# Molecular cloning and functional characterization of $V_2$ [8-lysine] vasopressin and oxytocin receptors from a pig kidney cell line

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[Arg<sup>8</sup>]vasopressin and oxytocin are the two main members of the neurohypophysial hormone family found to be present in nearly all mammals. [Lys<sup>8</sup>]vasopressin ([Lys<sup>8</sup>]VP) has been identified as the antidiuretic hormone in pig and some marsupial families. The porcine-derived kidney epithelial cell line, LLC-PK<sub>1</sub>, expresses both [Lys<sup>8</sup>]VP receptors coupled to the activation of adenylate cyclase (V<sub>2</sub> receptors) and oxytocin receptors. Here we report the molecular cloning of the V<sub>2</sub> [Lys<sup>8</sup>]VP receptor and the oxytocin receptor from LLC-PK<sub>1</sub> cells. The cloned V<sub>2</sub> [Lys<sup>8</sup>]VP receptor differs from human and rat V<sub>2</sub> [Arg<sup>8</sup>] receptors mainly in its N-terminal region, in residues located in the extracellular loops and in intracellular phosphorylation sites. When expressed in COS7 cells, the V<sub>2</sub> [Lys<sup>8</sup>]VP receptor exhibits the relative order of ligand affinity [Lys<sup>8</sup>]VP = [Arg<sup>8</sup>]VP  $\geq$  1deamino[D-Arg<sup>8</sup>]VP  $\geq$  oxytocin and adenylate-cyclase stimulation, expected for the porcine V<sub>2</sub> [Lys<sup>8</sup>]VP receptor but different from V<sub>2</sub> [Arg<sup>8</sup>]VP receptors. Adenylate-cyclase activation by [Lys<sup>8</sup>]VP was inhibited in COS7 cells by a V<sub>2</sub> antagonist. The cloned oxytocin receptor exhibits in COS7 cells a ligand specificity typical of mammalian oxytocin receptors. mRNA-distribution analysis revealed a single 5.5-kb transcript in the uterus from pregnant guinea pig.

Vasopressin-oxytocin peptides were first characterized as neurophypophysial hormones in mammals. In recent years the phylogenetic distribution of this family of peptides has been greatly expanded. At present vasopressin-oxytocin peptides have been characterized in over 50 vertebrate species (Acher, 1984 and 1985; Acher et al., 1985). The hormone superfamily comprises more than a dozen variants, mostly nonapeptides with several highly conserved residues including two cysteine residues at position 1 and 6 which form a disulfide bridge. Each species has usually two neurohypophysial hormones: one belonging to the oxytocin family which is involved in reproduction and one belonging to the vasopressin (VP) family, which is involved in hydroosmotic regulation. Placental mammalian species (Eutheria) have oxytocin and [Arg<sup>8</sup>]VP, except the family Suidae in which the pig has [Lys<sup>8</sup>]VP instead of [Arg<sup>8</sup>]VP (Popenoe et al., 1952). During evolution in Methateria, in some marsupial families two pressor hormones occurred: [Lys8]VP together with either [Phe2, Arg8]VP in australian macropodials or with [Arg<sup>8</sup>]VP in american opossums (Chauvet et al., 1983, 1985).

The molecular cloning of receptors for [Arg<sup>8</sup>]VP (Morel et al., 1992; Birnbaumer et al., 1992b; Lolait et al., 1992) and oxytocin (Kimura et al., 1992) has been recently accomplished and has demonstrated that these receptors form a sub-family among the large number of guanine-nucleotide-bind-

ing-regulatory-protein (G protein)-coupled receptors with seven transmembrane domains. Cloning of receptors for other neurohypophysial hormones will provide an opportunity to study the relationship of species-specific hormone variants to variations in receptor sequences.

The porcine-kidney-derived epithelial cell line, LLC-PK<sub>1</sub> (Hull et al., 1976), expresses [Lys<sup>8</sup>]VP receptors of the V<sub>2</sub> type coupled to the activation of adenylate cyclase (Ausiello et al., 1980). This established cell line has been extensively used as a cellular model system to study the V<sub>2</sub>-receptor adenylate-cyclase system (Ausiello et al., 1980; Roy et al., 1981; Jans et al., 1989). A small number of oxytocin receptors has been detected on these cells (Stassen et al., 1988; Cantau et al., 1990). The low density of oxytocin receptors as compared to V<sub>2</sub> receptors and the overlapping ligand specificity of vasopressin and oxytocin receptors have limited a precise pharmacological and functional characterization of each receptor on LLC-PK<sub>1</sub>cells.

The purpose of the present work was to clone and characterize the  $[Lys^8]VP V_2$  receptor and the oxytocin receptor from LLC-PK<sub>1</sub> cells, and to find common and divergent structural domains in the sequence of the vasopressin-oxytocin-receptor family, which are related to their functional properties.

### MATERIALS AND METHODS

#### Polymerase chain reaction (PCR)

Two degenerate oligonucleotides corresponding to transmembrane domains (TM) 2 and 6 were designed based on the cDNA sequences of the cloned vasopressin and oxytocin receptors (Morel et al., 1992; Birnbaumer et al., 1992b;

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Abbreviations. TM, transmembrane domain; PCR, polymerase chain reaction; G protein, guanine-nucleotide-binding regulatory protein.

Note. The novel nulceotide sequence data published here have been deposited with the EMBL sequence data banks and are available under accession numbers X71795 and X71796.

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3	1	CTGC	CCA	GCT	GCC	CAC	GAG	GCC	GGGG	AGC	сто	GCC	AGG	CAC	TCT	GCA	CGC	ста	cco	AGC	cc
	61	CACO	M	L	R	GCC	T	ACC T	S	A	V	P	R	GCC A	L	TCG	W	P	A	GCA A	P
	121	CGGC	AAC	GGC	AGC	GAC	AGG	GAG	ccc	СТС	GAC	GAC	cgg	GAC	ccc	ста	CTO	GCC	CGG	GTG	GA
		G	N A	G	s	E	R	E	P	г	D	D	R	U	P	г	<u>г</u>	A	ĸ	v	£
	181	GCTO	GCC A	CTG	CTG L	TCC	ACG T	GTC V	TTT F	GTG V	GCC A	GTG V	GCC	CTG L	AGC S	AAT N	000' 0	L	GTC V	CTG L	00 0
	241	GGCC	CTG	GTG	cgg	cgo	seec	cee	cee	GGC	cec	TGG	GÇG	ccc	ATG	CAC	GTO	TTC	ATC	GGC	CA
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	301	CTTG L	TGT C	CTG L	GCC A	GAC	CTG L	GCC A	GTG	A	L	F	Q	GTG V	CTG L	P	Q	L	GCC A	TGG W	GA D
	361	TGCC	ACC	TAC	CGC	ттс	CGT	GGG	сст	GAT	GCC	CTG	TGC	CGG	GCG	GTC	AAC	TAC	CTO	CAG	AT
		A	т	Y	R	F	R	G	P	D	A	L	с	R	A	v	ĸ	Y	L	Q	м
	421	GGTA	GGC	ATG	TAC	GCC	TCC	тсс	TAC	ATG	ATC	CTG	GCC	ATG	ACG	CTG	GAC	cec	CAC	cec	GC
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	481	CATC	TGC C	CGC R	CCC P	ATG M	CTG L	GCA A	TAC	CGC R	CAC H	GGA G	GGT G	GGA G	GCT A	CGC R	TGC: W	SAAC N	CGC R	CCG P	GT V
	541	COTO	CTC.		TCC		TTC	TCC	CTC	CTT	CTC	ACC	CTG	<u></u>	CAG	<u><u><u></u></u></u>	-	NTC.		1000	<u></u>
		L	v	A	W	A	F	s	L	L	L	S	L	P	Q	L	F	I	F	A	Q
	601	GCGC	GAC	GTG	GGA	GAC	GGC	AGC	GGG	бтс	стс	GAC	тсс	TGG	GCC	AGC	TTI	GCC	GAG	ccc	TG
		R	D	v	G	D	G	s	G	v	L	D	с	w	A	s	F	A	E	P	W
	661	GGGC	CTG	CGT	scc	TAC	GTC	ÂCG	TGG	ATC	GCC	CTG	ATG	GTG	TTT	GTG	GCG	CCT	GCC	TTG	GG
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	721	CATC	GCC A	GCC' A	rgc C	CAG Q	GTG V	CTC L	ATC I	TTC F	CGG R	GAG E	ATT I	CAC. H	ACC T	AGC S	CTG L	GTC V	CCG P	GGGG G	CC P
	781	0000	GAG	AGG	GCT	666	GGG	CAC	cGC	ممم	GGG	CGC	cGG	GCG	GGC	AGC	ccc	CGC	GAG	GGA	GC
		A	E	R	A	G	G	н	R	G	G	R	R	A	G	s	P	R	E	G	A
	641	CCGG	GTG'	rcg s	GCG	GCC	ATG M	GCT A	AAG K	ACG T	GCG A	AGG	ATG.	ACG	CTG L	GTG V	ATC	GTG	GCT	GTG V	TA Y
		CETC	-	PC C	TCC			TTC	TTC	•	CTC	CNC	CTT C	TCC	TCC	CTC	TCC		~~~~		~~
	901	v	L	C	W	A	P	F	F	L	v	Q		W	S	V	W	D	P	K	A
	961	GCCT	CGG	GAA	scci	ccc	сст	TTT	GTG	CTG	CTC	ATG	CTG	CTG	GCC	ÅGC	CTC	AAC	AGC	TGT	AC
		Р	R	E	G	Р	P	F	v	L	L	м	L	L	A	S	L	N	S	с	т
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		N	P	TGG. W	I	Y	A	s	F.	S	S	S	I	s	s	E	L	R	S	L	L
1	081	CTGC	CCC P TGC	IGG. W	I	Y AGG	A	s acc	F.	s	S	s ctc	I	s	S	E	GAG	R	s	L	L AC
1	081	CTGC	P TGC C	IGG W CCC P	I CGG R	Y AGG R	A CGC R		F. CCG	S CCC P	AGC S	S CTC L	I AGG R	s ccc P	S CAA Q	E GAG E	L GAG E	R TCC S	TGC	L GCC A	L AC T
1	081	CTGC C CGCC A	CCC <sup>C</sup> P TGC C AGC S		I CGG R TTC F	AGG R TCG	GCC A CGC R GCC A	ACC T AGG R	F. CCG P GAC D	S CCC P ACC T	AGC S TCG	S CTC L TCC S	I AGG R TGA	S CCC P GAA	S CAA Q GCC	GAG E AGG	GAG E GGC	R TCC S TGC	TGC C C TGC	L GCC A	L AC T TC
1	081	CTGC C CGCC A CAGG	CCC <sup>C</sup> P TGC C AGC S		TTC R TTC F		GCC R GCCC A GCCC	ACC T AGG R	F. CCG P GAC D	ACC P ACC T	AGC S TCG S CCT	S CTC L TCC S GGG	I AGG R TGA TCT	GAA	S CAA Q GCC CTG	GAG E AGG	GAG E GGC GCC	R TCC S TCC	TGC CTG	L GCC A CCT	L AC T TC
1	.081 .141 .201 .261	CTGC C CGCC A CAGG GGGC			I CGG R TTC F GGG		GCC R GCC A GCC A GCC A GCC A	ACC T AGG R AGG	GAC D GAC	ACC P ACC T	AGC S TCG S CCT	S CTC L TCC S GGG	I AGG R TGA TCT	GAA GGC CCG	S CAA Q GCC CTG GCA	GAG E AGG GGA	GAG GGC GGC AGC	R TCC S TGC S CTGC	GGG GGG TTG	L GCC A A GCC A GCC	L AC T TC
1	081	CTGC C CGCC A CAGG GGGC GGGC	CCC <sup>C</sup> P TGC C AGC S GGC GCG GCG	TCC P TCC S TCC GGG	I CGG R TTC F GGG AGG		GCC R GCC A GCC A GCC A GCC A GCC A GCC A GCC A	ACC T ACC T ACC R ACC R ACC R ACC	GAC D GAC D GAC D GAC	ACC P ACC T	AGC S TCG S CCT GTC CTC	S CTC L TCC S GGG AGG CAG	I AGG R TGA TCT GGC GCT	GAA GGC CCG CCG	GCC GCC GCC GCA GCA GCA	GAG E AGG GGA GGC GCC	GAG E GGC GGC GCC	R TCC S TGC		AGA GCC AGA GCG	L AC T TC AG CCA

-			M	E	G	v	L	λ	٨	N	W	8	λ	E	A	v	N	5	5	x
61	GGCG	CCG P	CCA P	GAG	GCC	GAG	GCC	AAC N	CGC R	ACC T	GCC	GCG G	CCG P	CCG P	CAG Q	CGC. R	AAC N	GAG E	5CC	CT L
121	GGCG	CGT	GTG	GAG	GTG	GCG	GTG	TTG	TGC	CTC	ATT	cic	TTC	CTA	GCT	CTG	AGC	GGCI	AATO	SC.
101	λ	R	V CTG	E	v	A (776)	v	L	ACG		г слс	AAG	CAT!	rcG		CTC	TTC	TTC	TTC	AT N
101	c	v	L	L	A	L	R	T	T	R	H	K	н	s	R	L	F	F	P	M
241	GAÁA K	CAC	L	AGC S	ATA I	GCC A	GAC	CTG L	GTG V	GTG V	A	GTG V	F	Q	GTG V	L	P	Q	L	L
301	GTGG W	GAT D	ATC I	ACC T	TTC F	CGC R	TTC F	TAC Y	GGA G	CCC P	GAC	TTG L	CTG L	TGC C	CGC R	L	GTC V	AAG' K	Y	Ľ
361	CCAG	GTG V	GTG V	GGC	ATG M	TTC F	GCT	TCC	ACT	TAC	CTG L	CTG L	CTG L	CTC L	ATG M	TCG S	CTC L	GAC	CGC R	TG C
421	TCTG	GCC	ATC	TGC	CAG	CCG P	TTG	CGC R	GCG	CTG L	icgo R	CGC	CCC P	GCG A	GAT	CGT R	CTG L	GCA A	GTG V	CT L
481	AGCO	ACA	TGO	CTG	GGC	TGC	CTG	GTG	GCC	AGO	GCC	ccc	CAG	GTO	CAC	ATC	TTO	TCC	cro	cg
541	A	Т СТО	w	L	G	с	L 	V GAT	A TGC	TGO	A 5600	P GTC	TTC:	N ATC	n CAC	-	TGO	5 5660		
	E	v	A	D	G	v	F	D	c	W	A	v	F	I	Q	P	W	G	P	K
601	GGCC A	Y	I	T	W	I	T	L	A	V	Y	I	V	P	V	I	V	L	A	A
661	CTGC	TAC Y	G	L	I	AGC S	F	K K	I	TGO	Q	SAAC N	L	R	L	:AAG K	T	rgcα λ	GCG A	GA E
721	AGCG À	GCA A	GAG	GCG	ATC I	GCG	GGA Ç	ACC T	GAG E	G G	GCC A	GCC A	GCG A	6000 G	CAGO S	CGG R	2002 0	CGA R	GCG A	GC
781	TCTC L	GCC A	CGC R	GTC	AGC	AGC S	GTC V	AAG K	CTC	ATC	rrco s	CAAC K	GCC A	AAC K	I I	CGC R	ACC	GTC V	AAG K	AT M
841	GACO	TTO	ATC	ATI	GTG	cro	GCC	TTC	ATC	GTI	TGO	TGO	ACC	icci	TTO	TTT	TTO	GTO	CAC	AT
901	GTGG	AGO	GTO	TGO	GAC		GAT	rGCO	-	-	GGA		TCI	GC	FTT	CATO	CAT	TGC/	ATC	
	W	s	v	W	D	A	D	A	P	ĸ	E	A	s	A	F	I	I	A	N	L
961	L	A	S	L	N	S	C	c	N	P	W	I	Y	M	L	F	T	G	н	Ľ
1021	CTTC F	CA1	GAA	L	GTG V	Q	R	TTC F	L	TGC C	CTGC C	S	rtcc s	AGC S	CAC H	CTC L	K K	T	AGC S	CG R
1081	GCCC P	G G	GAG E	ACG	AGT S	GTC V	AGC S	K K	AAG K	AGC S	CAAC N	STCC S	STCC S	T CAC	F	CGTC V	L	SAGO	CAC Q	CA H
1141	CAGO S	TCC S	AGC S	CAG	AAG K	AGC S	TGC	TCC S	TAG	cc	TCC	ACC	GGGG	TG <i>i</i>	\cc/	TCG	AGO	CTO	AGA	СТ
1201	GCCT	ccc	TGO	CTI	GGG	TTO	TGI	GAC	AGG	GAC	AGT		GCC	CT	GGI	GGT	GGG	TGC	GTC	cc
1321	CCAG	GGG	ALT	TCC	ACC	TGG	GGT	GGG	GAA	GCC	CATO	TC	GAG	GGG	GA	GAT	GAC	AGO	GTG	AC
1381	TCAG	CC.	TTO	GAC	AAT	ccc	TGG	ACC	TCC	cci	GGG	CTO	CCA	CCI	TAC	TCC	TGO	TAC	CCT	GT
1441	CCCC	NC1	GCI	GCC	CCT	GGC	TGC	TGG	GTG	GG1	rggi	TTT	TTT	TCI	CTC	AAG	:001	CT!	ATT	TT
1501	AATC	CAG	TGT	CCI	TTT	AGI	CCI	TTG	TCC	TGC	GAI	CT	GTC	AA	GG	GGT	AAC	TAT	NGG	AT
1561	TGGT	GAC	CN	ATG	GGC	TCC	:	GCC	TAG	TG	TC	GAC	CT/	GGG	GTO	AAG	AA	ATC	ACC	TC
1681	CACT	601	GG1	GCC	CTT.	010	CON	CATC	000	TT	AGC/	ACTO	TAG	CC	110	IGCC	ACC	5661	100	TC
1741	CTAT	GAN	AGC 2	TCT	000	ACI	GCC	AGC	AGA	TT	200		rcc	TT	AC		TT	TAC	CAAC	cc
1801	AGTA	CTT	AA	AAG	CAC	TTO	GGI	AAC	AAA	AAT	CAN	AA.	CAT	TC	AA.	TGC		AAA	~~~	AA
1861	***																			
VD	-	nto		n	AL	~1	0.74	D	KO	1	(a)		d	the		ww.	oci	n_r	000	mt

AGCCAACTGGAGCGCCGAGGCGGTCAACTCGAGCGC

Fig. 1. Nucleotide and deduced amino acid sequence of the V2-[Lys8]VP receptor cL clone **/I (a) a** oxyı cDNA (b) from LLC-PK<sub>1</sub> cells. The putative transmembrane domains are overlined. (**A**), Potential N-glycosylation sites; (**O**), possible phosphorylation sites. The position of the intron in PK12 cDNA is indicated by an arrow.

Lolait et al., 1992; Kimura et al., 1992). The 5' primer was 5'-TTCCA ( $_{G}^{\circ}$ ) GT ( $_{T}^{\circ}$ ) ( $_{T}^{\circ}$ ) TGCC ( $_{S}^{\circ}$ ) GAGCT-3' and the 3' primer was 5'-GAAGAA (<sup>o</sup><sub>A</sub>) GG (<sup>c</sup><sub>c</sub>) GCCCAGCA (<sup>c</sup><sub>A</sub>)A-3'. PCR was performed using LLC-PK, cDNA as a template under the following conditions, 30 cycles 94°C 1 min; 55°C, 2 min; 72°C, 3 min. The PCR product was gel-purified, cloned into pGEM-7Zf(+) and partially sequenced.

#### Screening of cDNA library

mRNA was isolated from LLC-PK<sub>1</sub> cells grown to about 90% confluency by the standard guanidinium-thiocyanate method followed by oligo(dT)-cellulose chromatography. Oligo(dT)-primed cDNA was synthesized with Superscript reverse transcriptase (Gibco BRL), size-fractionated on an agarose gel and ligated to  $\lambda$ gt10 EcoRI arms. To isolate V<sub>2</sub>-VPreceptor cDNA clones, the  $\lambda$ gt10 cDNA library was screened with the <sup>32</sup>P-labeled PCR fragment under highly stringent conditions: hybridization in 6×NaCl/Cit. (NaCl/Cit., 0.15 M NaCl, 15 mM sodium citrate, pH 7.0), 5×Denhardts' solution, 0.5% SDS at 65°C; final washing in 0.1×NaCl/Cit., Fig. 2. Nucleotide sequence of the 293-bp segment in PK12 cDNA. The exon sequences flanking the segment are shown by uppercase letters.

0.1% SDS at 65°C. To search for oxytocin-receptor cDNA clones, screening was done with two <sup>32</sup>P-labeled oligonucleo-5'-ATCCA(8)GGGTTGCAGCAGC-3' and CCAtides CATCTG (E)ACGAAGAAGAAGAA-3', complementary to the human oxytocin-receptor mRNA (Kimura et al., 1992) within TM7 and the third extracellular loop, respectively. The conditions for hybridization and washing were as described previously (Gorbulev et al., 1992).

# **DNA** sequencing

cDNA inserts from recombinant phages were recloned into pUC19 or pGEM-7Zf(+) and sequenced on both strands

	IM 1
HSOTR	MEGALAANWSAEAANASAAPPGAEGNRTAGPP RRNEALARVEVAVLCLILLLALSGNA
SSOTR	MEGVLAANWSAEAVNSSAAPPEAEGNRTAGPP QRNEALARVEVAVLCLILFLALSGNA
RNAV1	MSFPRGSQDRSVGNSSPWWPLTTEGSNGSQEAARLGEGDSPLGDVRNEELAKLBIAVLAVIFVVAVLGNS
RNAV2	MLLVSTVSAVPGLFSPPSSPSNSSQEELL DDRDPLLVRABLALLSTIFVAVALSNG
HSAV2	MLMASTTSAVPGHPSLPSLPSNSSQERPL DTRDPLLARAELALLSIVFVAVALSNG
SSLV2	MLRATTSAVPRALSWPAAPGNGSEREPL DDRDPLLARVELALLSTVFVAVALSNG
	TM 2 ***** *** TM 3
HSOTR	CVLLAL RTTROKH SRLFFFMKHLSIADLVVAVFOVLFOLLWDITFRFYGPDLLCRLVKYLOVVGMFAS
SSOTR	CVILAL RTTRHKH SRLFFFMKHLSTADLVVAVFOVLPOLLWDITFRFYGPDLLCRLVKYLOVVGHFAS
RNAV1	SVILAL HETPEKT SEMHLETEHLSLADLAVAFFOVLPOLCWTSP SFRGPDWLCRVVKHLOVFANFAS
RNAV2	LVLGALIERGERGEWAPMHVFISHLCLADLAVALFOVLFOLAWDATDEFHGPDALCRAVKYLOMVGHYAS
HSAV2	LVLAALAREGREGHWAPIHVFIGHLCLADLAVALFOVLPOLAWKATDRFRGPDALCRAVKYLOMVGNYAS
SSLV2	LVLGALVRRGRRGRWAPMHVFIGHLCLADLAVALFOVLPOLAWDATYRFRGPDALCRAVKYLOMVGWYAS
	TM /
HCOTTR	
SCOTP	TIDE DESTRUCTION OF DESTRUCT DA LAVIATED CONTRACT ADDRESS AND AND ADDRESS ADDR
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DNAVI	CIVIT AND TO DO TO DOM A VOUCCCA DINID DUI VANA FOI LIGI DOL FINA OND V CANGGUEDOM
NIVAV2	
HSAV2	SIMILARI DANKATCHEMATANGGGARANKE VIVARAT SILLABLEVI I I RAUTA DASSO VIDERA
22045	SIMILARI LURANCERICA I KAGGGARANKE VUVANAI SUUUBLE QUI ZEAQADV GOSSOVULCAR
	*** TM5
HSOTR	VFIQPWGPKAYITWITLAVYIVPVIVLATCYGLISFKIWQNLRLKTAAAAAA EAPEGAAAGDGGRVAL
SSOTR	VFIQPWGPKAYITWITLAVYIVPVIVLAACYGLISFKIWQNLRLKTAAEAAEAIAGTEGAAAGSRGRAAL
RNAV1	TFIQPWGTRAYVTWMTSGVFVAPVVVLGTCYGFICYHIWRNIRGKTASSRHSKGDKGSGEAVGPFHKGLL
RNAV2	RFAEPWGLRAYVTWIALMVFVAPALGIAACQVLI FREIHASLVPGPSERAG TPQRAPDR SPSEGA
HSAV2	CFAEPWGRRTYVTWIALMVFVAPTLGIAACQVLI FREIHASLVPGPSERPG GRRRGRRTGSPGEGA
SSLV2	SFAEPWGLRAYVTWIALMVFVAPALGIAACQVLI FREIHTSLVPGPAERAG CHRGGRRAGSPREGA
	TM6 TM7
HSOTR	AR VSSVKLISKAKIRTVKMTFIIVLAFIVCWTPFFFVQMMSVWDAN APKRASAFIIVMLLASLNS
SSOTR	AR VSSVKLI <b>SKA</b> KIRTVKMTFIIVLAFIVCMTPFFFVQMWSVWDAD AFKBASAFIIAMLLASLNS
RNAV1	VTPCVSSVKSISRAKIRTVKMTFVIVSAYILCWAPFFIVOMMSVMDENFIWTDSENPSITITALLASLNS
RNAV2	HVSAAMAKTVRMTLVIVIVVLCWAPFFLVQLWAAMDPE APLERPPFVLLMLLASLNS
HSAV2	HVSAAVAKTVRMTLVIVVVVLCWAPFFLVQLWAAWDPE APLEGAPFVLLMLLASINS
SSLV2	RVSAAMAKTARMTLVIVAVYVLCWAPFFLVQLWSVWDPK APREGPPFVLLMLLASLNS
HSOTR	CCNPWIYMLFTGHLFHELVQRFLCCSASYLKGRRLGETSASKKSNSSSFVLSHRSSSQRSCSQPSTA
SSOTR	CCNPWIYMLFTGHLFHELVORFLCCSSSHLKTSRPGETSVSKKSNSSTFVLSOHSSSOKSCS
RNAV1	CCNPWIYMFFSGHLLODCVOSFPCCHSMAQKFAKDDSDSMSRKTD FLF
RNAV2	CTNPWIYASFSSVSSE LRSLLCCAORHTTHSLGPODESCATASS SLMKDTPS
HSAV2	CTNEWIYASESSVSSE LRSLLCCARGRTPPSLGPODESCTTASS SLAKDTSS
SSLV2	CTNPWIYASFSSISSE LRSLLCCPRRTPPSLRPQEESCATASS FSARDTSS

Fig. 3. Comparison of the amino-acid sequences of receptors from the oxytocin-vasopressin subfamily according to Feng and Doolittle (1990). Invariant residues are shown in boldface type. (\*), Invariant amino acids conserved only within the oxytocin-vasopressin subfamily but not in other G-protein-coupled receptors. The putative transmembrane domains (TM1-TM7) are overlined. HSOTR, human oxytocin receptor; SSOTR, pig oxytocin receptor; RNAV1, rat V<sub>1</sub> receptor; RNAV2, rat V<sub>2</sub> AVP receptor; HSAV<sub>2</sub>, human V<sub>2</sub> AVP receptor; SSLV2, pig V<sub>2</sub> LVP receptor.

by the dideoxynucleotide chain-termination method using a Sequenase version 2.0 kit (United States Biochemicals) and utilizing both the exonuclease III/mung bean nuclease approach (Henikoff, 1984) and appropriate oligonucleotide primers.

## Northern hybridization

mRNA from frozen tissues or from cultured cells were isolated as described above.  $1-5 \mu g$  of the mRNA preparations was resolved on a formaldehyde-agarose gel, transferred to Hybond-N membrane (Amersham) and hybridized with <sup>32</sup>P-labeled V<sub>2</sub> [Lys<sup>8</sup>]VP-receptor cDNA or oxytocin-receptor cDNA under the conditions described previously (Gorbulev et al., 1992). The washing conditions are given in the figure legends.

## Expression in COS cells and binding experiments

The entire cDNA inserts were subcloned into the expression vector, pCDM8 (Seed and Aruffo, 1987) using *BstXI* adaptors. The resulting DNA was transfected into COS7 cells during 4.5 h using the DEAE-dextran method (Seed and Aruffo, 1987). After 70 h cells were harvested and treated as described previously (Gorbulev et al., 1992). The saturation analysis was performed by incubation of 200 µl cell suspension with varying concentrations of [3H] [Arg8]VP (26 Ci/ mmol, NEN) or [3H]oxytocin (36.6 Ci/mmol, NEN). Nonspecific binding was determined in the presence of a 100fold excess of non-radioactive hormone. The displacement experiments were performed with 10 nM [<sup>3</sup>H] [Arg<sup>8</sup>]VP (for V<sub>2</sub> receptor) or [<sup>3</sup>H]oxytocin (for oxytocin receptor) in the presence of different concentrations of competitors. All incubations were performed for 30 min at 30°C in duplicate. Bound ligand was separated from free ligand by filtering the incubation mixture through Whatman GF/B filters soaked in 0.05% bovine serum albumin. The binding data were analysed using the Ligand program (Munson, 1983). The same conditions were used for binding experments with LLC-PK<sub>1</sub> cells grown to 90% confluency (Luzius et al., 1991).

#### cAMP assay

Transfected COS7 cells were harvested and disrupted to prepare cell homogenates as described (Birnbaumer et al.,

1992a). Cell homogenates were incubated with ligands in the presence of 0.2 mM isobutylmethylxanthine for 10 min at 30°C as described (Fahrenholz et al., 1980). Reactions were stopped with the addition of HCl to 0.2 M and the samples were frozen. After thawing and neutralization, cAMP accumulation was measured by using a cAMP assay kit (Amersham).

# RESULTS

To amplify the cDNA sequences coding for the  $V_2$  VP receptor in LLC-PK<sub>1</sub> cells, we utilized PCR with two degenerate oligonucleotides (see Materials and Methods). The most prominent PCR product was gel purified and cloned. Partial sequencing revealed significant similarity to  $V_2$  [Arg<sup>8</sup>]VP receptors. This PCR fragment was used to screen a LLC-PK<sub>1</sub> cDNA library. After two rounds of screening we isolated 18 strongly hybridizing clones from about 200000 bacteriophage plaques. Restriction analysis of DNA from four independent recombinant phages revealed that they represent two cDNA populations with different insert sizes, 1.5 kb and 1.8 kb. Two clones, PK91 (1.5-kb insert) and PK12 (1.8-kb insert), were chosen for further analysis.

The sequencing of these cDNA showed that they are related to each other, share the same nucleotide sequence, but that PK12 cDNA has in addition an extra G at the 5' end, a poly(A) tail just after the 3' terminal nucleotide of PK91 cDNA and a 293-bp segment between nucleotides 86 and 87 of the PK91 cDNA is shown in Fig. 1a. It contains 1494 bp and has an open reading frame for 370 amino acids. The deduced amino-acid sequence shows about 84% identity to human and rat V<sub>2</sub> [Arg<sup>8</sup>] receptors (Birnbaumer et al., 1992b; Lolait et al., 1992) and only 40% identity to the rat V<sub>1</sub> [Arg<sup>8</sup>]VP receptor (Morel et al., 1992), which is coupled to the activation of the phospholipase-C system. The position of the 293-bp segment in PK12 cDNA coincides with that of the first intron found in the human V<sub>2</sub> [Arg<sup>8</sup>]VP-receptor gene (Seibold et al., 1992). The terminal sequences of the 293-bp segment and flanking sequences fit well to the consensus splicing signal on the exon-intron boundary (TCGG/ GTGAGT...CCCAG/CT) (Breathnach and Chambon, 1981; Fig. 2). Thus, PK12 cDNA seems to represent an incompletely spliced transcript of the  $V_2$ -[Lys<sup>8</sup>]-VP receptor gene.

When screening the same  $\lambda gt10$  cDNA library with two oxytocin-receptor-specific oligonucleotides (see Materials and Methods), we found one strongly hybridizing clone with an insert of about 1.9 kb. The complete sequencing of the cDNA insert from this clone revealed an open reading frame encoding a 386-amino-acid protein with 92% identity to the human oxytocin receptor (Fig. 1b). The oxytocin-receptor gene from LLC-PK<sub>1</sub> cells contains at the position corresponding to Gln385 of the human oxytocin receptor (Kimura et al., 1992) a substitution C $\rightarrow$ T resulting in a stop-codon five amino acids before the C-terminus of the human oxytocin receptor.

The cloned  $V_2$  [Lys<sup>8</sup>]VP and oxytocin receptors share most of the structural features common for this receptor subfamily (Fig. 3) including an unique stretch of seven amino acids (Phe-Gln-Val-Leu-Pro-Gln-Leu) at the end of TM2, one sequence in the first putative extracellular loop (Gly-Pro-Asp) and two sequences in the second extracellular loop (Asp-Cys-Trp-Ala and Pro-Trp-Gly). These sequences are conserved exclusively within this subfamily and may play an important role in ligand-receptor recognition. The  $V_2$ 



Fig. 4. Binding of [<sup>3</sup>H] [Arg<sup>8</sup>]VP to COS7 cells transfected with PK91 cDNA. (a) Saturation analysis. The results shown are the means of duplicate determinations and are representative of three independent experiments. Inset shows Scatchard plot of the binding data. (b) Displacement analysis. The data shown are the means of duplicate values and are representative of two [for 1-deamino[D-Arg<sup>8</sup>]VP (dDAVP) and oxytocin] or three [for [Arg<sup>8</sup>]VP (AVP) and [Lys<sup>8</sup>]VP (LVP)] independent experiments.

[Lys<sup>8</sup>]VP receptor has only one potential N-glycosylation site in its N-terminal segment, whereas the oxytocin receptor has three such sites. N-glycosylation has been shown to play a role in biosynthesis and internalization of the  $V_2$  [Lys<sup>8</sup>]VP receptor in LLC-PK<sub>1</sub> cells (Jans et al., 1992).

To functionally characterize the cloned V<sub>2</sub> [Lys<sup>8</sup>]VP and oxytocin receptors, their cDNA were recloned into the eukaryotic expression vector pCDM8 and used to transfect COS7 cells. COS7 cells transiently express not only PK91 cDNA, but also PK12 cDNA indicating that the 293-bp intron of PK12 cDNA is properly spliced during the propagation of monkey kidney cells. Saturation analysis of COS7 cells transfected with PK91 cDNA (Fig. 4a) shows a dose-dependent binding of [<sup>3</sup>H] [Arg<sup>8</sup>]VP with an apparent dissociation constant  $(K_d)$  of 2.3 nM, which is similar to that determined for LLC-PK<sub>1</sub> cells (Jans et al., 1986). Competitive-binding studies with [<sup>3</sup>H] [Arg<sup>8</sup>]VP gave the inhibition constants  $(K_i)$ of 5.0 nM for [Arg8]VP, 7.3 nM for [Lys8]VP, 0.27 µM for 1deamino [D-Arg<sup>8</sup>]VP and 0.6 µM for oxytocin (Fig. 4b). The low affinity of the cloned V<sub>2</sub> [Lys<sup>8</sup>]VP receptor for oxytocin and the specific V<sub>2</sub> agonist 1-deamino [D-Arg<sup>8</sup>]VP is consistent with the values obtained on membranes derived from pig kidney (Roy et al., 1975b; Stassen et al., 1985). Our results confirm the observation that replacement of L-Arg in



Fig. 5. Displacement of [<sup>3</sup>H] [Arg<sup>8</sup>]VP binding to LLC-PK<sub>1</sub> by [Lys<sup>8</sup>]VP (LVP) (----) and [Arg<sup>8</sup>]VP (AVP) (----). The data shown are the means of duplicate values and are representative of two independent experiments.



Fig. 6. Adenylate-cyclase stimulation assay for COS7 cells transfected with PK91 cDNA (a) and oxytocin-receptor cDNA (b). The ligands used are as follows;  $[Lys^{8}]VP$  (LVP) 100 nM;  $[Arg^{8}]VP$  (AVP), 100 nM;  $V_{2}$  antagonist d(CH<sub>2</sub>)<sub>5</sub>[D-Phe<sup>2</sup>, Val<sup>4</sup>, Arg<sup>8</sup>]VP (Manning et al., 1982), 50  $\mu$ M;  $V_{1}$  antagonist d(CH<sub>2</sub>)<sub>5</sub>[MeAla<sup>7</sup>, Arg<sup>8</sup>]VP (Fahrenholz et al., 1984), 50  $\mu$ M; oxytocin, 100 nM. Control is cAMP accumulation without additives. Isoproterenol (Iso) was added to a final concentration of 1  $\mu$ M. Incubations were performed in duplicates and the data shown are means  $\pm$  SEM of two experiments.

position 8 by D-Arg, as in 1-deamino[D-Arg<sup>8</sup>]VP, leads to a drastic decrease in affinity for the  $V_2$  receptor in pig, whereas the human and rat  $V_2$  receptors retain one to two orders higher affinity (Guillon et al., 1982; Butlen et al., 1987).

Contradictory data about the relative binding affinities of  $[Lys^{8}]VP$  and  $[Arg^{8}]VP$  in pig kidney have been reported (Roy et al., 1975a; Butlen et al., 1987). Therefore, we examined their affinity on LLC-PK<sub>1</sub> cells and obtained  $K_{i}$  values



Fig. 7. Binding of [<sup>3</sup>H]oxytocin (OT) to COS7 cells transfected with oxytocin-receptor cDNA. (a) Saturation analysis. The results shown are the means of duplicate determinations and are representative of two independent experiments. Inset shows Scatchard plot of the binding data. (b) Displacement analysis. The data shown are the means of duplicate values and are representative of two independent experiments. dSOT, 1-dearnino[7-sarcosine]oxytocin; AVP, [Arg<sup>8</sup>]VP.

of 1.0 nM for  $[Arg^8]VP$  and 1.7 nM for  $[Lys^8]VP$  (Fig. 5), i.e. the same order of affinities as for the cloned V<sub>2</sub> receptor.

The cloned [Lys<sup>8</sup>]VP receptor stimulated cAMP accumulation in response to [Lys<sup>8</sup>]VP or [Arg<sup>8</sup>]VP (Fig. 6a). The adenylate-cyclase activity was inhibited in the presence of a V<sub>2</sub> specific antagonist and was not affected by a V<sub>1</sub>-specific antagonist. Control experiments using COS7 cells transfected with oxytocin-receptor cDNA revealed no cAMP accumulation in response to oxytocin (Fig. 6b) as has been expected taking into account that oxytocin receptors couple to the phosphatidylinositol second-messenger system (Schrey et al., 1986). The saturation and displacement analysis of COS7 cells transiently expressing oxytocin receptors after transfection with oxytocin receptor cDNA gave a  $K_d$  value of 4.6 nM for [3H]oxytocin (Fig. 7a) and the relative order of ligand affinities: oxytocin = 1-deamino[7-sarcosine]oxytocin (Grzonka et al., 1983)  $\geq$  [Arg<sup>8</sup>]VP with  $K_i$  of 4.6, 5.6 and 15.5 nM, respectively (Fig. 7b). These binding characteristics of the cloned oxytocin receptor are in accordance with those reported for the pig oxytocin receptor (Cantau et al., 1990; Jans et al., 1993).

Northern-blot analysis was performed to analyse the expression of  $V_2$  [Lys<sup>8</sup>]VP and oxytocin receptors in different tissues and cell lines.  $V_2$  [Lys<sup>8</sup>]VP-receptor mRNA was detected neither in liver, nor in brain (Fig. 8). Hybridization



Fig. 8. Northern-blot analysis of  $V_2$  VP mRNA expression. Lanes contain mRNA from VPR1 cells, 5 µg (lane 1), from LLC-PK<sub>1</sub> cells, 5 µg (lane 2) from pig brain, 4 µg (lane 3), from pig liver, 4 µg (lane 4) from pig kidney, 4 µg (lane 5). The filter was hybridized to <sup>32</sup>Plabeled V<sub>2</sub>-[Lys<sup>8</sup>]VP-receptor cDNA, washed to a final stringency 0.25 × buffer A (buffer A, 0.18 M NaCl, 10 mM sodium phosphate, pH 7.7, 1 mM EDTA), 0.1% SDS at 60°C and exposed for 30 h.



Fig. 9. Northern-blot analysis of oxytocin receptor mRNA expression. Lanes contain mRNA from LLC-PK<sub>1</sub> cells, 2  $\mu$ g (lane 1), from guinea pig pregnant uterus, 4  $\mu$ g (lane 2) and from human pregnant uterus, 2.5  $\mu$ g (lane 3). The filter was hybridized to <sup>32</sup>P-labeled oxytocin-receptor cDNA, washed to a final stringency of 0.25×buffer A, 0.1% SDS at 55°C and exposed for 20 h.

with  $V_2$ -[Lys<sup>8</sup>]VP-receptor cDNA revealed a transcript of 1.5 kb in LLC-PK<sub>1</sub> cells and 1.8 kb in kidney. The mRNA level of V<sub>2</sub> [Lys<sup>8</sup>]VP receptor in kidney was significantly lower than in LLC-PK<sub>1</sub> cells. In VPR1 cells, a mutant of LLC-PK<sub>1</sub> cells defective in  $V_2$  VP binding and function (Luzius et al., 1991), we found  $V_2$ -[Lys<sup>8</sup>]VP-receptor mRNA at the same position as in LLC-PK<sub>1</sub> cells. This indicates that the mutation causing the V<sub>2</sub>-receptor defect in VPR1 cells does not affect gene transcription. LLC-PK<sub>1</sub> cells have, however, an additional hybridizing band of about 3.4 kb, that is absent in VPR1 cells. Oxytocin receptor mRNA was not detected in pig kidney, pig liver and pig brain, but it was identified in LLC-PK<sub>1</sub> cells and in human and guinea pig pregnant uteri (Fig. 9). Human oxytocin-receptor mRNA is of 4.4 kb (Kimuara et al., 1992), whereas guinea pig uterus and LLC-PK<sub>1</sub> cells contain larger transcripts of 5.5 kb and 5.4 kb, respectively. As the cloned oxytocin-receptor cDNA has a polyadenlyation signal and a poly(A) tail, the results of

Northern-blot analysis may suggest an unusually long 5' nontranslated region of pig oxytocin mRNA.

# DISCUSSION

The cloned  $V_2$  [Lys<sup>8</sup>]VP receptor binds the two hormones [Lys<sup>8</sup>]VP and [Arg<sup>8</sup>]VP with roughly equal affinity. In this respect it is interesting to note that both [Lys<sup>8</sup>]VP-like and [Arg<sup>8</sup>]VP-like peptides have been detected in pig ovaries and testis (Nicholson et al., 1988). AVP synthesized in extrahypothalamic areas of this species might therefore bind to the  $V_2$  receptor with the same high affinity as [Lys<sup>8</sup>]VP. In contrast,  $V_2$  receptors in mammalian species with [Arg<sup>8</sup>]VP as the only VP show significantly higher binding for [Arg<sup>8</sup>]VP compared to [Lys<sup>8</sup>]VP (Hechter et al., 1978; Guillon et al., 1982).

The V<sub>2</sub> [Lys<sup>8</sup>]VP receptor and human V<sub>2</sub> [Arg<sup>8</sup>]VP receptor demonstrate more than 90% identity within their putative transmembrane domains, 80% identity for their C-terminal regions and a relatively low identity of 62% for their Nterminal regions. This divergence cannot be attributed only to species differences, because oxytocin receptors, that belong to the same subfamily and have been cloned from the same species, have a similar identity for both N-terminal and C-terminal regions, of 86.5% and 81%, respectively. Studies primarily performed on adrenergic and muscarinic receptors suggest that the N-terminal part of G-protein-coupled receptors for catecholamines and acetylcholine is responsible for the transport of receptors to the cell membrane, but contains no determinants for ligand binding (Ostrowski et al., 1992). However, for receptors that recognize peptide hormones, the importance of the N-terminal region for ligand binding has been shown: for the lutropin-choriogonadotropin receptor (Xie et al., 1990; Nagayama et al., 1991) and recently for tachikinin and endothelin-receptor subfamilies (Yokota et al., 1992; Hashido et al., 1992). Thus, we suggest that the Nterminus of  $V_2$  VP receptors may be involved in determining the relative affinity for [Lys<sup>8</sup>]VP and [Arg<sup>8</sup>]VP. Other differ-ences between [Lys<sup>8</sup>]VP and [Arg<sup>8</sup>]VP V2 receptors are Tyr102 (in the first extracellular loop), Lys298 and Arg301 (in the third extracellular loop) in  $[Lys^8]VP V_2$  receptor instead of Asp, Glu and Leu, respectively, located in the corresponding positions of [Arg<sup>8</sup>]VP V<sub>2</sub> receptors. The importance of these substitutions for binding selectivity should be tested in future experiments.

Comparison of the three  $V_2$  VP receptors also shows that only the porcine  $V_2$  receptor contains three potential phosphorylation sites for protein kinase C (Woodget et al., 1986) and cAMP-dependent protein kinase (Kennelly and Krebs, 1991) in its C-terminal region. This difference in phosphorylation sites might explain the observation that cAMP-dependent protein-kinase activation induces down-regulation of the  $V_2$  [Lys<sup>8</sup>]VP receptor in LLC-PK<sub>1</sub> cells (Jans and Hemmings, 1991) but not of the human  $V_2$  [Arg<sup>8</sup>]VP receptor in stably transfected murine cells (Birnbaumer et al., 1992a).

Surprisingly, we could not detect oxytocin-receptor mRNA in lactating mammary glands from guinea pig and rat, although this tissue is known to express oxytocin receptors (Soloff, 1982). An explanation could be that mammary glands possess another subtype of oxytocin receptor and our non-homologous probe (pig oxytocin-receptor cDNA) was not able to detect it.

In conclusion, we cloned  $V_2$  [Lys<sup>8</sup>]VP and oxytocin receptors form LLC-PK<sub>1</sub> cells. The two receptors belong to the

same subfamily of G-protein-coupled receptors, bind related hormones and analogues, but couple to different second-messenger systems. One of them, the V<sub>2</sub> [Lys<sup>8</sup>]VP receptor, discriminates well between VP and oxytocin, the other, the oxytocin receptor, binds both hormones with a similar high affinity (Figs 4b and 7b). These related receptors cloned from the same species, but having different selectivity for their ligands, and different G-protein-coupling specificity will provide suitable tools to determine their structural domains important for ligand binding and G-protein-mediated signal transduction.

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#### REFERENCES

- Acher, R. (1984) Nova Acta Leopold 56, 137-151.
- Acher, R. (1985) Peptides (NY) 6, 309-314.
- Acher, R., Chauvet, J., Chauvet, M. T. & Hurpet, D. (1985) Current trends in Comparative Endocrinology (Lofts, B. & Holmes, W. N., eds) pp. 1147-1152, Hong Kong University Press, Hong Kong.
- Ausiello, D. A., Hall, D. H. & Dayer, J. M. (1980) Biochem. J. 186, 773-780.
- Birnbaumer, M., Antaramian, A., Themmen, A. P. N. & Gilbert, S. (1992a) J. Biol. Chem. 267, 11783-11788.
- Birnbaumer, M., Seibold, A., Gilbert, S., Ishido, M., Barberis, C., Antaramian, A., Brabet, P. & Rosenthal, W. (1992b) Nature 357, 333-335
- Breathnach, R. & Chambon, P. (1981) Annu. Rev. Biochem. 50, 349-383.
- Butlen, D., Guillon, G., Rajerison, R. M., Jard, S., Sawyer, W. M. & Manning, M. (1987) Mol. Pharmacol. 14, 1006-1017.
- Chauvet, M. T., Colne, T., Hurpet, D., Chauvet, J. & Acher, R. (1983) Biochem. Biophys. Res. Commun. 116, 258-263. Chauvet, J., Hurpet, D., Colne, T., Michel, G., Chauvet, M. T. &
- Acher, R. (1985) Gen. Comp. Endocrinol. 57, 320-328.
- Cantau, B., Barjon, J. N., Chicot, D., Baskevitch, P. P. & Jard, S. (1990) Am. J. Physiol. 258, F963-F972.
- Fahrenholz, F., Thierauch, K.-H. & Crause, P. (1980) Hoppe-Seyler's Z. Physiol. Chem. 361, 153-167.
- Fahrenholz, F., Boer, R., Crause, P., Fritzsch, G. & Grzonka, Z. (1984) Eur. J. Pharmacol. 100, 47-58.
- Feng, D. F. & Doolittle, R. F. (1990) Methods Enzymol. 183, 659-669.
- Gorbulev, V., Akhundova, A., Büchner, H. & Fahrenholz, F. (1992) Eur. J. Biochem. 208, 405-410.
- Guillon, G., Butlen, D., Cantau, B., Barth, T. & Jard, S. (1982) Eur. J. Pharmacol. 250, 291-304.
- Grzonka, Z., Lammeck, B., Gazis, D. & Schwartz, J. L. (1983) J. Med. Chem. 26, 1786.

- Hashido, K., Gamou, T., Adachi, M., Watanabe, T., Furuichi, Y. & Myamoto, C. (1992) Biochem. Biophys. Res. Commun. 187, 1241 - 1248
- Hechter, O., Terada, H., Spitsberg, V., Nakahara, T., Nakagawaga, S. H. & Flouret, G. (1978) J. Biol. Chem. 253, 3230-3237.
- Henikoff, S. (1984) Gene (Amst.) 28, 351-359.
- Hull, R. N., Cherry, W. R. & Weaver, G. W. (1976) In vitro (Rockville) 12, 670-677.
- Jans, D. A., Resink, T. J., Wilson, E. R., Reich, E. & Hemmings, B. A. (1986) Eur. J. Biochem. 160, 407-412.
- Jans, D. A. & Hemmings, B. A. (1991) FEBS Lett. 281, 267-271.
- Jans, D. A., Peters, R., Zsigo, J. & Fahrenholz, F. (1989) EMBO J. 9.2481 - 2488
- Jans, D. A., Jans, P., Luzius, H. & Fahrenholz, F. (1992) Arch. Biochem. Biophys. 294, 64-69.
- Jans, D. A., Pavo, J. & Fahrenholz, F. (1993) FEBS Lett. 266, 1555-1558.
- Kennelly, P. J. & Krebs, E. G. (1991) J. Biol. Chem. 266, 1555-1558.
- Kimura, T., Tanizawa, O., Mori, K., Brownstein, M. J. & Okayama, H. (1992) Nature 356, 526-529
- Lolait, S. J., O'Carroll, A.-M., McBride, O. W., Konig, M., Morel, A. & Brownstein, M. J. (1992) Nature 357, 336-339.
- Luzius, H., Jans, D. A., Jans, P. & Fahrenholz, F. (1991) Exp. Cell Res. 195, 478-484.
- Manning, M., Klis, W. A., Olma, A., Seto, J. & Sawyer, W. H. (1982) J. Med. Chem. 25, 419-419.
- Morel, A., O'Carroll, A.-M., Brownstein, M. J. & Lolait, S. J. (1992) Nature 356, 523-526.
- Munseon, P. J. (1983) Methods Enzymol. 92, 543-576.
- Nagayama, B., Waldsworth, H. L., Chazenbalk, G. D., Russo, D., Seto, P. & Rapoport, B. (1991) Proc. Natl Sci. USA 88, 902-905.
- Nicholson, H. D., Smith, A. J., Birkett, S. D., Denning-Kendall, P. A. & Pickering, B. T. (1988) J. Endocrinol. 117, 441-446.
- Ostrowski, J., Kjelsberg, M. A., Caron, M. G. & Lefkowitz, R. J. (1992) Annu. Rev. Pharmacol. Toxicol. 32, 167-183.
- Popenoe, E. A., Lawler, H. C. & du Vigneaud, V. (1952) J. Am. Chem. Soc. 74, 3713-3714.
- Roy, C., Barth, T. & Jard, S. (1975a) J. Biol. Chem. 250, 3149-3156.
- Roy, C., Barth, T. & Jard, D. A. (1975b) J. Biol. Chem. 250, 3157-3168.
- Schrey, M. P., Reed, A. M. & Steer, P. (1986) Biosci. Rep. 6, 613-616.
- Seed, B. & Aruffo, A. (1987) Proc. Natl Acad. Sci. USA 84, 3365-3369.
- Seibold, A., Brabet, P., Rosenthal, W. & Birnbaumer, M. (1992) Am. J. Hum. Genet. 51, 1078-1083.
- Soloff, M. S. (1982) J. Dairy Sci. 65, 326-337.
- Stassen, F. L., Heckman, D. C., Schmidt, D. B., Stefankiewicz, J., Sulat, L., Huffman, W. F., Moore, M. M. & Kinter, L. B. (1985) in Vasopressin (Schrier, R. W., ed.) pp. 145-154, Raven Press, New York.
- Stassen, F. L., Heckman, G., Schmidt, D., Papadopoulus, M. T., Nambi, P., Sarau, H., Aiyar, N., Gellai, M. & Kinter, L. (1988) Mol. Pharmacol. 33, 218-224.
- Woodget, J. R., Gould, K. L. & Hunter, T. (1986) Eur. J. Biochem. 161, 177-184.
- Xie, Y.-B., Wang, H. & Segaloff, D. L. (1990) J. Biol. Chem. 265, 21411-21414.
- Yokota, Y., Akazawa, C., Ohkuba, H. & Nakanishi, S. (1992) EMBO J. 11, 3585-3591.