor and thus provides a link to the fibrinolysis system (Ichinose *et al.*, 1986; Hauert *et al.*, 1989). Probably the second most important function of PK, next to its participation in the contact activation system, is its action as a kinin-liberating enzyme in the kallikrein-kinin system (Bhoola *et al.*, 1992).

Recently, Ciechanowicz *et al.* (1993) and our group (Hermann *et al.*, 1996) found by reverse transcription PCR that PPK mRNA is present in RNA preparations from whole tissues of kidney, adrenal gland and placenta. Thus, in contrast to the generally accepted concept that plasma kallikrein is synthesized solely in the liver, one has to conclude that synthesis of PPK also takes place outside the liver and that the enzyme may have special, hitherto unknown local function(s). The surprising new finding of extrahepatic PPK mRNA synthesis and its potential importance with respect to functions of PPK/PK have obviously been overlooked, since no subsequent work on these topics has been reported.

The previous results (Ciechanowicz *et al.*, 1993; Hermann *et al.*, 1996) did not indicate whether PPK mRNA is expressed by ubiquitous cells like fibroblasts, endothelial cells, and white blood cells, or by tissue-specific cells. Therefore, in order to clarify this issue and to find a model system for investigating the physiological significance of extrahepatic PPK synthesis, we undertook a study on PPK mRNA expression in cultured human fibroblasts and epithelial cells originating from different human tissues. Additionally, a variety of human tissues was examined for expression of PPK mRNA.

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# Results

# Expression of PPK mRNA in Human Cell Lines and Tissues

In order to examine whether tissues other than those reported as well as cultured cells express mRNA of plasma prekallikrein, a semi-nested RT-PCR was performed with total RNA of a variety of human tissues and cell lines. To confirm the identity of the amplicons, restriction fragments were prepared by incubating aliquots of the PCR mixtures with the endonuclease Sacl. The sizes of the PCR products and restriction fragments were determined by polyacrylamide gel electrophoresis. The silver stained gel of a typical experiment representing the result for RNA from HeLa cells is shown in Figure 1. For comparison samples of a PCR with leukocyte genomic DNA before and after digestion with Sacl (lanes 4 and 5) were also applied onto the gel. Figure 1 demonstrates that with RT-PCR of mRNA of HeLa cells a product of the size predicted for plasma prekallikrein mRNA (394 bp, R in lane 1; cf. Fig-



**Fig. 1** Expression of Plasma Prekallikrein mRNA in HeLa Cells Demonstrated by Semi-Nested RT-PCR.

Total RNA was extracted from cultured HeLa cells and reverse transcribed using a specific primer for PPK. Semi-nested PCR was performed with the cDNA and genomic DNA. Aliquots of these reaction mixtures with and without *Sacl* digestion were subjected to PAGE. DNA bands were visualized by silver staining. Lane 1: cDNA; lane 2: cDNA after *Sacl* digestion; lane 3: DNA size standard; lane 4: genomic DNA after *Sacl* digestion; lane 5: genomic DNA; lane 6: negative control (semi-nested PCR was performed with a sample where in the reverse transcription step the RNA solution had been replaced by water). Designations of bands: G, G-F1, G-F2: amplicon of genomic DNA and its *Sacl* digestion products (for details see Figure 2 and Results).

ure 2) is obtained, and that by digestion with *Sac*I fragments of the expected lengths are produced (312 bp + 4 b and 78 bp + 4 b, R-F1 and R-F2 in lane 2; the cleavage site of *Sac*I in the upper strand of the cDNA is located between bases 1195 and 1196, Figure 2). Figure 1 indicates that the RNA preparation was not absolutely free of genomic DNA since, in addition to the amplicon of the cDNA, a PCR product (G in lane 1,  $\approx$  900 bp) and *Sac*I digestion fragments (G-F1,  $\approx$  663 bp + 4b, and G-F2, 233 bp + 4b, in lane 2) of the sizes expected for genomic DNA (lanes 4 and 5) were obtained.

Further proof that the amplicons R and G resulted indeed from mRNA and genomic DNA of PPK was gained by DNA sequence analysis of the PCR-products of HeLa cells using the forward primer hPK-29F. Except for some ambiguities, the readable sequences (Figure 2) were identical to the known sequences of PPK cDNA (880-1273 of GenBank accession number M13143) and intron 8 (Kunapuli et al., 1995). The amplicon G must contain the three introns 8, 9 and 10 inserted after the positions 961, 1124 and 1237 of the cDNA sequence (Asakai et al., 1987; Beaubien et al., 1991; Kunapuli et al., 1995); the sequences of intron 8 and 10 have been elucidated previously (Kunapuli et al., 1995). The full-length intron 8 was found at the reported site (Figure 2). The exon-9/intron-9 junction is expected at position 367-368 of amplicon G where, in fact, the sequence started to differ from the known cDNA sequence (Figure 2). As calculated from the sizes of amplicon G (about 900 bp, cf. Figure 1), of introns 8 and 10 (158 and

Table 1Expression of Plasma Prekallikrein mRNA in Fibroblastand Epithelial Cell Lines Derived from Various Human Tissues.

Cell line or tissue	Comments	PPK mRNA	
WI 38	Fibroblasts (lung)	+	
Colo 668	Fibroblasts (colon)	+	
Decidua	Fibroblasts	+	
	(primary culture)		
HaCaT	Keratinocytes	+	
HaCaT II-4RT	Keratinocytes	+	
PancTu	Pancreatic tumor	+	
	(epithelial)		
BT 20	Mammary tumor	+	
	(epithelial)		
SW 480	Colon carcinoma	+	
	(epithelial)		
HT 29	Colon carcinoma	+	
	(epithelial)		
Kato III	Stomach carcinoma	+	
	(epithelial)		
HeLa	Cervix carcinoma	+	
	(epithelial)		
HUVEC	Primary culture	+	
Neutrophils	Freshly isolated	+	
Brain	Whole tissue	+	
Heart	Whole tissue	+	
Lung	Whole tissue	+	
Trachea	Whole tissue	+	
Liver	Whole tissue	+	
Kidney	Whole tissue	+	

	880	AGTGGCACAC	CAAGTTCCTC	TACTCCTCAA	GAAAACACCA	TATCTGGATA	TAGCCTTTTA
G	1				ACCA	TATCTGGATA	TAGCCTTTTA
R	1		•		CACCA	TATCTGGATA	TAGCCTTTTA
	940	ACCTGCAAAA	GAACTTTACC	TG			
G	25	ACCTGCAAAA	GAACTTTACC	TG			
R	26	ACCTGCAAAA	GAACTTTACC	TG			
	1	gtaatgtgat	ttgataataa	tattacataa	aatgtaacct	atttcatgac	ttttaacagc
G	47	gtaatgtgat	ttgataataa	tattacgtaa	ratgtracct	atwrcatgac	tttraacrgc
	61	aacagtgatg	aaacaatcct	caaggtaaca	gaaacttgtg	taaatgtcgt	tcattgcttt
G	107	aacagtgatg	aapcaatcct	craggtaaca	paaacttgtg	taaatgtcgt	qcattgcttt
	121	tcccatctga	tatcttttg	tgtttataat	tgacacag		
G	167	tcccatctga	tatctttttg	tgtttataat	tgacacag		
	962	AACCCTGCCA	TTCTAAAATT	TACCCGGGAG	TTGACTTTGG	AGGAGAAGAA	TTGAATGTGA
G	205	AACCCTGCCA	TTCTAAAATT	TACCCGGGAG	TTGACTTTGG	AGGAGAAGAA	TTGAATGTGA
R	48	AACCCTGCCA	TTCTAAAATT	TACCCGGGAG	TTGACTTTGG	AGGAGAAGAA	TTGAATGTGA
1	022	CTTTTGTTAA	AGGAGTGAAT	GTTTGCCAAG	AGACTTGCAC	AAAGATGATT	CGCTGTCAGT
G	265	CTTTQGTTAA	AGGAGTGAAT	GTTTGCCANG	AGACTTGCNC	AAAGATGATT	CGCTGTCAGT
R	108	CTTTTGTTAA	AGGAGTGAAT	GTTTGCCAAG	AGACTTGCAC	AAAGATGATT	CGCTGTCAGT
1	082	TTTTCACTTA	TTCTTTACTC	CCAGAAGACT	GTAAGGAAGA	GAA	
G	325	TTTTCNCTTA	TTCTTTACTC	CCANAAGACT	GTAAGGAANA	GAAgttanag	gaattnttn
R	168	TTTTCACTTA	TTCTTTACTC	CCAGAAGACT	GTAAGGAAGA	NAA	
1	125	GTGTAAGTGT	TTCTTAAGAT	TATCTATGGA	TGGTTCTCCA	ACTAGGATTG	CGTATGGGAC
R	211	GTGTNAGTGT	TTCTTAAGAT	TATCTATGGA	TGGTTCTCCN	ACTAGGATTG	CGTATGGGAC
1	185	ACAAGGGAGC	TCTGGTTACT	CTTTGAGATT	GTGTAACACT	GGGGACAACT	CTGTCTGCAC
R	271	ACAAGGGAGC	TCTGGTTACT	CTTTGANATT	GTGTTNCACT	GGGGACAACT	CTGTCTGCAC
1	245	ААСАААААСА	AGCACACGCA	TTGTTGGAG			
R	331	AACNAANACA	AGCNCTCGCN	TTGTTGGAG			

**Fig. 2** Comparison of the Sequences Determined for Amplicons G and R with Known Sequence Data. The sequences determined for amplicons G and R (lines with prefixes G and R, respectively) are aligned to the segment 880–1273 of the cDNA sequence of human plasma kallikrein (GenBank accession number M13143) with the intron 8 (Kunapuli *et al.*, 1995) inserted. Intron sequences are in lower case letters. The primer hPK-29F used for sequencing corresponds to bases 880–898. The cleavage site for *Sacl* is between bases 1195 and 1196. Symbols for not clearly identified nucleotides: P: A/G; Q: C/T; R: A/T; W: G/T; N: A/C/G/T.

155 bp, Kunapuli *et al.*, 1995) and of the cDNA segment (394 bp), intron 9 has a length of about 200 bp. Intron 10 is not contained in the sequenced section of amplicon G.

The same electrophoresis results as for HeLa cells, demonstrating the expression of plasma prekallikrein mRNA were obtained for total RNA from all cell lines investigated (Table 1) and from human liver, kidney, brain, heart, lung, trachea, HUVECs (human umbilical vein endothelial cells) and leukocytes.

# Discussion

# Specificity of RT-PCR

The primers for reverse transcription and PCR were selected in such a manner that amplicons of genomic DNA contained three introns and, therefore, were easily distinguishable from the 394 bp PCR product of cDNA by electrophoretic size determination. Indeed, when PCR was performed with genomic DNA a single product of about 900 bp was obtained (Figure 1, Iane 5), demonstrating that the amplicon of genomic DNA cannot be confused with that of cDNA and, in addition, that the human genome does not contain retropseudogenes of PPK that could give rise to PCR products of similar size as that of PPK cDNA.

Further confirmation that the amplicon resulted from mRNA was achieved by digestion with *Sac*I which produced fragments of the correct lengths of 312 bp and 78 bp (Figure 1, lane 2). Like the undigested PCR products, these fragments were unequivocally distinguishable from those resulting from genomic DNA with predicted chain lengths of  $\approx$  663 bp and 233 bp (lanes 2 and 4). In addition,

in the case of HeLa cells the identity of the cDNA amplicon was verified by sequence determination (Figure 2).

Partial DNA sequence determination of the  $\approx$  900 bp PCR product revealed that it indeed represented the amplicon of the respective PPK gene segment (Figure 2). The sequence was as expected for cDNA but with intron sequences inserted in the positions reported by Kunapuli et al. (1995), and as deduced from the homology of PPK with human factor XI (Asakai et al., 1987) and rat PPK (Beaubien *et al.*, 1991). The  $\approx$  900 bp band was observed in several RT-PCR experiments indicating some contamination of the RNA preparations with genomic DNA (cf. Figure 1, lane 1). However, since both the RT-PCR product of PPK mRNA and its restriction fragments are unequivocally distinguishable from those of genomic DNA, no efforts were undertaken to achieve complete removal of genomic DNA from the RNA preparations. Taken together, the method of semi-nested RT-PCR combined with chain length estimation by PAGE and restriction fragment analysis provides a sensitive and specific means for the detection of PPK mRNA. It guarantees unambiguous results even in the presence of genomic DNA; further confirmation by DNA sequence analysis is not required.

# Expression of PPK mRNA in Nonhepatic Tissues and Cell Lines

Plasma kallikrein is a serine proteinase that is present in blood as the inactive zymogen plasma prekallikrein. It is generally accepted that PPK is synthesized in hepatocytes and secreted into the blood where it circulates mainly as a heterodimer complex with high molecular weight kininogen, and is available for participating in multiple physiological processes (see Introduction). Its synthesis in the liver was originally deduced from the fact that circulating levels of PPK were reduced in patients with liver cirrhoses (Colman and Wong, 1979). Direct evidence of hepatic PPK synthesis came from immunocytochemical studies using confocal laser scanning microscopy which demonstrated PPK in hepatocytes (Henderson et al., 1992). Recently, Ciechanowicz et al. (1993) and our group (Hermann et al., 1996) provided evidence for extrahepatic synthesis of PPK: by employing RT-PCR, expression of PPK mRNA was detected in human liver, kidney, adrenal gland and placenta. The aim of the present study was to clarify whether PPK mRNA is expressed in tissues other than the liver and in tissue-specific cells, and to find a model for studying extrahepatic PPK synthesis and its regulation. We therefore analyzed RNA from various tissues, from leukocytes, from primary culture of human umbilical vein endothelial cells and from cell lines of fibroblasts and epithelial cells. We found that PPK mRNA is present in all of them (Table 1). The presence of PPK mRNA in the ubiquitously occurring fibroblasts, leukocytes and endothelial cells may suggest that these cell types are the only ones expressing PPK mRNA extrahepatically. However, the PPK gene is transcribed also in epithelial cell lines originating from different organs (Table 1). Thus, the results indicate that expression of PPK mRNA and assumingly also of the protein takes place not only in ubiquitous cells but also in tissue-specific epithelial cells throughout the organism. The final answer to the question as to which of the cells in the various tissues express PPK mRNA will be arrived at from in situ hybridization or in situ RT-PCR studies.

#### **Extrahepatic Synthesis of PPK**

Expression of a mRNA provides strong indication, but does not necessarily prove synthesis of the respective protein. Synthesis of PPK has until now been shown only in the liver by Henderson *et al.* (1992) who demonstrated immunoreactive PPK in isolated human hepatocytes by confocal laser scanning microscopy and in sections of liver by light microscopy. Seidah *et al.* (1988) isolated plasma kallikrein from human pituitary gland, however, they could not exclude the possibility that the enzyme resulted from blood or the extravascular bed. Even though no unambiguous evidence of extrahepatic synthesis of PPK has been presented, the transcription of the gene in various tissues and cell lines demonstrates that extrahepatic synthesis of PPK is possible, but final proof will have to come from studies at the protein level.

# Possible Physiological Functions of Extrahepatically Synthesized PPK

The physiological function of PPK synthesized in extrahepatic tissues can only be speculated upon. It may contribute to the PPK pool in blood plasma or cause local activation of various proenzymes (cf. Introduction). Also, concluding from *in vitro* studies, local processing of prohormones seems possible (Seidah et al., 1988). We favor the idea that PPK synthesized in extrahepatic tissues may function as a local kininogenase, acting similar to glandular (tissue) kallikrein in an autocrine- or paracrine-like manner. Glandular kallikrein is completely different from PK (Bhoola et al., 1992); the two enzymes have in common that they liberate kinin from kininogens, PK releases bradykinin, glandular kallikrein lysyl-bradykinin. Consistent with this hypothesis are our studies on mRNA expression of other components of the kallikrein-kinin system which demonstrate that the genes of high molecular weight kininogen (manuscript submitted) and FXII (unpublished results) are transcribed also in many nonhepatic tissues and cells. Thus it appears reasonable to assume that many tissues are furnished with a complete plasma kallikrein-kinin system that can become activated and exert local effects.

Our results confirm the reports of Ciechanowicz *et al.* (1993) and Hermann *et al.* (1996) and demonstrate that the PPK gene is transcribed in an even larger variety of tissues and cells than reported by these authors. Inferring from the results with epithelial cell lines, not only ubiquitously occurring cells are the origin of PPK mRNA but also tissue-specific cells. The next steps will be to verify extrahepatic PPK protein synthesis by immunocytochemical methods and to perform studies on cell lines with respect to PPK synthesis and its regulation.

# Materials and Methods

#### **Cell Lines and Cell Culture Conditions**

Human keratinocyte cell lines HaCaT, a spontaneously immortalized aneuploid cell line (Boukamp *et al.*, 1988) and HaCaT II-4RT, derived from HaCaT by transfection with the c-Ha-ras (EJ) oncogene (Boukamp *et al.*, 1990) were kind gifts of Dr. N. E. Fusenig (German Cancer Research Center, Heidelberg). PancTu cells (Kalthoff *et al.*, 1993) were kindly provided by Dr. K. Pantel (Institute of Immunology, University of Munich), primary cultures of decidua cells by Dr. J. Rehbock (Hospital of Gynecology, University of Munich) and primary cultures of human umbilical vein endothelial cells (HUVEC) and leukocytes by Dr. I. Müller (our institute). WI 38, SW 480, Kato III, BT 20 and HT 29 were from American Type Culture Collection, Colo 668 cells from the German Collection of Microorganisms and Cell Culture (Braunschweig, Germany), HeLa cells from EACC, Salisbury, UK.

Cells were cultured at 37 °C in a humidified atmosphere of 5% CO<sub>2</sub> and 95% air. HaCaT, HaCaT II and WI 38 were grown in DMEM medium containing 1 g/l glucose (PAA, Cölbe, Germany), supplemented with 0.35 g/l L-glutamine, 0.05 g/l gentamicin sulfate (Biowhittaker, Boehringer Ingelheim, Germany) and 10% fetal calf serum (PAA, Cölbe, Germany). Colo 668, decidua, BT 20, HT 29, SW 480 and PancTu cells were grown in RPMI medium (Biowhittacker), supplemented with 0.35 g/l L-glutamine, 100 U/ ml penicillin, 0.1 g/l streptomycin (Sigma, Deisenhofen, Germany) and 10% fetal calf serum (PAA).

After reaching confluence cells were harvested by incubation with 0.5 g/l trypsin and 0.2 g/l EDTA (Sigma) for 10 min. The proteolytic activity of trypsin was stopped by the addition of medium containing fetal calf serum. The cells were isolated from the suspensions (centrifugation at 200 g for 10 min) and washed twice

with phosphate buffered saline. Aliquots of about  $10^7$  cells were stored at – 70 °C in RNase-free plastic tubes until use.

# **RNA** Preparation

Total RNA was extracted from cultured cells and leukocytes by using the method described by Chomzynski and Sacchi (1987). Briefly, about 10<sup>7</sup> cells were lysed with 500 µl nucleic acid extraction buffer (4 M guanidine isothiocyanate, 5 g/l N-laurylsarcosine sodium salt, 0.1 M 2-mercaptoethanol, 25 mM sodium citrate, pH 7). 50  $\mu l$  of 2  $\mbox{M}$  sodium acetate pH 5.2 were added to the homogenates. The suspension was extracted with 500 µl phenol and 100  $\mu$ l of a chloroform/isoamyl alcohol mixture (49:1, v/v) and centrifuged at 13000 g. The RNA dissolved in the aqueous phase was precipitated with ethanol and suspended in diethylpyrocarbonate-treated water. The isolated RNA was quantified spectrophotometrically at 260 nm. A negative control was obtained by performing the whole procedure without adding cells. Total RNA from human liver, kidney, brain, heart, lung, and trachea was purchased from Clontech Laboratories (Paolo Alto, USA; catalog number K4000-1).

#### **Reverse Transcription and PCR**

Primers for semi-nested RT-PCR were selected from the cDNA sequence of human PPK (GenBank accession number M13143) taking into consideration the partially known exon-intron organization (Kunapuli *et al.*, 1995) that the PCR product included three exon-exon transitions. All primers were synthesized by MWG-Biotech (Ebersberg, Germany). The primers used were: hPK-29F, 5'-AGTGG CACAC CAAGT TCCT-3' (880–898, in exon 8), hPK-422R, 5'-CTCCA ACAAT GCGTG TGCT-3' (1273–1255, in exon 11), hPK-486R, 5'-TGAGC TGTCA GCTTC ACCT-3' (1337–1319, in exon 11). The quality of mRNA and cDNA preparations was validated by RT-PCR amplifying a segment of the ribosomal protein RPS4X (GenBank accession number M58458) using the forward primer RPX-1, 5'-TTCCT TGCCT AACGC AGC-3', and the reverse primer RPX-2, 5'-GATCT CACAT GTCAC CCA-3'.

cDNA of plasma prekallikrein and RPS4X was synthesized using a First Strand cDNA Synthesis Kit (Pharmacia, Freiburg, Germany). 1  $\mu$ g of total RNA or a corresponding volume of the negative control was incubated with 1  $\mu$ l of 200 mM dithiothreitol, 50 ng of the outer reverse primer hPK-486R, 50 ng of primer RPX-2 and RNase-free water in a final volume of 10  $\mu$ l. The incubation was performed at 65 °C for 10 min and then the mixture was placed on ice. 5  $\mu$ l of the 'Bulk First Strand Reaction Mix' (Pharmacia, Freiburg, Germany), containing murine reverse transcriptase, RNAguard, bovine serum albumin and deoxynucleotide triphosphates, were added and the mixture incubated at 37 °C for 2 h.

The first PCR was performed with 1  $\mu$ l of cDNA synthesis reaction mixtures, of the negative control, and of genomic DNA. To these samples 5  $\mu$ l of *Taq* DNA polymerase buffer (100 mM Tris-HCl pH 9.0, 500 mM KCl, 15 mM MgCl<sub>2</sub>, 1 g/l gelatin, 1% Triton X-100), 1  $\mu$ l of 20 mM dNTP solution (Pharmacia, Freiburg, Germany), 50 ng of the outer reverse primer (hPK-486 R) and 50 ng of the forward primer (hPK-29F), autoclaved water up to 50  $\mu$ l and 0.5 units of *Taq* DNA polymerase (Boehringer Mannheim, Germany) were added. 20 cycles of PCR amplification were performed each one consisting of a denaturation step at 92 °C for 30 s, an annealing step at 52 °C for 30 s and an elongation step at 72 °C for 30 s.

The second PCR with the forward primer hPK-29F and the inner reverse primer hPK-422R was performed with 1  $\mu$ l of the 1:10 diluted reaction mixture of the first PCR. 35 cycles were run with the profile described above. PCR for RPS4X cDNA amplification was performed only in the second PCR using the primers RPX-1 and RPX-2.

#### **Restriction Analysis**

Aliquots of  $4 \mu l$  of the PCR mixtures of cDNA and genomic DNA were incubated at 37 °C for 2 h with 7.5 U of the restriction enzyme *Sacl* (Boehringer Mannheim, Germany) and  $0.4 \mu l$  cleavage buffer. A third aliquot remained undigested and was diluted to the same volume.

#### Size Determination by PAGE

For size determination the PCR products, restriction fragments and a DNA size marker (G1751, Promega, Mannheim, Germany) were subjected to PAGE on a 14.5% acrylamide gel in 44.5 mM Tris, 44.5 mM boric acid, 1.0 mM EDTA, pH 8.0. DNA bands were visualized by silver staining (Riesner *et al.*, 1989).

#### **Sequence Analysis**

PCR products were purified by agarose gel electrophoresis and subsequent isolation of the respective band from the gel using a QIAquick gel extraction kit (Qiagen, Hilden, Germany). Nucleotide sequences were analyzed by the dideoxynucleotide chain-termination method using the Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin-Elmer, Weiterstadt, Germany) and an ABI PRISM 377 DNA sequencer (Perkin-Elmer).

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