CLONING AND EXPRESSION OF THE RAT AND HUMAN PROLACTIN RECEPTORS

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RESUME

Afin d'élucider la structure et la fonction du récepteur de la prolactine, nous avons eu recours aux techniques de biologie moléculaire. Après l'échec du criblage de banques d'expression des ADN complémentaires avec des anticorps poly- et monoclonaux, nous avons utilisé des sondes d'oligonucléotides correspondant aux séquences en acides aminés de fragments du récepteur pour isoler l'ADN complémentaire du récepteur de la prolactine du foie de rat. A l'aide de ce clone, la régulation de l'expression du récepteur de la prolactine par l'oestrogène et durant l'ontogénèse a été étudiée dans le foie de rat femelle et a révélé un double contrôle pré-traductionnel et traductionnel. Finalement, l'ADN complémentaire du récepteur humain a été isolé. Il code pour une longue forme de récepteur (598 a.a.) comparativement à celle du foie de rat (291 a.a.). Les deux formes présentent des régions d'identité marquée avec le récepteur de l'hormone de croissance. Ceci suggère que les gènes des récepteurs de la prolactine et de l'hormone de croissance forment une famille.

ABSTRACT

To elucidate the structure and the function of the prolactin receptor, I have used molecular biological techniques. After unsuccessful screening of expression cDNA libraries with poly- and monoclonal antibodies, synthetic oligonucleotide probes, deduced from tryptic fragment sequences of the purified receptor, were used to isolate the rat liver prolactin receptor cDNA. Using this cDNA,the regulation of prolactin receptor expression by estrogen treatment and during ontogenesis was studied in female rat liver and revealed both pre-translational and translational control of the receptor. Finally, the human prolactin receptor cDNA was isolated. It encodes a long form of receptor (598 aa) compared to the rat liver receptor (291 aa). Both forms present regions of strong identity with the growth hormone receptor, suggesting that prolactin and growth hormone receptor genes form a family.

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LIST OF ABBREVIATIONS

MEMBRANE RECEPTORS

ANP-C:	Atrial natriuretic peptide clearance receptor
ASGP-R:	Asyaloglycoprotein receptor
CD M6P-R:	Cation-dependent mannose-6-phosphate receptor
CSF-R:	Colony-stimulating factor receptor
DHP-Ca^{2+} channel:	Dihydropyridine-sensitive calcium channel molecule
EGF-R:	Epidermal growth factor receptor
GABA-R:	γ-Aminobutyric acid receptor
GC/ANP-R:	Guanylate cyclase/atrial natriuretic peptide receptor
GH-R:	Growth hormone receptor
GT:	Glucose transporter
HER 2:	Human erb-B-related gene
5-HT-R:	5-hydroxytryptamine receptor
IFN-γ-R:	Interferon receptor
IGF-I-R:	Insulin growth factor-I receptor
IGF-II-R:	Insulin growth factor-II receptor
IL-I-R:	Interleukin-1 receptor
IL-2-R:	Interleukin-2 receptor
IL-6-R:	Interleukin-6 receptor
I-R	Insulin receptor
IRGT:	Insulin-regulable glucose transporter
LDL-R:	Low Density Lipoprotein receptor
LEC-CAM:	Lectin, EGF, complement - cellular adhesion molecule
LRP:	LDL receptor-related protein
mACh-R:	Muscarinic acetylcholine receptor

M6P-R: Mannose-6-Phosphate receptor

nACh-R: Nicotinic acetylcholine receptor

NGF-R: Nerve growth factor receptor

PDGF-R: Platelet-derived growth factor receptor

PRL-R: Prolactin receptor

RTK: Receptor tyrosine kinase

REAGENTS

BSA: Bovine serum albumin

CHAPS: 3-[(3-cholamidopropyl)-dimethylammonio]-1-propane sulfonate

DMSO: Dimethylsulfoxide

DTT: Dithiothreitol

EDTA: Ethylenediaminetetra-acetic acid

PAGE: Polyacrylamide gel electrophorsis

PMSF: Phenyl methyl sulfonyl fluoride

SDS: Sodium dodecyl sulfate

TRIS: Tris-(hydroxymethyl)-aminomethane

MISCELLANEOUS

a.a.: Amino acid

ATP: Adenosine 5'-triphosphate

b.p. Base pair

C-: Carboxyl-

cAMP: Cyclic adenosine 3':5'-monophosphate

cDNA: Complementary deoxyribonucleic acid

cGMP:	Cyclic guanosine 3':5'-monophosphate
DAG:	1,2-diacylglycerol
Ig:	Immunoglobulin
K_a:	Affinity constant
kb:	Kilobase
kD:	Kilodalton
mAb:	Monoclonal antibody
Mol. wt:	Molecular weight
M_r:	Relative molecular mass
mRNA:	Messenger ribonucleic acid
N-:	Amino-
ODC:	Ornithine decarboxylase
Poly (A)+:	Polyadenylated
Poly Ab:	Polyclonal antibody
TK:	Tyrosine kinase
TPA	Tumor-promoting agent

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MANUSCRIPTS AND AUTHORSHIP INSTRUCTIONS

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It is acceptable for thesis to include as chapters authentic copies of papers already published, provided these are duplicated clearly on regulation thesis stationery and bound as an integral part of the thesis. Photographs or other materials which do not duplicate well must be included in their original form. In such instances, connecting texts are mandatory and supplementary explanatory material is almost always necessary.

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PREFACE

In accordance with guidelines concerning thesis preparation, the candidate has taken the option of writing the experimental part of the thesis (Chapters 4 to 7 inclusively) in the form of original papers. Except for Chapter 4, the other chapters represent manuscripts published or in press. Thus each of these chapters bears its own Summary, Introduction, Materials and Methods, Results, Discussion and References section. A preface to each chapter has been added to provide logical bridges between the different manuscripts. A general Abstract, a full Introduction and literature review and a general Discussion have also been included. The manuscripts published or in press are as follows:

Chapter 5: Boutin J-M, Jolicoeur C, Okamura H, Gagnon J, Edery M, Shirota M, Banville D, Dusanter-Fourt I, Djiane J, Kelly P.A. (1988) Cloning and expression of the rat prolactin receptor, a member of the growth hormone/prolactin receptor gene family. Cell 53: 69-77

Chapter 6: Jolicoeur C, Boutin J-M, Okamura H, Raguet S, Djiane J, Kelly PA (1989) Multiple regulation of prolactin receptor gene expression in rat liver. Molecular Endocrinol. In press.

Chapter 7: Boutin J-M, Edery M, Shirota M, Jolicoeur C, Lesueur L, Ali S, Gould D, Djiane J, Kelly PA (1989) Identification of a cDNA encoding a long form of PRL receptor in human hepatoma and breast cancer cells. Molecular Endocrinol. In press.

In Chapter 4, which constitutes unpublished observations, the candidate was responsible of all experiments except for the Northern blot analysis of figures 4 and 8 and for the cDNA synthesis and library construction from the enriched mRNA. These parts were done by Dr. Jolicoeur. The candidate prepared the text and figures of the manuscript.

In Chapter 5, the candidate was responsible for the enrichment of mRNA by immunopurification of polysomes, the preparation of the mRNA for the Clontech Library, screening this library, translation of mRNA in frog oocytes and stable transfection studies. He also sequenced the E1, and E2 clones and helped Dr. Shirota and Dr. Banville in the sequencing of the F3 clone. Dr. Jolicoeur completed the construction of the enriched library and screened it with the oligonucleotide probes. She also did the Northern blot of Figure 5. Dr. Okamura and Dr. Gagnon were responsible of the purification of the PRL-R to homogeneity. Finally our collaborators in France (Dr. Edery, Dusanter-Fourt and Djiane) did the transient transfection studies.

In Chapter 6, the candidate helped Dr. Jolicoeur with Northern blot analysis. Dr. Jolicoeur prepared the manuscript and the candidate revised it. The mRNA was prepared by Miss Raguet and the PRL-R binding studies were done by Dr. Okamura.

In Chapter 7, the candidate was responsible of all the experiments except for the initial screening of the human hepatoma library which was done by Dr. Edery and Miss Lesueur who obtained the HI cDNA clone. The candidate was helped by Dr. Shirota for sequencing, by Dr. Jolicoeur who prepared the T-47D library, by Mr. Gould who prepared the T-47D cells and

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PART I

INTRODUCTION AND REVIEW OF LITERATURE

CHAPTER 1

TECHNIQUES OF MEMBRANE RECEPTOR CLONING

The development and function of multicellular organisms depends on the ability of cells to communicate with each other. Three ways of communication are used by the cells: (1) indirect signalling by secreted chemicals (local chemical mediators, hormones or neurotransmitters); (2) direct signalling by plasma membrane-bound molecules, and (3) direct signalling via gap junctions. On the other hand, to respond to these signals, cells must possess high affinity receptors that are very specific. Most receptors are cell surface proteins since the extracellular signal cannot penetrate the cell. The main exceptions are intracellular receptors that bind the steroid and thyroid hormones, since these signalling molecules are lipid-soluble and can pass through the cell membrane. As prolactin is a water-soluble hormone, we will focus the present review on cell surface receptors. The steroid and thyroid hormone receptor superfamily has been the subject of an excellent recent review (Evans, 1988).

Our knowledge of the structure and function of the receptors has literally exploded during the last five years due to the use of recombinant DNA technology. Indeed, receptors are proteins that are difficult to purify or characterize due to their low level of abundance. However, the isolation of receptor cDNAs has permitted the determination of the primary structure of more than a hundred receptors (see table I). It is interesting to note, however, that when we undertook the isolation of the PRL receptor cDNA in 1984, the field was in its infancy and only the structure of the following eight membrane receptors had been obtained: the EGF receptor (Lin et al., 1984; Ullrich et al., 1984; Xu et al., 1984); the LDL receptor (Yamamoto et al., 1984); the

transferrin receptor (Schneider et al., 1984; McLelland et al., 1984); the IL-2 receptor (Leonard et al., 1984; Nikaido et al., 1984; Cosman et al., 1984); the nicotinic acetylcholine receptor (Noda et al., 1982, 1983a, 1983b; Ballivet et al., 1982); the Na channel EI (Noda et al., 1984); the Rhodopsin (Nathans and Hogness, 1984); and the T cell receptor (reviewed in Davis and Bjorkman, 1988). The first intracellular receptor to be isolated was the glucocorticoid receptor in 1985 (Hollenberg et al., 1985), which opened the field of the steroid and thyroid hormone receptor superfamily. The Nobel prize in Medicine was awarded to two of these groups: Brown and Goldstein in 1985 for the LDL receptor and Tonegawa in 1987 for the T cell receptor.

The isolation of receptor cDNAs has been achieved in many cases in the absence of any protein purification by using innovative techniques, taking advantage of known biological properties of the receptor and using tricks of molecular biology. In fact the methodology used to clone receptor cDNAs can be divided into two general classes: (1) the nucleic acid hybridization techniques, and (2) the gene expression techniques.

1.1. NUCLEIC ACID HYBRIDIZATION

1.1.1. Partial amino acid sequences

The classical method that has been used most successfully to isolate the majority of the cDNA clones requires purification of the receptor to homogeneity. Peptide fragments are obtained by tryptic or cyanogen bromide digestion and the amino acid sequence of several peptides is obtained. Complementary oligonucleotides can be synthesized either as a mixture of short oligonucleotides (~14-25 nucleotides) representing most

or all the mRNA sequences that may code for the corresponding stretch of the peptide or as a single long oligonucleotide (~25-60 nucleotides), where the bases are chosen in accordance to the published frequency tables of codon usage for a particular species. The oligonucleotides are labelled and used as probes to screen cDNA libraries. The libraries are usually made from mRNAs extracted from tissues or cells enriched in the receptor of interest and size-selected to obtain full length cDNAs. λgt10 is the vector most often used, since it is very efficient (Huynh et al., 1985). Two more rounds of screening are usually necessary to obtain isolation of the positive cDNA clones. The advantages of this method are its reliability and its specificity. Its main disadvantage is the difficulty of obtaining a pure receptor and a usable peptide fragment for which the degeneracy of the genetic code is not too great.

1.1.2 Homologous cDNA or oligonucleotides

It is now clear that receptors can be grouped into different families with common sequences or structures. By using complementary synthetic oligonucleotides corresponding to those regions, it is possible to screen genomic or cDNA libraries at different stringencies to obtain hybridizing clones representing different members of a receptor family. A typical example of such a method is the recent isolation of the androgen receptor using an oligonucleotide corresponding to the highly conserved DNA binding region of other steroid hormone receptors (Chang et al., 1988; Lubanu et al., 1988). Alternatively, a fragment or an entire homologous cDNA clone can be used as a probe at low stringency. A β_2 adrenergic cDNA probe permitted the isolation of the serotonin 5-HT_{1A}

receptor cDNA (Kobilka et al., 1987) and the D_2 dopamine receptor cDNA (Bunzow et al., 1988). Moreover, receptor cDNA or oligonucleotide probes are frequently used to obtain the same receptor cDNA but in a different species by using low-stringency conditions. The advantage of this technique is the ease with which the probes can be obtained, but the problem is the divergence in sequences found between species evolutionarily distant, or between members of the same receptor family, rendering the cross-hybridization difficult.

1.1.3. Differential hybridization

In this method a cDNA library, usually made with a plasmid vector, is screened on duplicates with two different probes: the "positive" cDNA probe made from mRNAs containing the receptor mRNA and the "negative" cDNA probe made from mRNAs in which the receptor mRNA is absent. After hybridization with labelled probes, the autoradiographs of both replicates are matched in search for signals reacting only with the positive probe and not with the negative probe. The positive clones are picked, grown and rescreened. This technique was used to clone the cDNA encoding the γ subunit of Torpedo acetylcholine receptor (Ballivet et al., 1982). In this case, the positive probe was prepared from Torpedo electric organ 18-20S poly(A)+ RNA, enriched for the subunit of interest, while the negative probe was prepared from poly(A)+ RNA from Torpedo brain, devoid of this protein. For cloning the transferrin receptor, Schneider et al. (1983) enriched the mRNA used for the positive probe by immunopurification of polysomes and used the polysomal RNA which failed to bind to the protein-A column as template for the negative probe.

Although this method is attractive for receptors for which no purification has been achieved or no antibody is available, it remains cumbersome and very difficult to apply for low abundance receptors, where screening of a large number of clones is necessary to find a positive clone.

1.1.4. cDNA subtraction

The principles of this method are the same as for the preceding technique except that the differential hybridization is made at the level of the probe instead of at the level of the screening. A positive cDNA probe is prepared and hybridized with an excess of the negative poly(A)$^+$ mRNA. The single-stranded, subtracted cDNA is separated from the non-specific hybrid double-stranded cDNA by using hydroxyapatite chromatography, and used to screen a cDNA library. The isolation of the T-cell receptor followed this experimental strategy, exploiting the fact that B and T-cells differ in only a small fraction of their gene expression (Hedrick et al., 1984; Chien et al., 1984). The cDNA probe made from T-cell mRNA was hybridized and hydroxyapatite-selected with B-cell mRNA. Many runs of subtraction are usually necessary to make specific probes.

The advantage of this technique is the same as for the differential hybridization and allows screening of a large number of clones in a library. However, its success depends on the specificity of the subtracted cDNA probe. In other words, the perfect match between the "positive" and the "negative" mRNAs except for the receptor mRNA which should be present only in the "positive" RNA.

1.1.5. Chromosome walking

This is a well-known technique which has been applied only recently in the field of receptors to isolate the K^+ channel cDNA (Kambet et al., 1987; Bauman et al., 1987; Papazian et al., 1987). In fact, it is the genetics of Drosophila melanogaster that allowed this approach. The Shaker locus, which was known to be important for K^+ channel function was first cytologically localized on a region on the X chromosome (Tonouye et al., 1981). A cDNA clone, unrelated to Shaker but which hybridized in this region, was used to begin a chromosomal walk, that is, to obtain from libraries of genomic DNA, a set of overlapping clones containing DNA from this region. The walk was oriented by in situ hybridization of cloned DNA to polytene chromosomes. Rearrangements in Shaker mutants have been mapped to a sequence of the genome by Southern blot analysis and in situ hybridization. Coding sequences were then identified by using the genomic DNA of this segment to screen cDNA libraries and to isolate cDNA clones for the K^+ channel. This is a very useful technique to detect a low-abundance receptor but limited to more primitive organisms where the genetic studies are well-detailed.

1.2. EXPRESSION SYSTEMS

1.2.1. Immunoscreening

Mainly two E.Coli expression vectors have been used for this purpose: the plasmid pUC8 (Helfman et al., 1983) and the phage λgt11 (Huynh et al., 1985) which has supplanted the former for its high efficiency. Both vectors utilize the promoter of the Lac Z gene which is a strong E.coli promoter. In the case of λgt11, a unique EcoRI cloning

site is located at the 3'end of the Lac Z gene. When a library is made and plated on E.coli, cDNA inserts cloned into this site in the proper orientation and reading frame, result in the production of exogenous protein fused to β-galactosidase. The antigens produced are transferred onto nitrocellulose filters and then probed with antibody to detect the desired recombinant. This technique has been used successfully for at least a dozen of receptors (see Table I) and, remarkably, four of them by the group of Lodish (Spiess et al., 1985; Spiess and Lodish, 1985; Mueckler et al., 1985; Kopito and Lodish, 1985). This approach is rapid and useful for isolating cDNAs corresponding to receptors for which good antibodies are available. However, it is dependent on the specificity of the antibody and its capacity to recognize a fusion β-gal-receptor protein. Moreover, its efficiency is only one-sixth that of the hybridization techniques, since to be expressed, the cDNA must be in the right orientation (one-half) and in the right coding frame (one third).

1.2.2. Gene transfer

This method is now well-established and has been the subject of excellent reviews (Ruddle, 1985; Kavathas, 1985). It has been especially popular for T-cell surface antigens, for which specific monoclonal antibodies were available (Kavathas and Herzenberg, 1983; Kavathas et al., 1984; Littman et al., 1985; Maddon et al., 1985). However, only two hormone receptors (transferrin and NGF receptors) have been isolated using this technique. Typically mouse L cells deficient in thymidine kinase activity are used as recipients for DNA-mediated gene transfer. These cells are cotransformed with human, high-molecular weight DNA, and the cloned herpes thymidine kinase gene. The transformants are first

selected for the expression of donor TK activity. This population is enriched from cells that have taken up large amounts of human DNA. The TK^+ transformants are screened for the expression of the receptor of interest. In all the cases, except for the Na^+/H^+ antiporter, monoclonal antibodies were used in combination with an immunological rosette assay or with a fluorescence-activated cell sorter (FACS) to isolate the positive transformants. In the case of the Na^+/H^+ antiporter, Sardet et al. (1989) selected the positive cells by an acid-loading test. An additional round of transformation and selection is necessary to purge the human DNA fragments unrelated to the receptor of interest from the cells. A genomic DNA library is then made from the secondary transformant DNA and probed with human middle repetitive DNA (ALU), in order to locate the human DNA fragments encoding the receptor. These genomic fragments can be used as probes to screen a cDNA library to isolate the receptor cDNA. Alternatively, the cDNA library can be screened with a subtracted cDNA probe that is produced by using the DNA made from the RNA of the secondary transformants hybridized to an excess of mRNA of the non-transformant cells. The advantages of this method are the direct isolation of the receptor genomic sequences and the generation of expression transformants that permit study of structure-function relationships at an early time of the cloning process. On the other hand, the application of this approach is possibly limited by the size of the gene and the possibility that the simultaneous transfer of more than one gene is required to ensure the expression of the selected phenotype.

1.2.3. Direct expression in Xenopus oocytes

A new method was recently devised to avoid the use of antibodies or oligonucleotide probes to clone receptor cDNAs (Levitan, 1988). In this approach, poly $(A)^+$ mRNA is size-fractionated and used to make a cDNA library in a plasmid or phage containing a RNA polymerase promoter. This allows the synthesis of RNA in vitro from the cDNAs. Synthetic RNA derived from pools of clones are injected into oocytes that are assayed for sensitivity to the ligand, usually by voltage-clamp recording of ligand activated currents. A pool of cDNA clones giving a positive response is identified and progressively subdivided into smaller pools (sib selection) until a single positive clone is obtained. This method has been successfully applied for two neurotransmitter receptors and the Na^+/glucose co-transporter (Masu et al., 1987; Julius et al., 1988; Hediger et al., 1987). However, not all receptors can be easily cloned by this approach, since the oocyte does not allow expression of all receptors. Another problem arises with multi-subunit receptors and receptors with a long RNA size, for which expression would be impractical in this system.

1.2.4. Direct expression in COS cells

This method combines some advantages of the two preceding ones. It appeared in 1987 (Seed 1987; Seed and Aruffo, 1987), and is based on a vector designed to direct high-level expression of cloned cDNA molecules in mammalian cells and also used to transform E. coli. A cDNA library is constructed using this vector. The library plasmid DNA is transfected transiently in COS-7 cells. Cells expressing the receptor of interest are usually selected with monoclonal antibodies and selected by FACS or

panning. The episomal DNA of the positive transfectants are recovered, expanded in bacteria, subjected to three or more rounds of expression and selected to obtain a pure cDNA clone. This has been successfully used for some receptors of the immunoglobulin superfamily (Seed and Aruffo, 1987; Yamasaki et al., 1988; Sims et al., 1989) as well as for some adhesion proteins (Bevilacqua et al., 1989; Stamenkovic et al., 1989). A major convenience of this method and the preceding one is the recovery of the DNA of interest in a form that contains the necessary sequences for surface expression. Moreover, the use of a high-efficiency cDNA expression vector allows the receptor to accumulate at high levels on cell surface, compared to the gene transfer technique, where the genomic sequences might fail to generate sufficient levels of protein. Compared to the expression in Xenopus oocytes, expression in COS cells can have wider applications, but has the same limitations regarding the expression of multi-subunit receptors or high molecular weight receptors.

TABLE - 1: MEMBRANE RECEPTOR FAMILIES

A] SINGLE MEMBRANE-SPANNING RECEPTORS

Receptor	Species	Source	Cloning strategy	Protein M_r	Protein (a.a.)	No. of mRNAs	Chrom.	References
1) Receptor Tyrosine Kinases (RTK)								
SUBCLASS I								
EGF/TGF-α-R	Human	A431	Immunoscreening[5]	-	-	3	7	Lin et al., (1984)
	Human	A431	Oligo[1]	131,360	(1186)	10	-	Ullrich et al., (1984)
	Human	A431	Homology[3]	-	-	6	-	Xu et al., (1984)
HER2/neu	Human	Placenta	Homology[3]	137,828[10]	(1234)	4	17	Coussens et al., (1985)
SUBCLASS II								
Insulin-R	Human	Placenta	Oligo[2]	153,917[11] (α:735) (β:620)		5	19	Ebina et al., (1985)
	Human	Placenta	Oligo[1]	152,784[11] (α:719) (β:620)		6	-	Ullrich et al., (1985)
IGF-1-R	Human	Placenta	Oligo[1]	151,869[11] (α:706) (β:627)		2	15	Ullrich et al., (1986)
SUBCLASS III								
PDGF-R								
β-type	Mouse	NR6	Oligo[2]	120,000	(1067)	1	5	Yarden et al., (1986)
	Human	Fibroblast	Homology[4]		(1106)[10]	2	-	Gronwald et al., (1988)
α-type	Human	Fibroblast brain	Homology[3]	120,000	(1065)	1	4	Matsui et al., (1989)
c-fms/CSF-1-R	Human	Placenta	Homology[3]	106,000[10]	(972)[10]	1	5	Sherr et al., (1985)
c-Kit	Human	Placenta	Homology[4]	109,740[10]	(976)[10]	1	4	Yarden et al., (1987)
2) Single membrane-spanning transporters								
LDL-R	Bovine	Adrenal	Oligo[2]	Incomplete	(264)	1	-	Russel et al., (1983, 1984)
	Human	Fetal adrenal	Homology[3] & Oligo[2]	93,102	(839)	1	19	Yamamoto et al., (1984)
LRP	Human	Liver	Homology[4]	503,000	(4525)	1	-	Herz et al., (1988)

Receptor	Species	Source	Cloning strategy	Protein M_r	(a.a.)	No. of mRNAs	Chrom.	References
IGF-2/M6P-R	Human	Hep G2	Oligo[1]	274,353[10]	(2491)[10]	1	-	Morgan et al., (1987)
	Rat	Placenta	Oligo	Incomplete	(2060)	-	-	MacDonald et al., (1988)
	Human	Placenta	Immunoscreening[4]	270,000	(2451)	1	-	Oshima et al., (1988)
	Bovine	Calf Liver	Oligo[1]	269,953	(2455)	-	-	Lobel et al., (1987,1988)
CDM6P-R	Bovine	Fetal Liver	Oligo[1]	28,746	(257)	3	-	Dahms et al., (1987)
	Human	Placenta	Immunoscreening[4] & Oligo[2]	27,910	(257)	1	12	Pohlmann et al., (1987)
Transferrin-R	Human	Placenta	Differential hybridization	84,910	(760)	-	-	Schneider et al., (1983, 1984).
	Human	Fibroblasts	Gene Transfer		(760)	1	3	Kühn et al., (1984) McClelland et al., (1984)
ASGP-R (H1)	Human	Hep G2	Immunoscreening[4]	33,122	(291)	1	-	Spiess et al., (1985)
(H2)	Human	Hep G2	Immunoscreening[4]		(311)	1	-	Spiess and Lodish, (1985)
3) Miscellaneous								
IL-2-Rα	Human	Hut-102B2	Oligo[2]	28,437	(251)	2	10	Leonard et al., (1984, 1985)
	Human	MT-1	Oligo[2]	28,463	(251)	2	-	Nikaido et al., (1984)
	Human	Hut-102	Oligo[1]	28,428	(251)	2	-	Cosman et al., (1984)
HGF	Human	A-875	Gene Transfer	49,689	(399)	1	17	Chao et al., (1986) Johnson et al., (1986)
	Rat	PCNA	Gene Transfer	42,478	(396)	-	-	Radeke et al., (1987)
IFN-γR	Human	Raji	Immunoscreening[4]	54,000	(472)	1	6	Aguet et al., (1988)
ANP-C	Bovine	BASM	Oligo[1+2]	55,701	(496)	>4	-	Fuller et al., (1988)
GC/ANP-R	Rat	Brain	Homology[3]	115,852	(1028)	-	-	Chinkers et al., (1989)
GH-R	Rabbit	Liver	Oligo[1]	70,000	(620)	1	-	Leung et al., (1987)
	Human	Liver	Homology[3]	70,000	(620)	1	-	
4) Ig Superfamily[12]								
Lymphokine Receptors								
IL-1-R	Mouse	EL46.1C10	Expression[9]	64,598[10]	(576)[10]	1	-	Sims et al., (1988)
IL-6-R	Human	HK-like	Expression[9]	-	(467)[10]	1	-	Yamasaki et al.,(1988)

Receptor	Species	Source	Cloning strategy	Protein Mr	Protein (a.a.)	No. of mRNAs	Chrom.	References
Viral Receptors	Human	Several	Gene Transfer	Variable	-	-	-	White and Littman (1989)
5) Adhesion Proteins								
I- Integrins	Several	Several	Various	Heterodimers (α/β)		-	-	Hynes (1987)
II-LEC-CAM	Several	Several	Oligo[1,2] Expression[3]	-	-	-	-	Reviewed by Stoolman, (1989)
III-Hermes/CD44	Several	Several	Expression[3] Immunoscreening[4]	-	-	-	-	Reviewed by Stoolman, (1989)

B) MULTIPLE MEMBRANE-SPANNING RECEPTORS

Receptor	Species	Source	Cloning strategy	Protein M_r	(a.a.)	No. of mRNAs	Chrom.	References
1) Seven-helix receptors								
Adrenergic receptors								
α_1	Hamster	DDT, MF-2	Oligo[1]	56,000	(515)	1	-	Cottecchia et al., (1988)
α_2	A Human	Platelet	Oligo[1]	-	(450)	-	10	Kobilka et al., (1986b)
	B Human	Kidney	Homology[3]	-	(461)	-	4	Regan et al., (1988)
β_1	Human	Placenta	Homology[3]	51,220	(477)	1	-	Frielle et al., (1987)
	Turkey	Fetal RBCs	Oligo[1]	54,078	(483)	-	-	Yarden et al., (1986b)
β^2	Hamster	DDT1-MF2	Oligo[1]	46,000	(418)	-	-	Dixon et al., (1986)
	Human	Placenta	Homology[3]	46,000	(413)	1	5	Kobilka et al., (1987a)
Rhodopsin	Bovine	Retina	Oligo[1]	-	(348)	1	-	Nathans and Hogness, (1983)
	Human	Genomic	Homology[3]		(348)	-	3	Nathans and Hogness, (1984)
Blue Pigment	Human	Genomic	Homology[3]	-	(348)	-	7	Nathans et al., (1986)
Green Pigment	Human	Genomic	Homology[3]	-	(348)	-	X	Nathans et al., (1986)
Red Pigment	Human	Genomic	Homology[3]	-	(348)	-	X	Nathans et al., (1986)
Muscarinic Acetylcholine Receptors (mACh-R)								
M1	Porcine	Cerebrum	Oligo[2]	51,416	(460)	1	-	Kubo et al., (1986a)
	Rat	Brain	Homology[4]		(458)	-	-	Stein et al., (1988)
	Human	Genomic	Homology[4]	-	(460)	1	-	Peralta et al., (1987b)
	Rat	Cerebrum	Homology[4]	-	(458)	1	-	Bonner et al., (1987)
M2	Porcine	Heart	Oligo[2]	51,670	(466)	1	-	Kubo et al., (1986b)
	Rat	Heart	Homology[4]	51,543	(466)	-	-	Gocayne et al., (1987)
	Porcine	Heart	Oligo[1]	51,700	(466)	-	-	Peralta, (1987a)
	Human	Genomic	Homology[4]	-	(466)	1	-	Peralta et al., (1987b)
	Human	Genomic	Homology[4]	-	(466)	-	-	Bonner et al., (1987)
M3	Rat	Cerebrum	Homology[4]	-	(589)	1	-	Bonner et al., (1987)
	Human	Genomic	Homology[4]	53,049	(479)	1	-	Peralta et al., (1987b)

Receptor	Species	Source	Cloning strategy	Protein M_r	Protein (a.a.)	No. of mRNAs	Chrom.	References
Muscarinic Acetylcholine Receptors (mACh-R)								
M4	Human	Genomic	Homology[4]	66,127	(590)	1	-	Peralta et al., (1987b)
	Rat	Cerebrum	Homology[4]	-	(478)	1	-	Bonner et al., (1987)
	Human	Genomic	Homology[4]	-	(478)	-	-	Bonner et al., (1987)
M5	Rat	Genomic	Homology[3]	-	(532)	-	-	Bonner et al., (1988)
	Human	Genomic	Homology[3]	-	(531)	-	-	Bonner et al., (1988)
Serotinine receptors								
5-HT_{1A}	Human	Genomic	Homology[3]	-	(421)	1	5	Kobilka et al., (1987c) Fargin et al., (1988)
5-HT_{1C}	Mouse	Choroid plexus Papilloma	Expression[8]	Incomplete		1	-	Lübbert et al., (1987)
	Rat	Choroid plexus	Expression[8]	51,899	(460)	1	-	Julius et al., (1988)
5-HT-2	Rat	Forebrain	Homology[4]	-	(449)	-	-	Pritchett et al., (1988a)
Substance K receptor	Bovine	Stomach	Expression[8]	43,066	(384)	-	-	Masu et al., (1987)
D_2 Dopamine receptor	Rat	Brain	Homology[3]	47,064	(415)	1	-	Bunzow et al., (1988)
Angiotensin receptor	Human	Genomic	Homology[3]	-	(325)	1	-	Young et al., (1986) Jackson et al., (1988)
2) Ligand-dependent ion channels								
Nicotinic acetylcholine receptors (nACh-R)								
Muscle nACh-R								
$\alpha 1$	Torpedo Californica	Electroplax	Oligo[2]	50,116	(437)	1	-	Noda et al., (1982)
β_1	Torpedo Californica	Electroplax	Oligo[2]	53,681	(469)	1	-	Noda et al., (1983a)
γ	Torpedo Californica	Electroplax	Differential hybridization	-	-	1	-	Ballivet et al., (1982)
	Torpedo Californica	Electroplax	Oligo[2]	56,279	(489)	-	-	Noda et al., (1983b)

Receptor	Species	Source	Cloning strategy	Protein M_r (a.a.)	No. of mRNAs	Chrom.	References
Muscle nACh-R							
δ	Torpedo Californica	Electroplax	Oligo[2]	57,565 (501)	1	-	Noda et al., (1983a)
Nerve nACh-R							
α2	Rat	Brain	Homology[3]	55,480 (484)	-	-	Wada et al., (1988)
α3	Rat	PC12	Homology[3]	54,780 (474)	2	-	Boulter et al., (1986)
α4	Rat	Hypothalamus & Hyppocampus	Homology[3]	67,124	-	-	Goldman et al., (1987)
β2	Rat	PC12	Homology[3]	57,321 (503)	2	-	Deneris et al., (1988)
β3	Rat	Brain	Homology[3]	53,300[19] (464)[19]	-	-	Deneris et al., (1989)
$GABA_A$ receptors							
α_1	Bovine	Brain	Oligo	48,800 (429)	1	-	Schofield et al., (1987)
α_2	Bovine	Brain	Homology[4]	48,000 (423)	1	-	Levitan et al., (1988)
α3	Bovine	Brain	Homology[4]	52,000 (464)	1	-	Levitan et al., (1988)
β	Bovine	Brain	Oligo	51,400 (449)	-	-	Schofield et al., (1987)
γ_2	Human	Fetal Brain	Homology[4]	48,000 (428)	-	-	Pritchett et al., (1989)
Glycine receptor							
Strychnine-binding subunit	Rat	Spinal Cord	Oligo[2]	48,383 (421)	5	-	Grenningloh et al., (1987)

Receptor	Species	Source	Cloning strategy	Protein M_r (a.a.)	No. of mRNAs	Chrom.	References
3) Voltage-dependent ion channels							
Na channel							
BI	Electrophorus Electricus	Electroplax	Immunoscreening[5]	208,321 (1820)	1	-	Noda et al., (1984)
RI	Rat	Brain	Homology[3]	228,758 (2009)	1	-	Noda et al., (1986)
RII	Rat	Brain	Homology[3]	227,840 (2005)	1	-	Noda et al., (1986)
RIII	Rat	Brain	Homology[3]	221,375 (1951)	2	-	Kayano et al., (1988)
DHP-sensitive Ca channel (L-type)							
α1	Rabbit	Skeletal Muscle	Oligo[1]	212,018 (1873)	-	-	Tanabe et al., (1987)
	Rabbit	Skeletal Muscle	Immunoscreening[7]	- (1873)	1	-	Ellis et al., (1988)
α2	Rabbit	Skeletal Muscle	Immunoscreening[6]	125,018[10](1106)[10]	2	-	Ellis et al., (1988)
K channel							
Type A	Drosophila	Head	Chromosome Walking	70,200 (616)	5	X	Kamb et al., (1987) Papazian et al., (1987) Baumann et al., (1987) Tempel et al., (1987)
	Mouse	Brain	Homology[3]	56,400 (495)	1	-	Tempel et al., (1988)
	Rat	Brain	Homology[4]	56,400 (495)	-	-	Christie et al., (1989)
4) Multiple membrane-spanning transporters							
Glucose transporters							
Brain/HepG2 GT	Human	Hep G2	Immunoscreening[6]	54,117 (492)	1	1	Mueckler et al., (1985)
	Rat	Brain	Immunoscreening[6]	56,133 (492)	2	-	Birnbaum et al., (1986)
Liver GT	Human	Liver	Homology[3]	57,499 (524)	5	3	Fukomoto et al., (1988)
	Rat	Liver	Homology[3]	57,000 (522)	2	-	Thorens et al., (1988)

Receptor	Species	Source	Cloning strategy	Protein M_r (a.a.)	No. of mRNAs	Chrom.	References
Glucose transporters							
GT-like	Human	Fetal Skeletal muscle	Homology[3]	53,933 (496)	2	12	Kayano et al., (1988)
IRGT	Rat	Adipocyte heart	Homology[3]	54,860 (509)	1	-	James et al., (1989)
	Rat	Skeletal muscle	Homology[3]	54,832 (509)	1	-	Birnbaum, (1989) Charron et al., (1989)
Na/glucose antiporter	Rabbit	Intestinal mucosa	Expression[8]	73,080 (662)	1	-	Hediger et al., (1987)
Anion antiporter							
Band 3	Mouse	Spleen	Immunoscreening[4]	103,000 (929)	-	-	Kopito and Lodish, (1985)
Cation antiporter							
Na^+/H^+ antiporter	Human	Genomic	Gene Transfer	99,354 (894)	1	-	Sardet et al., (1989)
5) Ion Pumps							
$(Na^+ - K^+)$ATPase							
α	Sheep	Kidney	Oligo[2]	112,177 (1016)	-	-	Shull et al., (1985)
	Rat	Brain	Homology3	112,573 (1018)	1	-	Shull et al., (1986a)
α(+)	Rat	Brain	Homology[3]	111,736 (1015)	1	-	Shull et al., (1986a)
αIII	Rat	Brain	Homology[3]	111,727 (1013)	1	-	Shull et al., (1986a)
β	Sheep	Kidney	Oligo[2]	34,937 (302)	-	-	Shull et al., (1986b)

Recepter	Species	Source	Cloning strategy	Protein M_r (a.a.)	No. of mRNAs	Chrom.	References
Ion Pumps							
$(H^+ - K^+)$ATPase							
α	Rat	Stomach	Homology[4]	114,012 (1033)	-	-	Shull and Lingrel, (1986)
Ca^{2+} ATPase							
Slow twitch	Rabbit	Muscle	Oligo[2]	109,763 (997)	2	-	MacLennan et al., (1985)
Fast twitch	Rabbit	Muscle	Homology[3]	110,331 (1001)	3	-	Brandl et al., (1986)
6) GAP Junction							
Connexin 43	Rat	Heart	Homology[3]	43,036 (382)	1	-	Beyer et al., (1987)
Connexin 32	Rat	Liver	Immunoscreening[4]	32,007 (283)	1	-	Paul (1986)
	Human	Liver	Oligo[1]	32,022	-	-	Kumar and Gilula, (1986)

FOOTNOTES

[1] Single oligonucleotide representing the best predicted sequence

[2] Mixture of oligonucleotides representing all or several possible sequences

[3] Homologous cDNA

[4] Homologous oligonucleotide

[5] Screening a library prepared with the expression vector pUC8 using polyclonal antibodies

[6] Screening a library prepared with the expression vector λgt11 using polyclonal antibodies

[7] As [6], except that monoclonal antibodies were used for screening.

[8] Direct expression system in Xenopus oocytes

[9] Direct expression system in COS cells

[10] Includes the signal peptide

[11] M_r of the proreceptor

[12] The immunoglobulin superfamily includes many other families of membrane proteins:

- immunoglobulins
- T cell receptor complex
- MHC antigens
- β_2-m associated antigens
- T cell adhesion molecules
- T subset antigens
- brain/lymphoid antigens
- immunoglobulin receptors
- neural molecules
- tumor antigen (CEA)
- Subclass III of TKR

The readers are referred to the excellent reviews of Williams and Barclay, 1988 for the immunoglobulin superfamily and Davis and Bjorkman, 1988 for the T cell receptors.

CHAPTER 2

STRUCTURE AND FUNCTION OF MEMBRANE RECEPTORS

Our view of membrane receptors has changed considerably during the last few years, following the discovery of the primary structure of many receptors. In this review, I tentatively classify them into different families according to their structure and function (See Table I). However, overlap between these classes is often present, and modifications will certainly become necessary as more receptor sequences are determined. I first divide the membrane receptors into two general groups according to the number of transmembrane region(s): single membrane-spanning and multiple membrane-spanning receptors. Single membrane-spanning receptors can be divided into at least 5 superfamilies: the receptor tyrosine kinase molecules (RTK), the transporters, the immunoglobulin superfamily, the adhesion proteins and a miscellaneous class of hormone and growth factor receptors. The multiple membrane-spanning receptors, on the other hand, include at least six superfamilies: the seven-helix receptors, the ligand dependent ion channels, the voltage dependent ion channels, the transporters, the ATPase pumps and the GAP junctions. In this Chapter, I will give a general overview of each family of membrane receptors and put more emphasis on those binding hormones, growth factors or neurotransmitters.

2.1 Single membrane-spanning receptors

2.1.1. Receptor tyrosine kinases (RTK)

The common structural characteristics of this class of receptors include: a highly glycosylated, cysteine-rich, extracellular domain which makes them protease resistant, a single transmembrane domain of 23-26 amino acids; and an intracellular portion which displays extensive

sequence homology with the protein tyrosine kinase family (Hunter and Cooper, 1985). Yarden and Ullrich (1988a) further subdivided this family into three groups of receptors according to shared structural features and homologous primary structures (see Fig. 1):

Subclass I includes the EGF receptor and the HER2/neu gene product and is characterized by two cysteine-rich sequence repeat regions within the ectodomains.

Subclass II includes the insulin and IGF-1 receptors which are heterotetrameric structures, comprising two α and two β subunits that are connected by disulfide bonds. The α chains contain the ligand binding domain and a single cysteine-rich sequence. The β chains span the membrane and carry the TK function.

Subclass III includes the two PDGF receptors α and β, the CSF-1 receptor and the c-kit gene product. Instead of the cysteine-rich repeat clusters, another type of repeat structure including cysteine residues is found in the ectodomain of these receptors. Moreover, the tyrosine kinase domain of the cytoplasmic domain is interrupted by a hydrophilic insertion sequence of 77-107 amino acids which is not found in the other classes.

It has been shown for subclasses I and II that within a specific subclass, the receptors share antigenic cross-reactivity and some binding of each other's specific ligand. The RTKs bind diverse growth factors and transduce a broad range of signals leading to cell growth and proliferation. In addition, non-proliferative metabolic and cellular effects are described for some ligands. Ligand binding activates the cytoplasmic kinase domains leading to autophosphorylation and to phosphorylation of various cellular

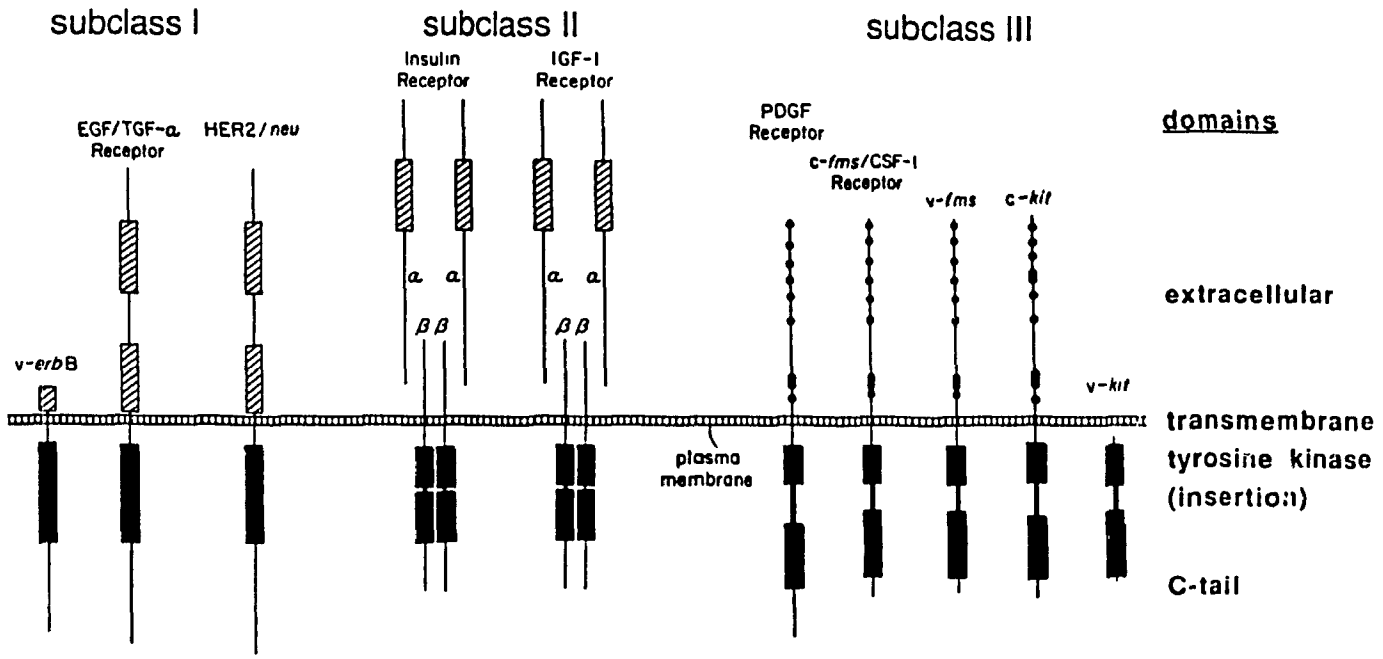

FIG. 1 Receptor tyrosine kinase family

All members and their known viral oncogenic counterparts are schematically shown. Hatched regions represent cysteine-rich repeat domains, and closed boxes indicate the tyrosine kinase domains. Closed circles represent individual cysteine residues in the extracellular portions of subclass III RTKs (Yarden and Ullrich, 1988, p. 3114).

substrates (Hunter and Cooper, 1985). The discovery of retroviral oncogenes products which are modified RTKs was a major step toward the understanding of the crucial role played by RTKs in cell regulation (see Fig. 1 and Table 1).

Structure-function relationships have been studied in detail for the EGF receptor by using mutagenesis and chimeric receptors coupled to cell transfection studies. Such studies have revealed that domain III, which is flanked by the two cysteine-rich repeat clusters, is a major ligand-binding domain (Lax et al, 1989). It has been suggested that the extracellular domain has a negative regulatory role on the kinase activity. Indeed, amino-terminal truncations identified in v-erb B (Downward et al., 1984; Ullrich et al., 1984) and v-kit (Yarden et al., 1987) lead to constitutive kinase activation. The tyrosine kinase activity can be completely abolished by replacement mutations of the lysine residue at the ATP binding site of both the EGF receptor (Chen et al., 1987; Honegger et al., 1987) and the insulin receptor (Ebina et al., 1987; McClain et al., 1987; Chen et al., 1987). Analyses of these mutants have shown that a functional tyrosine kinase is essential for the diverse biochemical effects of EGF and insulin, including rapid alterations in intracellular calcium, activation of gene transcription and the ultimate stimulatory effects on cell proliferation.

While autophosphorylation of tyrosine residues appears to play a role in activation of the receptor-associated tyrosine kinase activity for the insulin receptor (Rosen et al., 1983; Yu and Czech, 1986; Klein et al., 1986), the situation is less clear for the EGF receptor. Gill and colleagues (Bertics and Gill, 1985) have presented evidence

suggesting that autophosphorylation enhances the kinase activity, whereas Waterfield and colleagues have reported that it has no effect (Downward et al., 1985; Gullick et al., 1985). On the other hand, a negative feedback loop induced by EGF is produced by phosphorylation of the receptor at serine and/or threonine residues. The juxtamembrane threonine residue (thr 654) appears to be the major target site of protein kinase C phosphorylation and heterologous regulation (Hunter et al., 1984; Davis and Czech, 1985).

Finally, evidence is growing in favour of an allosteric receptor oligomerization model for the signal transduction of the EGF receptor and the other RTKs (Schlessinger, 1988a). In such a model, receptor-receptor interactions, stabilized by ligand binding, would bring about the activation of the tyrosine kinase by allosteric subunit interaction. Moreover, it has been demonstrated that autophosphorylation can be mediated by intermolecular cross-phosphorylation within an oligomeric receptor complex (Honegger et al., 1989). Schlessinger (1988b) further proposed a more general model that includes activation by heteroligomeric interactions between receptors with different ligand specificities or with accessory proteins which would modulate receptor activity. This is particularly interesting for the PDGF-receptor, for which two homologous forms bind the three PDGF dimers AA, AB and BB, with different affinities offering many different interactions.

Exactly how the receptor kinases ultimately transmit their signal is still uncertain. The first possibility involves a phosphorylation cascade where the activated RTK phosphorylates one or more cellular substrates. Several endogenous substrates for the insulin receptor have

been uncovered (Kahn and White, 1988; Czech et al., 1988). However, no clear, physiologically relevant, candidate has emerged. The second possibility is the mediator hypothesis where the receptor autophosphorylation is a requisite step for allosteric interaction of the receptor with an effector system which mediates signalling pathways. In the case of the I-R, this secondary effector can be a glycosyl-phosphatididyl inositol-phospholipase C that has been recently shown to generate phospholipid-derived intracellular signalling substances (Low and Saltiel, 1988; Farese, 1988; Standaert and Pollet, 1988). In the case of PDGF-R, the activated receptor is physically associated with a phosphatidylinositol kinase that is involved in the production of novel inositol phosphate second messengers mediating the mitogenic action of PDGF (Coughlin et al., 1989; Auger et al., 1989).

It is probable that many mechanisms of action are necessary to explain the various effects elicited by the binding of the growth factor receptor tyrosine kinases.

2.1.2 Single membrane-spanning transporters

This class of membrane receptors constitutes an heterogenous structural group where the functional aspect is the common denominator. Indeed the main function of these receptors is the translocation of proteins by endocytosis of the ligand-receptor complex via coated pits. The ligand is dissociated from the receptor in the acidic environment of endosomic vesicles (Tycko and Maxfield, 1982) and following segregation of receptor from the ligand in tubular vesicles (Geuze et al., 1983). These vesicles divide, delivering ligand to lysosomes and receptors back

to the cell surface (Willingham and Pastan, 1980). Except for the IGF-II/M6P-R, none of these receptors is thought to transduce a signal by binding the ligand. The determination of the primary structure of these transporters did not reveal a striking consensus sequence, but all display a very short cytoplasmic domain, which suggests that the transmembrane and the cytoplasmic domains are only involved in anchoring the receptor to the membrane. However, some subclasses can be distinguished according to the amino acid sequence. The first includes the LDL receptor and the LDL-receptor-related protein (LAP). The LDL receptor is a multidomain protein. More than one-half of the extracellular domain is composed of two types of cysteine-rich repeats (Südhof et al., 1985). One type is present in seven copies at the N-terminal and is homologous with complement. The first repeat binds calcium (Van Driel et al., 1987), and the other six bind regions of the apo-B100 and apo-E that are enriched in basic residues (Esser et al,., 1988). The second type of repeat, present in three copies, is homologous to EGF and is required for acid-dependent ligand dissociation and recycling of the receptor (Davis et al., 1987). A region near the transmembrane domain is highly glycosylated and the cytoplasmic domain contain a signal for clustering in clathrin-coated pits (Russell et al., 1984). The LAP is thought to be the apo-E receptor. It contains similar domains to the LDL receptor (see Fig. 2). These are arranged in a manner resembling four copies of the ligand binding and the EGF-precursor homologous region of the LDL-receptor. The EGF precursor, LDL receptor and LAP are all remarkably similar over a 400-amino acid region (see Fig. 2). Surprisingly, many other membrane-bound or secreted

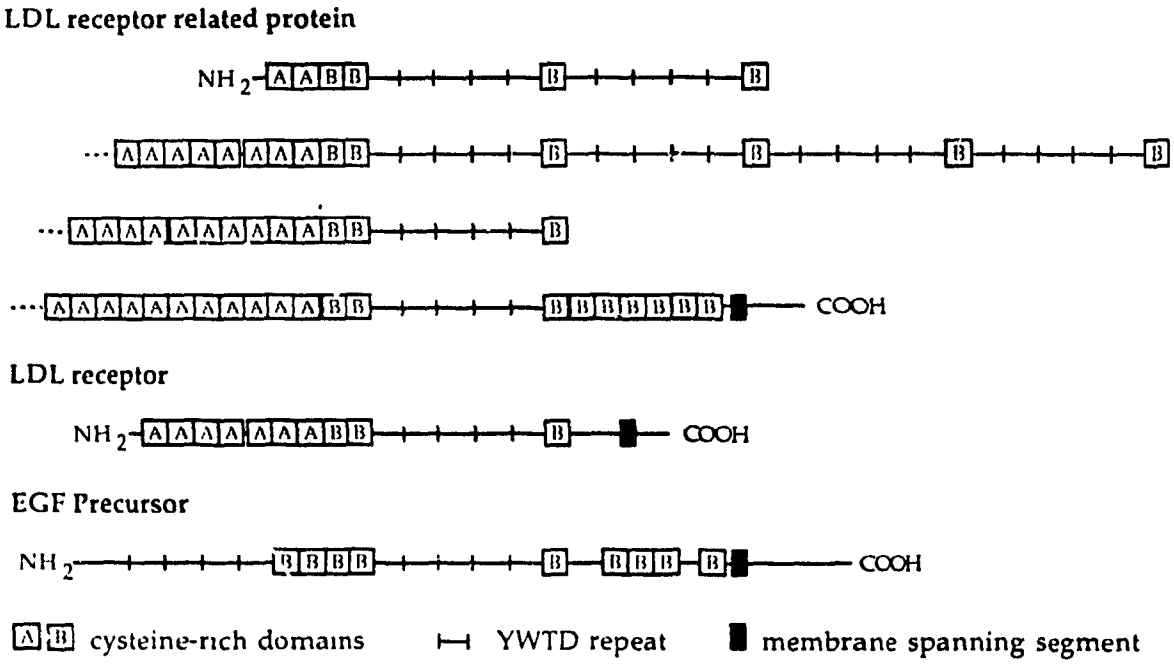

FIG. 2 Homologous domains between LRP, LDL-R and EGF precursor

The LRP has been broken into four pieces to emphasize the similarity with the other molecules. Boxes A represent the class A cysteine-rich motifs, homologous to complement, boxes B, the class B cysteine-rich motifs, homologous to EGF and the vertical lines, Tyr-Trp-Thr-Asp repeats (Herz et al., 1989, p. 4122).

glycoproteins contain EGF repeats including a subclass of adhesion proteins called lectin, EGF, complement-cellular adhesion molecules (LEC-CAM, see Table I). Although the role of the EGF domain in most cases remains unknown, some have been shown to bind directly to other proteins (Johnston et al., 1989).

A second subclass of transporters include the IGF-II/M6P receptor and the cation-dependent M6P receptor (see Table 1). Molecular cloning and biochemical studies have revealed that the IGF-II receptor and the cation-independent M6P receptor are the same protein (Morgan et al., 1987; Roth et al., 1987; Tong et al., 1988; MacDonald et al., 1987; Kiess et al., 1988). The primary structure of this receptor reveals a large ectodomain made up of fifteen repeat sequences containing a highly conserved pattern of 8 cysteine residues and a small region homologous to the collagen-binding domain of fibronectin. Despite the fact that IGF-II has a similar structure to insulin and IGF-I and can interact with their receptors, its receptor has nothing in common with the RTKs. IGF-II and M-6-P bind to separate sites on the protein and can reciprocally modulate the binding of the other ligand (Roth et al., 1987; MacDonald et al., 1987; Kiess et al., 1989; Rogers and Hammerman, 1989). Moreover, there is recent evidence suggesting that IGF-II can stimulate a response through its own receptor (Roth, 1988; Rogers and Hammerman, 1989). A serum form of this receptor representing a truncated form in the carboxyl-terminal domain has been detected recently (MacDonald et al., 1989). The CD M6P receptor is a small receptor with an extracellular domain made up of a sequence that closely resembles one repeat unit of the IGF-II/M6P receptor.

Two other transporters, the transferrin receptor and the asialoglycoprotein receptor, do not belong to a specific subclass, but each is among those rare membrane proteins with the carboxyl-terminus outside and the amino-terminus inside. Neither contain a cysteine-rich region. Two related peptides, H1 and H2, comprise the human ASGP-R, and transfection studies have shown that the co-expression of both forms is necessary to bind or internalize the ligand, suggesting that the ASGP-R is a multichain hetero-oligomer (Shia and Lodish, 1989). On the other hand, the primary structure involved in the endocytosis of the transferrin receptor has been studied by mutagenesis studies by Kühn and colleagues (Rothenberger et al., 1987; Iasopetta et al., 1988). These studies revealed that the endocytosis of the transferrin receptor requires the cytoplasmic domain but not its phosphorylation site. The cytoplasmic domain plays an active role by providing an assembly site for coated pit formation.

2.1.3 Miscellaneous

Receptors in this category do not share sequence homology with other classes of receptors nor between each other. The mechanism of signal transduction upon binding to the ligand is not known for any member of this group. However, it appears that each receptor, for which functional transfection studies in a heterologous cell system have been performed, required the presence of another cell specific subunit or cofactor to form high affinity receptor. The best characterized of these receptors is the IL-2 receptor. The high affinity receptor on T lymphocytes constitutes a bimolecular complex consisting of a 55 kD and a 70-75 kD chain.

The 55 kD component (IL-2 Rα) is the well-defined Tac antigen present in large numbers on activated T and B cells. The receptor has been cloned (see Table I) and shown to bind IL-2 with low-affinity when transfected in non-lymphoid cells devoid of the 70-75 kD chain (Greene et al., 1985; Hatakeyama et al., 1985; Nishi et al., 1988). A 70-75 kD component (IL-2-Rβ) has been identified by crosslinking studies with labelled ligand and found to be expressed in limited numbers on both resting and activated T-cells (Sharon et al., 1986; Tsudo et al., 1986). Alone IL-2-Rβ binds IL-2 with intermediate affinity but interaction with the IL-2-Rα results in the high affinity receptor. Functional studies suggest that the 70-75 kD chain transduces the T-cell proliferation signal whereas the 55 kD chain serves as a helper binding site (Lowenthal and Green, 1987; Siegel et al., 1987). The eventual cloning of the 70-75 kD chain will clarify this question.

Similarly, the ability to generate high affinity NGF receptors by transfection of the cloned NGF receptor cDNA is limited only to selected cells that are derived from the neural crest. This suggests that another subunit present only in this population of cells may be required for the formation of the high affinity state (Johnson et al., 1986; Hempstead et al., 1989). It is possible that the IL-2-Rβ as well as this second NGF receptor chain will belong to the family of RTKs, since their ultimate effect on cell regulation is similar. The transfection of the human interferon-γ receptor cDNA is another example of the need for cell-specific cofactors in receptor function, since transfected mouse cells were not sensitive to human IFN-γ, although they displayed the same binding properties as human cells. This goes along well with the

observation that in mouse-human somatic cell hybrids, human IFN- effects were only observed when human chromosome 21 was present in addition to chromosome 6 (Jung et al., 1987), suggesting a requirement of additional elements for generating biologic response. Such a requirement is not necessary for atrial natriuretic peptide receptor, since its recent cloning has shown that it is composed of an extracellular ligand-binding domain and intracellular guanylate cyclase catalytic domain (Chinkers et al., 1989). The ANP-clearance (ANP-C) receptor is a truncated form of the ANP-R as it contains a homologous ligand binding domain with a short cytoplasmic tail that does not include the cyclase domain (Fuller et al., 1988), suggesting that this receptor is in fact a transporter. On the other hand, the cytoplasmic domain of the ANP-R is homologous to the soluble form of guanylate cyclase and to the protein tyrosine kinase domain of the PDGF receptor.

Finally, the cloning of the growth-hormone receptor failed to provide clues about its mechanism of signal transduction (Leung et al., 1987). This receptor sequence is not related to known tyrosine-kinase growth-factor receptors, or to any other known protein. On the other hand, the work of Leung et al. revealed that the serum protein that binds GH probably represents the extracelluar domain of the receptor produced either by proteolytic cleavage or altered processing of a precursor of the receptor mRNA. The establishment of functional studies should determine if this cloned receptor alone is sufficient to mediate GH action, or if additional subunits are required.

2.1.4. Immunoglobulin superfamily

Members of this superfamily contain one or many immunoglobulin-like domains recognized by sequence identity and by the formation of a common three-dimensional structure consisting of two antiparallel β sheets held together by a disulfide bond (Willians and Barclay, 1988). These structures are grouped into three categories called V-set, C1-set and C2-set, according to the putative pattern of β-strands approximating to a V- or C- domain. The distinction between the C1 and C2-sets is based on differences in some conserved sequence patterns . In most cases, the majority of the domain sequences are encoded within one exon, suggesting an important functional role for these structures. The common structure concerns only the extracellular sequence, since the transmembrane and cytoplasmic domains are mostly completely unrelated and can vary greatly in length.

The immunoglobulin-related receptors commonly form homo- or heterodimers that are often stably linked by disulfide bonds. These molecules present a diversity of functions but form undoubtedly one of the key groups in immunity and also in the mediation of cell surface recognition to control the behavior of cells in various tissues. A subclass of this family includes receptors binding growth factors and cytokines. Indeed PDGF receptors and related receptors of the subclass III of the RTKs described previously contain 5 Ig-like domains which belong mainly to the C2-set (Yarden et al, 1986). Recently two cytokine receptors were cloned and shown to contain such Ig-like domains. The IL-1 receptor possesses 3 of these domains of the C-set type (Sims et al., 1988) while the IL-6 receptor has only one C2-set domain (Yamasaki

et al., 1988). Interestingly, these receptors differ from the IL-2 receptor which does not contain an Ig-like domain. They differ also from the subclass III RTKs, since they do not have a cytoplasmic tyrosine kinase activity and their mechanism of signal transduction is unknown.

2.1.5. Adhesion proteins

This superfamily of transmembrane glycoproteins participate in cell-matrix and cell-cell adhesion in various physiologically important processes. Functionally speaking, it includes some members of the Ig superfamily but based on the structural organization it can be divided into at least three families. The first is the integrin family which is composed of heterodimeric adhesion molecules consisting of non-covalently associated α and β chains (Hynes, 1987). It can be subdivided into three subclasses based on the sharing of a common β chain (that is β_1, β_2 and β_3). α and β subunits of a given receptor are not closely related. The β subunit contains four repeats of a forty amino acid cysteine-rich motif and a short cytoplasmic domain, while the α-subunits are numerous and, for some of them, are cleaved post-translationally to give a heavy and a light chain linked by disulfide bonding. Some of these receptors recognize the tripeptide sequence Arg-Gly-Asp (RGD), which is common to many extracellular ligands (Ruoslahti and Pierschbacher 1986), but integrin specificity is not limited to this recognition signal.

More recently two new families of adhesion receptors have been discovered (Stoolman, 1989). They are involved in the regulation of inflammatory and immunological events by the adhesive interaction of blood cells and vessel wall. The first family has an unusual protein

mosaic architecture containing an amino terminal lectin-like domain, an EGF domain and tandem repetitive motifs related to those found in complement-regulatory proteins. For this reason it is called the LEC-CAM family (Lectin, EGF, complement-cellular adhesion molecule). The other family includes the Hermes/CD44 group and has a potentially significant N-terminal homology (~30%) with cartilage link proteins which may provide a structural basis for interactions with proteoglycans and collagen. As mentioned previously, all these receptors seem to play an interactive role at the cell surface and are not involved in the transmission of a signal through the cell membrane although their short intracellular domain can interact with the cytoskeleton.

2.2. MULTIPLE MEMBRANE-SPANNING RECEPTORS

2.2.1 Seven-helix receptors

Structurally this superfamily of receptors is characterized by seven hydrophobic transmembrane domains and functionally by interaction with guanine nucleotide binding (G) proteins. These receptors share a complex system of signal transduction where they are coupled to specific effector molecules via intermediary coupling proteins. At the external surface of the cell membrane, they interact with hormones, drugs or sensory stimuli such as light. On the other hand, effector molecules like the adenylate cyclase, the cGMP phosphodiesterase, the phospholipase C or the ion channels are exposed at the cytoplasmic surface. Interposed between both, the G proteins which bind and hydrolyze GTP, act as coupling proteins. Primary structure of these G proteins has revealed many types corresponding to different effectors (Gilman, 1987). The receptors

themselves can be divided into several subtypes that were first identified by their pharmacological differences. Determination of the DNA sequence of the different "isoforms" of a receptor type reveal important differences and shows that they are encoded by different genes. These subtypes are even found to be more numerous than would have been expected from classical pharmacological studies. For instance, five different genes have so far been identified that encode muscarinic receptors (Bonner et al., 1988), while only two subtypes were expected by pharmacological studies. These five subtypes correspond to two functional groups according to their effect on PI turnover, cyclase inhibition and channel couplings (Peralta et al., 1988; Fukuda et al., 1988; Bonner et al., 1988). The existence of subtypes allows tissue specialization and confers different sensitivities to the transmitter and to possible modulators, as well as alternative transduction pathways for different effector systems (Barnard, 1988). In the case of adrenergic receptors, the β_1 and β_2 subtypes are distributed in different tissues but both stimulate the enzyme adenylate cyclase by coupling to a G_s protein. The α_2 subtype inhibits this enzyme via a G_i protein and the α_1 subtype activates the phospholipase C through an as yet poorly characterized G protein (Lefkowitz and Caron, 1988).

Many receptors depicting seven membrane-spanning helices have been recently cloned (see Table I) and comparison of their primary structures shows an overall pattern of conservation including the seven hydrophobic transmembrane domains, one or more N-linked glycosylation sites on the extracellular domains, the first two cytoplasmic loops (CI and CII) and potential sites of regulatory phosphorylation on the cytoplasmic domains

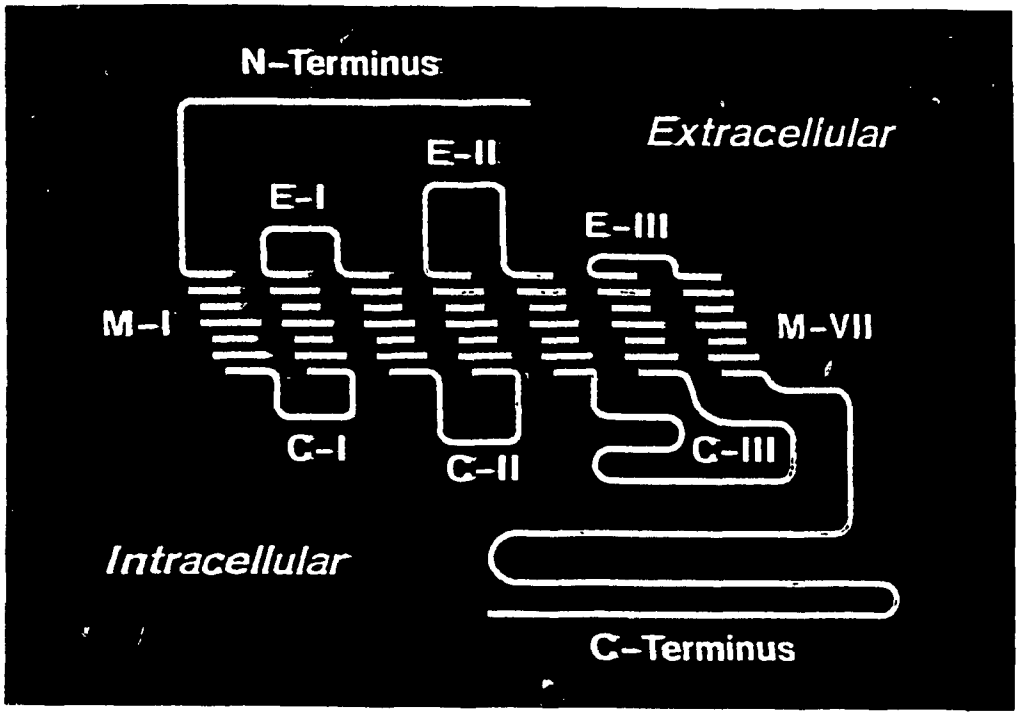

FIG. 3 Schematic representation of the seven-helix receptors

Segments M-I through M-VII are proposed as spanning the bilayer. E-I, E-II and E-III represent the extracellular connecting loops and C-I, C-II and C-III, the connecting loops exposed to the cytoplasmic side of the plasma membrane (Lefkowitz and Caron, 1988, p. 4994).

(Dohlman et al., 1987 - see Fig. 3). A series of mutagenesis studies of the β_2-adrenergic receptor and studies of chimeric β_1/β_2 and α_2/β_2-adrenergic receptors suggest that several of the membrane-spanning regions, in particular the seventh hydrophobic domain and the conserved Asp^{113} of the third helix, are involved in the determination of receptor subtype specificity. It is thought that there is formation of a ligand-binding pocket, with determinants for agonist and antagonist binding being distinguishable (Dixon et al., 1987; Kobilka et al., 1987a; Strader et al., 1987a; Strader et al., 1988; Fraser et al., 1988; Frielle et al., 1988; Kobilka et al., 1988). It has been speculated that the C-III loop and the carboxyl-terminus probably interact with the different G proteins, since they are the most variable cytoplasmic domains allowing a variety of specific couplings. Evidence favoring this hypothesis has recently been obtained by the groups of Dixon (Dixon et al., 1987; Strader et al 1987b) and of Lewfkowitz (Kobilka et al., 1988; O'Dowd et al., 1988). Their mutagenesis studies suggest that the C-terminal portion of the C-III loop and the N-terminal segment of the cytoplasmic tail are critical for productive receptor coupling to G-proteins. Moreover, it seems that the receptors coupled to G_s protein, like the β_1- and β_2-adrenergic receptors, have a short C-III loop and a long carboxyl-terminus, while the receptors coupled to G_i (α_2 and M_2) or to the phospholipase C pathway (M_1), have a long C-III loop and a short carboxyl-terminus (Lefkowitz and Caron, 1988).

Multiple serine and threonine residues located at the carboxyl-terminus or the C-III loop of the G-protein coupled receptors are phosphorylated and are thought to be responsible for the decrease in

responsiveness to hormonal stimulation. Such a process, called desensitization, can either be agonist-independent and triggered by different hormones or drugs that activate cAMP-dependent protein kinase and protein kinase C (heterologous desensitization) or agonist-dependent and triggered by the agonist itself that activates a specific receptor kinase (homologous desensitization) (Sibley et al., 1987). Such receptor kinases have been isolated for the rhodopsin receptor (Kuhn and Dreyer, 1972) and the β_2-adrenergic receptor (Benovic et al., 1986). Direct evidence that one molecular mechanism of desensitization of these receptors involves their agonist-induced phosphorylation has been obtained recently by the truncation or the replacement of the serine and threonine residues of the carboxyl segment of the β_2-adrenergic receptor (Bouvier et al., 1988).

Finally, an interesting feature of this receptor family concerns the structure of their genes. Indeed while the opsin, 5-HT_{1c}, substance K and D_2-dopamine receptor genes contain intronic sequences, the adrenergic, muscarinic, 5-HT_{1A}, and angiotensin receptor genes are intronless in the coding region and contain direct repeats bordering the genes. These unusual findings suggest that the intronless genes arise as processed genes and raise important questions about the evolution of these receptors.

2.2.2 Ligand-dependent ion channels

All ion channels, ligand- or voltage-dependent, are thought to be built on a similar structural plan consisting of a roughly symmetric arrangement of homologous subunits or domains around a central water-

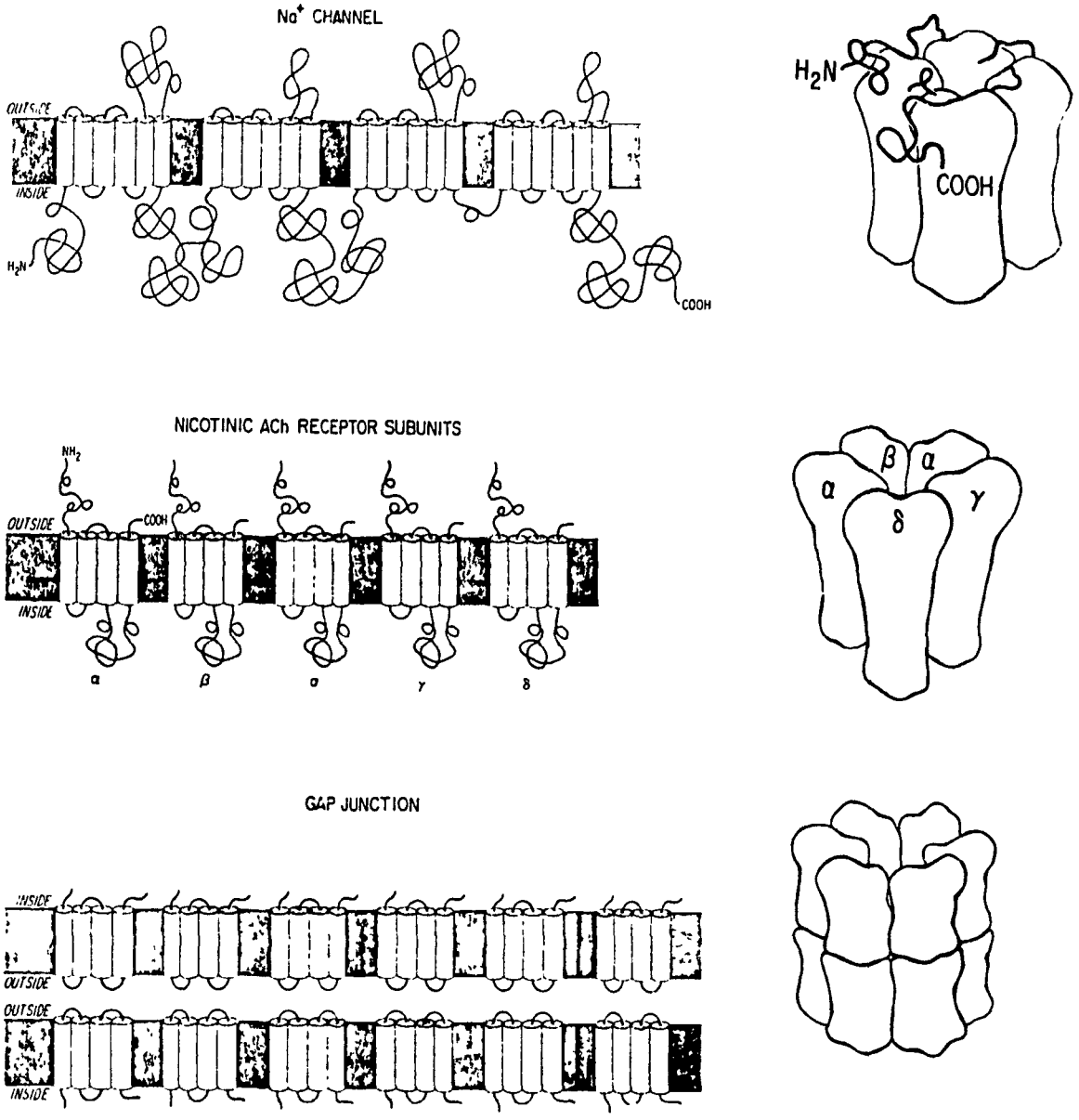

FIG. 4 Proposed models of functional ion channel complexes

On the left is shown the proposed transmembrane topology of the homologous domains or subunits of the channel with each cylinder representing a single transmembrane α helix. On the right is shown a hypothetical pattern of packing of the domains or subunits of the channel. (Top) The single polypeptide chain of the Na^+ channel α subunit, containing four homologous domains. (Middle) Pentameric structure of the muscle nicotinic ACh-R. (Bottom) Model of a 12-subunit complex of the liver gap junction channel (Miller, 1989, p. 1196).

filled pore (see Fig. 4). However, even if this general picture fits well with actual genetic and pharmacological information of a variety of channels, the muscle-type nicotinic ACh-R is the single case for which a functional channel has been shown rigorously to be constructed in this way (Changeux et al., 1984). The ligand-dependent ion channels include the muscle and neuronal nACh-R, the GABA receptor and the Glycine receptor. All these receptors are oligomers of M_r 250,000-300,000 formed by several homologous subunits of M_r 45,000-60,000 encoded by different genes. They all probably form a pentameric structure (Langosch et al., 1988), although a tetrameric, $\alpha_2\beta_2$, structure has been suggested for the GABA receptor (Mamalaki et al., 1987). Each subunit contains 4 transmembrane helices interconnected by hydrophilic regions. Binding of the ligand induces an allosteric conformational change of the oligomer that opens the ion channel. In the case of the nACh-R, a cationic channel, this produces depolarization and an excitation signal while in the case of the GABA and glycine receptors, both anionic channels, this produces hyperpolarization and an inhibitor signal. Neurotransmitter-gated ion channels provide the molecular basis for rapid signal transmission (in the millisecond range) at chemical synapses and do not employ a second messenger for this effect.

The muscle-type nACh-R is considered the prototype of this family of receptors. It consists of a pentameric structure made of 4 different subunits with a stoichiometry determined as $\alpha_2\beta\gamma\delta$. The stoichiometry for the other receptors is still uncertain. A summary of the primary amino acid structure of the subunits of the muscle type (α_1,β_1,γ,δ) and of the neuronal type (α_2, α_3,α_4, Nnα) of the nACh-R is given in Fig. 5.

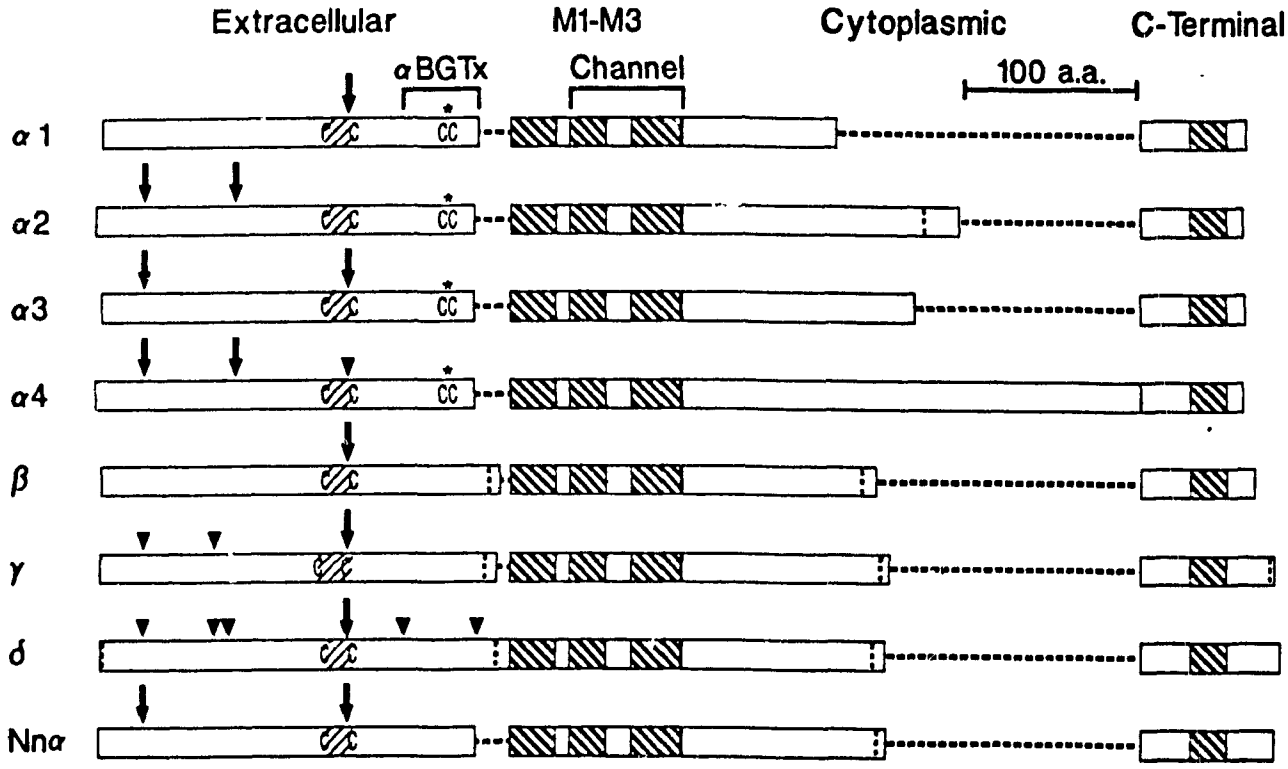

FIG. 5 Homologous domains of the vertebrate nicotinic ACh receptor subunits

The sequences are schematized and aligned to emphasize the similarities of the four regions. The broken lines indicate gaps introduced to permit alignment of sequences and broken vertical lines, the differences in size of subunits sequenced from different species. The hatched area between the two cysteine residues in the N-terminal portion delimit a highly conserved sequence found in all subunits. The star indicates the Cys-Cys pair found in all ACh-binding (α) subunits. The proposed region for α-neurotoxin (αBGTx) binding to the muscle α1 subunit is indicated. The hatched boxes represent the proposed membrane-spanning α-helices (M1 to M4). Potential sites for asparagine-linked glycosylation are indicated with arrows (arrowheads if not present in all examples of homologous subunits sequenced) (Steinbach and Hume, 1989 p. 5).

They all display a high homology in their structural organization. Indeed they all have an extracellular N-terminal end with one or more potential N-linked glycosylation site and 2 cysteine residues separated by 13 conserved amino acids. Another Cys-Cys pair is found only in the α-subunits and is proposed to be involved in ACh-binding. This is followed by a region containing the first three proposed membrane-spanning α-helices (M1, M2 and M3), the major cytoplasmic region, which is of variable length, and finally a region containing a proposed fourth helix (M4) followed by the C-terminal portion. Thus one muscle-type nACh-R will bind two molecules of ACh since it contains two α-subunits. The structure of the three types of subunits (α, β,γ) of the GABA receptor and of the strychnine subunit of the Glycine receptor that have been identified up to now (see Table I) is very similar.

It is interesting to note that transfection of a single subunit allows the formation of a functional homo-oligomeric channel complex capable of binding both agonist and allosteric modulator drugs. This has been shown by the expression in frog oocytes of either the α or β-subunit of the GABA receptor (Blair et al., 1988; Pritchett et al., 1988b), the α4 subunit of the neuronal nACh-R (Boulter et al., 1987) and the strychnine binding subunit of the glycine receptor (Schmieden et al., 1989). These results argue that the subunits are similar enough that they can substitute for each other. However, certain functions of these receptors necessitate the presence of specific subunits as demonstrated recently by the discovery of the γ_2 subunit, the co-expression of which with α_1 and β_1 subunits of the GABA receptor is required for the binding of benzodiazepines (Pritchett et al., 1989).

The four subunits of the muscle-type nACh-R have been shown to be phosphorylated in vivo (Vandlen et al., 1979). The phosphorylation is catalyzed by at least 3 known protein-kinases: protein kinase A,C and tyrosine kinase (Huganir and Greengard 1983; Huganir et al., 1984). The phosphorylated sites are all localized in the major cytoplasmic domain between M3 and M4. Moreover, it seems that such phosphorylations can increase the desensitization rate of the receptor and play an important role in the regulation of the receptor function (Huganir et al., 1986).

Much attention has been given to a model of the nACh-R ion channel with hypothetic regions of the transmembrane domains that might serve to line a water-filled pore (Guy and Hucho, 1987). A prominent amphipathic helix called "MA helix", found in equivalent positions of all four ACh-R subunits, was proposed to project both positively and negatively charged residues into the pore. However, experiments on mutant ACh-R channels, where complete deletion of the MA region did not impede the agonist-activated cation conduction, failed to confirm this hypothesis (Mishina et al., 1985). Recent studies based on covalent labeling of the protein with open-channel blockers (Hucho et al., 1986; Giraudet et al., 1987) or based on site-directed modifications of the ACh-R channel (Imoto et al., 1988; Leonard et al., 1988) support the notion that M2 is the most likely region to line the conduction pore. However, many questions remain unanswered, an important one being the mechanism used by the ACh-R to select specifically against anions.

2.2.3 Voltage-dependent ion channels

The voltage-sensitive ion channels mediate rapid, voltage-gated

changes in ion permeability during action potentials in excitable cells and also modulate membrane potential and ion permeability in many unexcitable cells (Catterall, 1988). Contrary to the ligand-gated channels, the channel-forming structure of the voltage-dependent ones are apparently encoded in a single, very large polypeptide chain (230-260 kD). This chain is composed of four homologous domains, each of which probably has 6 transmembrane helices designated S1 to S6 (see Fig. 4). Each of the four domains of the Na^+ and Ca^{2+} channels contains a sequence in which several basic residues (Arg or Lys) are present at every third position and interspersed with hydrophobic residues (see Fig. 6). This "S4 sequence" has been postulated to function as a voltage sensor (Catterall, 1988). The A-type K^+ channel is about one-fourth the size of the Na^+ and Ca^{2+} channel-forming subunits and contains the 6 transmembrane helices, including S4, corresponding to a single homologous domain of these two receptors (See Fig. 6). By analogy with the other two channels, it was proposed that the K^+ channel probably exists as an oligomer of four subunits.

Purification of the Na^+ and Ca^{2+} channels has yielded more than one subunit. In the case of the Na^+ channel, the large subunit α appears to be sufficient to form functional channels according to the transfection studies (Noda et al., 1986b; Suzuki, 1988). However, the co-expression of lower molecular weight subunits appeared to be necessary to obtain a normal rate of inactivation (Auld et al., 1988). On the other hand, the channel-forming subunit α_1 of the DHP-sensitive calcium channel is not sufficient to express the Ca^{2+} channel in frog oocytes, while it is when transfected in the skeletal muscle cells of a line of dysgenic mice

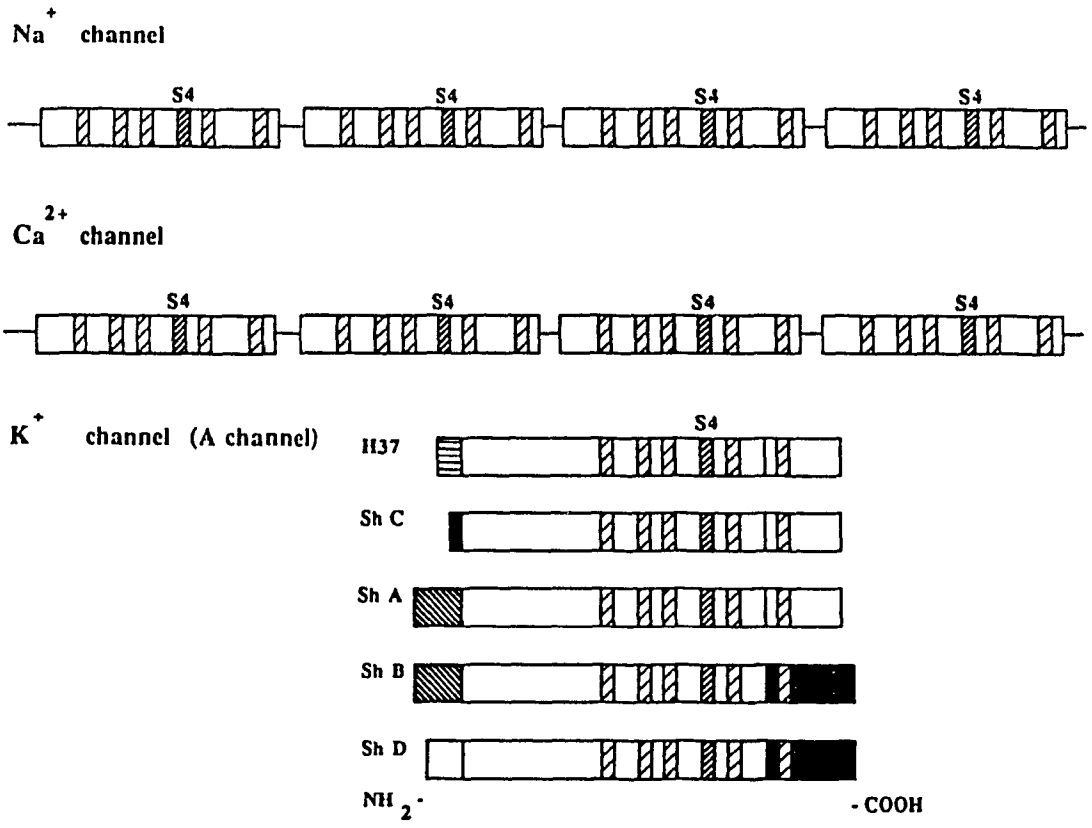

FIG. 6 Schematic diagram of voltage-sensitive ion channel primary structures

The boxes represent the four domains of the Na^+ and Ca^{2+} channels or the subunits of the K^+ channel. Each channel domain or subunit has five potentially hydrophobic membrane-spanning sequences (shaded) and one S4 sequence (marked above). The different amino and/or carboxyl-terminal regions of the various forms of the K^+ channel are generated by alternative splicing and are illustrated separately.

(Tanabe et al., 1988). This suggests that other subunits are necessary for the expression and function of the Ca^{2+} channel. One of these subunits (α_2) has recently been characterized and shown to be entirely different from the α_1 subunit, since it is a highly glycosylated protein with only three hydrophobic segments.

Unexpectedly, three genes coding for different Na^+ channels have been found in the rat brain (see Table 2) and multiple forms of the A-type K^+ channel are generated by alternative splicing at the Shaker locus (see Fig. 6). It seems that a multitude of different subtypes of the Na^+ and K^+ channels can correspond to different cell types and possibly different kinetic properties (Jan and Jan, 1989).

2.2.4 Multiple membrane-spanning transporters

Receptors in this superfamily consist of a single molecule with 10 to 12 putative transmembrane segments (see Table I). The glucose transporters of the Na^+-independent type constitutes an important family where the different members have 50% or more amino acid identity and are predicted to have similar structures including 12 hydrophobic, presumably membrane-spanning, regions together with an extracellular loop between the first two helices and a hydrophilic intracellular loop between helices 6 and 7. They differ primarily in the lengths and sequences of the putative extracellular loop and of the intracellular C-terminal domain, suggesting that these two regions confer functional specificity on individual glucose transporters (Kayano et al., 1988).

It is interesting to note that the Na^+-dependent glucose transporter does not share sequence identity with the other glucose transporters

(Hediger et al., 1987). In fact, it contains 11 putative membrane-spanning sequences five of which are amphipathic. A similar mixture of hydrophobic and amphipathic-sequences are found in both the Cl^-/HCO_3^- antiporter (Kopito and Lodish, 1985) and the Na^+/H^+ antiporter (Sardet et al, 1989). For the last two cases there is a pronounced dichotomy between the large hydrophilic and hydrophobic regions. It is postulated that the amphipathic helices can cluster to form an hydrophilic region involved in the ion-exchange process across the membrane bilayer (Kopito and Lodish, 1985). Mutagenesis studies should eventually clarify this question.

2.2.5 Ion pumps

These integral membrane proteins directly couple the hydrolysis of ATP to the vectorial transport of various cations across the plasma membrane (Ullrich, 1979). The (Na^+ - K^+)ATPase consists of a large catalytic subunit (α) and a smaller glycoprotein subunit (β) of unknown function. The α-subunit has extensive sequence identity with the (H^+ - K^+)ATPase and (Ca^{2+}-Mg^{2+})ATPase that have been recently cloned (see Table I). It consists of a protein with 6 to 10 putative transmembrane helices and includes a conserved sequence of $\sim$60 amino acids that spans the membrane and is bound at one end by the phosphorylation site and at the other by the apparent ouabain binding site. This region is likely to be a major component of the energy transduction system. The ATP binding domain precedes the transmembrane helix H5 and is well-conserved. The most substantial differences occur in the N-terminal region and in the transmembrane domains which probably confer ion specificity (Shull et

al., 1985; Shull and Lingrel, 1986; McLennan et al., 1985). The determination of the structural and functional domains of the (Na^+ - K^+)ATPase is of major interest, since the receptor binds cardiac glycosides, thus the inhibition of the Na^+/K^+ pump and subsequent inotropic action play a crucial role in the therapy of patients with heart failure (Allen et al., 1985).

2.2.6 GAP junctions

GAP junctions are formed by subunits present on two apposing cell membranes. They allow ions and small solutes to pass from one cell to the other, thus mediating intercellular communication (Loewenstein, 1987). The subunits (connexins) are assembled into hexamers (connexons), joined with connexons in adjacent cells (see Fig. 4). Two connexins have been cloned up to now: connexin 43 from the heart (Beyer et al., 1987) and connexin 32 from the liver (Paul, 1986; Kumar and Gilula, 1986). They are membrane proteins with sequence identity in their four putative membrane spanning domains and in their extracellular regions. Each short extracellular loops contains three conserved cysteine residues. The unique cytoplasmic primary structures of the connexins may confer different physiological actions. Recently, Swenson et al. (1989) have expressed the connexins in Xenopus oocytes and showed the formation of gap junctions between pairs of oocytes injected with the same connexin RNA (homomolecular channel) or with two different connexin RNAs (heteromolecular channel). The different pairs displayed different behaviour. With such molecular biologic tools it will be possible to explore the real function of these special channels.

CHAPTER 3

PROLACTIN AND ITS RECEPTOR

3.1 Functions of prolactin

A multitude of biological functions (over 85) have been attributed to PRL, an anterior pituitary hormone present in all vertebrates (Bern and Nicoll, 1968). These actions can be classified into different categories (Nicoll, 1980; Meites, 1988):

a) actions related to salt and water balance;

b) growth and development effects;

c) actions on reproductive functions;

d) metabolic effects;

e) behavioral effects;

f) immunoregulation functions; and

g) actions on ectodermal and intergumentary structures.

Certain functions like osmoregulation and nesting behavior are predominant in certain species (fish and birds, respectively), but of minor importance in mammals. On the other hand, PRL has few proven functions in mammals. Of these, its actions on mammary gland play a key role and have been well-studied. These actions include cell multiplication and differentiation, and synthesis of milk proteins, lactose, enzymes, lipid and antibodies (Shiu and Friesen, 1980). These effects are produced by an interaction with other hormones, such as estrogen, progesterone, corticosteroid, growth hormone, placental lactogen, insulin and other growth factors. The mitogenic effects on the mammary epithelium may be mediated by a growth factor-like substance produced by the liver: synlactin (Mick and Nicholl, 1985). The induction of milk protein synthesis (casein, α-lactalbumin, whey acidic protein) results from two independent phenomena: enhancement of milk protein mRNA

concentration and stimulation of their translation (Houdebine et al., 1985). Moreover, the accumulation of casein mRNA has been shown to be much more pronounced than the increased rate of transcription, suggesting that it results from both acceleration of transcription and from mRNA stabilization (Teyssot and Houdebine, 1981; Guyette et al., 1979).

There is no doubt that PRL is the key hormone involved in the induction and promotion of breast cancer in rodents (Welsch and Nagasawa, 1977). In rats, numerous spontaneous mammary tumors are seen in old females, reaching an incidence of 70% or more in Sprague-Dawley rats, and are correlated with the large elevations in PRL secretion with age. Growth of both spontaneous and carcinogen (DMBA)-induced breast cancers in the rat is PRL-dependent; raised PRL levels lead to progression and lowering of the PRL levels leads to tumor regression. In addition Fisher and Fisher (1963) reported that PRL promotes the metastasis of the cancer cells in spontaneous rat mammary carcinoma.

In human the role of prolactin in the development of breast cancer is controversial (Shiu et al., 1987). Clinical studies examining serum levels of PRL and the tumor remission by the use of prolactin-suppressing drugs in breast cancer patients, generated conflicting and inconclusive results. However, recently a significant report suggested the prognostic significance of the measurement of total PRL receptors in human breast cancer patients (Bonneterre et al., 1987). In addition, prolactin has been shown to induce a specific protein (PIP: prolactin-inducible protein) in human breast cancer cells (Shiu and Iwasiow, 1985). This protein is found in human breast cancer biopsies and in the serum of some

breast cancer patients (Murphy et al, 1987). No convincing stimulatory effect of PRL on growth of human breast cancer cell lines in vitro has been demonstrated. However, a recent report showed that PRL is capable of promoting the growth of primary human breast cancer cells taken from biopsies in a soft agar clonogenic assay in the absence of serum (Manni et al., 1986). This observation, coupled with the fact that both T-47D and MCF-7 cells respond to prolactin stimulation when grown as solid tumors in nude mice (Leung and Shiu, 1981; Welsch et al., 1981; Shafie and Grantham, 1981) suggested that something about the way in which human breast cancer cells were cultured may be responsible for the lack of any reported prolactin effect. A possible explanation came recently by the work of Biswas and Vonderhaar (1987) who showed the necessity of using charcoal stripped serum in culture medium to reveal a good response of MCF-7 cells to prolactin in long-term culture. This suggests that bovine PRL in fetal serum can camouflage the response to lactogen stimulation. Obviously more work is required to clarify the role of PRL in human breast cancer.

The liver contains high levels of PRL receptors and has provided one of the best systems in which to study the dynamic regulation of lactogen receptors (Hughes et al., 1985). Despite numerous reports demonstrating that the liver is a PRL-responsive target tissue, no truly important role of PRL in the physiological function of the liver has been established. PRL can modulate lipogenesis (Agius et al., 1979), RNA synthesis (Chen et al., 1972a); ornithine decarboxylase activity (Richards, 1975) and bile acid metabolism (Moltz and Leidahl, 1977). Moreover, prolactin stimulates the production of some proteins by the liver: IGF-1 (Francis

and Hill, 1975; Murphy et al., 1988a); synlactins (Nicoll et al., 1988) and a lactogenic factor (Frawley et al., 1988). These factors can potentially mediate growth-promoting and lactogenic effects of PRL on mammary gland.

There is growing evidence for the involvement of PRL in immunomodulation. Early work of Chen and coworkers (1972b) showed a relationship between the lymphoid trophic state and PRL. Studies by Berczi and Nagy (1986) showed the ability of lactogenic hormones as well as growth hormones to restore immunocompetence in hypophysectomized rats. They suggest that growth, nucleic acid synthesis and function of lymphoid organs are dependent on growth and/or lactogenic hormones. Similarly, lactogenic hormones stimulate growth of rat Nb2 lymphoma cells in a dose-dependent manner (Gout et al., 1980). This cell line has been used as a sensitive and specific bioassay to measure PRL and GH in human serum (Tanaka et al., 1980). PRL receptors are present on both B and T lymphocytes (Russell et al., 1984, 1985; Hiestand et al., 1986; Bellusi et al., 1987). A direct competitive inhibition of prolactin receptors on lymphocytes has been proposed as the mechanism of the immunosuppressive action of cyclosporin (Hiestand et al., 1986; Russell et al., 1987). Moreover, recently, macrophages have been shown to be activated by PRL and GH (Bernton et al., 1988; Edwards et al., 1988). There is even peripheral production of PRL and a PRL-like peptide by lymphocytes (DiMattia et al., 1988; Russell, 1988). These results, plus the fact that anti-PRL antibodies can block Con A-stimulated mitogenesis of lymphocytes (Montgomery et al., 1987), suggest possible autocrine regulation of lymphocytes by PRL.

3.2 Mechanism of action of PRL

As with GH, the postreceptor events that follow the binding of PRL to its cell surface receptor are poorly defined. No known second messengers have been shown to clearly mediate the PRL action. Cyclic AMP, which is involved in many hormonal transduction system, is unlikely to be a mediator of PRL action. Some studies have shown that its dibutyryl derivative does not mimic PRL action and in fact slightly inhibits its action in mammary gland explants (Rillema, 1980) and in Nb2 cells (Larsen and Dufau, 1988). Cyclic GMP, on the other hand, is able to partially mimic certain actions of PRL in cultured rat mammary tissues (Rillema, 1975) and in pigeon crop sac (Anderson et al., 1981). There is an absolute cation requirement for PPL's enhancement of guanylate cyclase (Vesely, 1984). However, the slight stimulation obtained with cGMP suggests at best, a limited participation in PRL action. Prostaglandins can cause a PRL-like increase in RNA synthesis in mouse mammary gland explants but cannot reproduce the stimulatory effect of PRL on casein synthesis, but the combination of polyamines (spermidine), prostaglandins and arachidonic acid can stimulate casein synthesis but the magnitude of this effect is low compared to PRL's effect (Rillema 1980a). Studies demonstrating a stimulatory effect of PRL on ornithine decarboxylase (ODC), the rate limiting enzyme in polyamine synthesis, suggested a role for the polyamines in PRL action (Oka and Perry 1976; Frazier and Costlow, 1982; Richards et al., 1982). Hughes et al., (1985) reported studies on the role of polyamines in Nb2 cells and concluded that they play a major supportive role in the PRL-mitogenic action but that they were incapable of any stimulation in the absence of lactogenic

hormones, suggesting the need for additional factors in mediating hormone action.

Activation of an amiloride-sensitive Na^+/H^+ exchange system by hPRL is the earliest post receptor response in Nb2 cells (Too et al., 1987). However, the failure of TPA, which activates the Na^+/H^+ exchange, to stimulate cell proliferation indicates that the Na^+/H^+ exchange is not sufficient to trigger cell proliferation.

Recent studies by the group of Dufau suggest that G-proteins, probably different from the classical G_s and G_i, are involved in the signal transduction mechanism of the PRL in Nb2 cells. They first showed that the proliferative effect of PRL was modulated by pertussis and cholera toxins (Larsen and Dufau, 1988) and then they demonstrated direct effects of PRL on two specific ADP-ribosylate membrane substrates of 38 kDa and 41.5 kDa (Barkey et al., 1988). No clues are provided in these studies concerning the transduction system to which these G proteins are linked.

Hormone binding to membrane receptors often results in inositol lipid turnover in the plasma membrane (Berridge, 1984). Phosphatidylinositol breakdown by phospholipase C results in the formation of two second messengers: inositol triphosphate which triggers Ca^{2+} mobilization and 1,2-diacylglycerol which activates protein kinase C. Recently there were many studies suggesting involvement of this transduction system and especially the protein kinase C route in PRL's actions. Exogenously added phospholipase C elicits PRL-like effects on ODC activity (Rillema et al., 1983) and RNA synthesis (Rillema, 1984) in mouse mammary gland explants. Ofenstein and Rillema (1987), by using

phospholipase inhibitors, abolished the PRL response in Nb2 cells, suggesting a possible involvement of phospholipase C in the PRL stimulation of mitogenesis. By itself, however, phospholipase C had no effect on the rate of cell division. Moreover, Etindi and Rillema (1988) showed that prolactin induces a phospholipase C-type hydrolysis of inositol phospholipids in mouse mammary gland explants, but this effect is transient and does not occur before 60 minutes after hormone exposure. This suggests that these changes are secondary to more primary actions of PRL on its target cells. Similar delayed effects of another lactogenic hormone (hGH) on phosphoinositide metabolism in Nb2 cells were observed (Gertler and Friesen, 1986).

There are several lines of evidence implicating Ca^{2+} in the action of PRL. Depletion of extracellular calcium by EGTA inhibits the synthesis of casein by mammary explants from mice (Rillema, 1980b) and pregnant rabbits (Wilde et al., 1981). A calmodulin inhibitor reduced by 80% the prolactin-induced differentiation in mouse mammary gland (Bolander, 1985). However, the calcium ionophore A23187 had no effect on differentiation and casein synthesis in mouse glands (Bolander, 1985; Cameron and Rillema, 1983) or on growth of Nb2 cells (Murphy et al., 1988b). These results suggest that Ca^{2+} is necessary but not sufficient to mediate PRL actions. However, it is possible that a small intracellular calcium pool may mediate the early actions of PRL in Nb2 cells, since the intracellular calcium antagonist TMB-8 inhibited both c-myc expression and DNA synthesis in these cells while the ionophore A23187 alone or in combination with TPA was without effect (Murphy et al., 1988).

Phorbol esters (TPA and others) have a structure similar to diacylglycerol and activate protein kinase C (Blumberg, 1988). They have PRL-like effects in the mammary gland (Rillema, 1985; Rillema and Waters, 1986) while they enhance PRL effects in Nb2 cells (Gertler et al., 1985; Buckley et al., 1986). Moreover, gossypol, an inhibitor of PKC, inhibits PRL-stimulated responses (Etindi and Rillema, 1987). More direct evidence for PKC involvement has been obtained recently by Waters and Rillema (1989) who showed that PRL treatment of mammary gland explants produces a transient, time-dependent translocation of PKC to the particulate-fraction. H7, a PKC inhibitor, inhibited the PRL-stimulated effects. Similar results were obtained by Buckley et al., (1986) in Nb2 lymphoma cells. Interestingly, a recent report indicates that GH triggers the formation of DAG within a few seconds and stimulates c-fos gene expression in the preadipose Ob 177 cells, but contrary to $PGF_{2\alpha}$, it did not increase the inositol lipid turnover (Doglio et al., 1989). The authors suggested that DAG is produced in response to GH by a phospholipase C-mediated mechanism which may involve other glycerophospholipids, including the phosphatidylinositol-glycan that was shown to be involved in insulin mechanism of action (Low and Saltiel, 1988; Farese, 1988; Standaert and Pollet, 1988). Buckley et al., (1988) reported that addition of PRL to purified rat liver nuclei results in a rapid activation by several hundred-fold of protein kinase C. This effect was blocked by an anti-PRL-R monoclonal antibody. They suggested a nuclear site of action for PRL that involves phospholipid metabolism.

Like many peptide hormones, PRL is internalized (Nolin and Witorsch, 1976; Josefberg et al., 1979). After internalization, the hormone-

receptor complex is thought to undergo proteolysis in lysosomes. This processing of PRL has been viewed by certain investigators as an obligatory step for the intracellular action of the PRL (Mittra, 1980). However, there are several studies suggesting that internalization of PRL is not a necessary step to its action. Lysosomotrophic agents such as chloroquine, which inhibit lysosomal proteolysis, do not alter the induction of casein gene expression by PRL (Houdebine and Djiane, 1980). Moreover, anti-PRL receptor antibodies that mimic hormone effects have been used to demonstrate that degradation of internalized PRL is not required for the lactogenic (Djiane et al., 1981) and the mitogenic (Shiu et al., 1983) actions of the PRL. These studies do not eliminate the possibility that the internalized PRL receptor itself, intact or processed, can activate intracellular processes involved in hormonal action.

In summary, although the mechanism of action of PRL remains obscure, there are growing evidence that PRL triggers a chain of reactions by binding to a cell surface receptor that may interact with a G protein. This protein is possibly linked to a phospholipase C-glycerophospholipid system which releases DAG, activating protein kinase C and subsequent phosphorylation of putative substrates. This hypothetic transduction system, if confirmed, would probably only partly explain the action of PRL. A thorough knowledge of the PRL receptor molecule is required to make progress in this field of research.

3.3 General characteristics of the PRL-R

PRL receptors are distributed in a wide variety of tissues as

TABLE II: TISSUE DISTRIBUTION OF PRL RECEPTORS[1]

Mammary gland: - normal
- tumors
- milk

Reproductive system:

Female

Ovary - corpus luteum
- granulosa cells

Uterus

Male

Testis - Leydig cells

Epididymis

Seminal vesicle & seminal vesicle fluid

Prostate - normal
- tumors

Lung

Liver

Kidney

Bladder

Pancreas

Chorion laeve

Pituitary

Brain: - choroid plexus
- hypothalamus
- cerebrospinal fluid

Lymphoid tissue: -lymphocytes
-Nb2 lymphoma

[1] Hughes et al., 1985 and Kelly et al., 1988

illustrated in Table II. This is not surprising when the numerous functions attributed to PRL are considered. However, the biological function of PRL in most of these tissues remains unknown. Thus far, characterization of PRL receptors has been performed mainly with rabbit mammary gland and rodent liver.

Human GH and ovine PRL bind to PRL receptors of both primates and non-primates with a comparable affinity, regardless of the preparation (whole cell, microsomes, solubilized or purified) of receptors. This affinity (K_a), assessed by Scatchard analysis, ranges from 10^9 to 10^{10} M^{-1}.

Like other hormones, the dissociation of the prolactin-receptor complex is very slow, in particular when hormones are allowed to associate with receptors for a longer time (Kelly et al., 1980; Van der Guyten et al., 1980). Moreover, there is a more than two-fold lower dissociation constant for PRL receptors in plasma membrane compared to those in Golgi, suggesting different forms of the receptor may exist in these different subcellular localization (Kelly et al., 1983).

In rat liver, the majority of receptors are found in endosomal compartments (Bergeron et al., 1978). These intracellular receptors are considered to consist of mixed population of internalized and newly synthesized receptors (Posner et al., 1979). Studies of the uptake of ^{125}I-oPRL into various organelles from rat liver revealed a maximum uptake at 15 min in the Golgi and at 30 min in both prelysosomes and lysosomes (Josefsberg et al., 1979; Khan et al., 1981; Ferland et al., 1984a). Moreover, chloroquine treatment markedly enhanced receptor levels in lysosomes, suggesting that degradation processes of the PRL

receptor may occur in these organelles (Ferland et al., 1984b). These experiments suggest either that the receptor-hormone complex is sequentially internalized into the Golgi and then reaches the lysosomal compartment or that there exist two independent paths of internalization, one toward the Golgi complex (for the recycling of the receptor) and the other going directly, although less rapidly, toward the lysosomes (for the degradation of the receptor) (Kelly et al., 1986).

3.4 Regulation of PRL receptors

The hormonal regulation of PRL receptors is complex. Indeed, binding studies revealed that steroid, thyroid and polypeptide hormones, including PRL, control the number of PRL receptors and interact in various ways depending on the tissue, sex and species tested (Hughes et al., 1985). As an example, testosterone increases PRL binding in rat prostate (Kledzik et al., 1976) whereas binding is decreased in liver, kidney and adrenal (Aragona et al., 1975; Marshall et al., 1976). On the other hand, estrogen acts as a stimulator of PRL binding activity in rat liver of both sexes, male mice and intact female mice (Posner et al., 1974; Marshall et al., 1978). In rat prostate, PRL receptor levels are decreased after estrogen treatment and in female mice, ovariectomy causes an increase in binding while estrogen replacement reduces binding to control levels or lower (Marshall et al., 1978). These <u>in vivo</u> effects of steroid hormones are in sharp contrast to the lack of either stimulatory or inhibitory effects of directly added estrogen or testosterone on the number of PRL binding sites in cultured hepatocytes (Barash et al., 1988). These results suggest an indirect role for sex

steroid hormones on receptor induction. It is thought that steroid hormones act via the pituitary secretion of PRL and GH, since hypophysectomy abolishes their modulatory effect (Posner et al., 1974; Aragona, 1975).

The importance of a pituitary factor in the maintenance of PRL binding sites in rat liver was first suggested when it was shown that hypophysectomy provoked the loss of PRL binding (Kelly et al., 1975). An inductive effect of PRL was postulated when it was observed that a capsular pituitary implant under the kidney in hypophysectomized rats augmented hepatic PRL binding sites approximately 3 days following the increase in serum PRL levels (Posner, 1975). Several reports confirmed PRL's induction of its receptors in tissues like rabbit mammary gland (Djiane and Durand, 1977) and rat liver (Manni et al., 1978; Amit et al., 1985), by the direct injection of PRL. It was even shown that oPRL, as well as anti-PRL receptor antibodies, were capable of maintaining PRL receptors in hepatocytes cultured in suspension (Rosa et al., 1982). On the other hand, hGH administration was also shown to increase PRL receptors in both hypophysectomized (Norstedt et al., 1981; Norstedt, 1982) and normal female (Baxter et al., 1984) rat liver. However, the fact that rat GH had similar effects to hGH on PRL receptors suggested that the inductive effect of hGH was not due to its lactogenic property (Baxter and Zaltsman, 1984; Norstedt et al., 1984). In order to resolve the controversy concerning the respective inductive roles of PRL and GH, Barash et al., (1988) evaluated their effect on rat hepatocytes cultured in serum-free medium. They found that both PRL and GH induced PRL receptors acting through their own receptor and suggested that this

explains the unusual potency of hGH which binds to both PRL and GH receptors.

A transient down-regulation of PRL receptors has been identified in rabbit mammary gland both *in vitro* (Djiane et al., 1979a) and *in vivo* (Djiane 1979b) following intravenous injection of PRL. A down-regulation of PRL receptors has also been observed in cultured rat hepatocytes, but only with supramaximal doses of PRL (Barash et al., 1988). Moreover, these authors have shown a heterologous down-regulation of these receptors by supramaximal concentrations of bGH. They proposed a model of homologous receptor regulation, where there is a balance between receptor production and receptor loss. Both processes are activated differently according to the concentration of the ligand, the first being predominant at low concentration of PRL and the second at high concentration. However, in mammary gland explants, down-regulation of the PRL receptor has been observed even at physiological PRL concentrations (Djiane et al., 1982). More studies are required to clarify this question.

The mechanisms underlying the regulatory changes in the number of PRL binding sites remain obscure. In fact, four factors are considered to be involved but it is unclear to what extent each one contributes to this dynamic regulation (Hughes et al., 1985):

1) de novo synthesis.

2) internalization and subsequent degradation

3) recycling from Golgi to cell surface

4) conversion between cryptic and active receptors.

This last factor does not require de novo protein synthesis and

depends on conversion from a non-binding to a binding state by an appropriate trigger mechanism. Evidence for such a mechanism came from studies in which agents that perturb membrane integrity produce an increase in PRL-binding activity (Alhadi and Vonderhaar, 1982; Koppelman and Dufau, 1982; Dave and Knazek, 1980).

3.5 Biochemical characteristics of the PRL receptors.

Isolation and purification of the PRL receptor is an important and necessary step for its characterization. All the purification methods described involved at least two steps: solubilization of particulate PRL receptors with a detergent and affinity chromatography. The first attempt to purify the PRL receptor came from Shiu and Friesen (1974) who used Triton X-100 for solubilization of rabbit mammary gland microsomal receptors. Because of the aggregation of PRL by this detergent, they had to use hGH as a ligand for affinity chromatography and as a tracer for radioreceptor assay. Using CHAPS as detergent, Liscia and Vonderhaar (1982) showed that they were able to use oPRL in affinity chromatography to purify the mouse liver receptor. The same tools were used to purify the rabbit mammary gland (Katch et al., 1985; Necessary et al., 1984) and the rat liver (Katoh et al., 1987) PRL receptors. Mitani and Dufau (1986) used a two-step affinity purification, combining concanavalin A-Sepharose and hGH-agarose affinity chromatography to purify the rat ovary PRL receptor. Although a relatively high degree of purification has been obtained on some occasions (Necessary et al., 1984; Mitani and Dufau, 1986), the yield was low and all the purification studies were at the analytical rather than the preparative level.

Molecular weight determination of the PRL receptor under non-dissociating conditions, such as gel-filtration chromatography, sucrose gradient sedimentation or non-denaturing electrophoresis, gave a high molecular weight, in the range of 99,800 to 340,000 (Kelly et al., 1988a), with the exception of a 37 kDa form in mouse liver (Liscia and Vonderhaar, 1982; Liscia et al., 1982).

The molecular structure of the PRL binding component has also been characterized by using SDS - polyacrylamide gel electrophoresis of the purified, immunoprecipitated or affinity-labeled PRL receptor. Affinity labeling or crosslinking of the receptor is an important technique in which a labeled hormone covalently linked to the receptor by a chemical reagent is identified on the SDS-gel followed by autoradiography. By these techniques, molecular weights determined ranged from 35,000 to 45,000 (Kelly et al., 1988a). Larger molecular weight forms ($M_r \sim 80,000$) have been found when detergent-solubilized receptors were analyzed using crosslinking techniques. This was reported for rat ovary (Bonifacino and Dufau, 1984) and for rat liver (Haldosén and Gustafson, 1987; Kelly et al., 1988b). These larger forms were not affected by the presence of a reducing agent and were not present when the membrane-bound PRL receptors were crosslinked (Kelly et al., 1988b). Recently immunoblot analysis of the PRL receptor using anti-rat liver PRL receptor monoclonal antibodies gave further insights into the structure of the PRL binding subunit. In the first report, Katoh et al., (1987) used the system of biotinylated second antibody-peroxidase staining to reveal bands while in the second, Okamura et al., (1989) used ^{125}I-labeled monoclonal antibodies as a direct approach that resulted in an increase of sensitivity of 100 to

1000-fold compared to the first technique. The great sensitivity of this technique allowed direct detection of PRL receptors in various tissues as well as in rat liver. Small M_r bands were obtained in rat liver and ovary (42,000), Nb2 cells (42,000), pig mammary gland (36,000) and rabbit mammary gland (36,000, 45,000). However, in addition to these forms, larger M_r bands were found in rat liver (84,000), rat ovary (51,000, 84,000), Nb2 cells (53,000, 64,000), pig mammary gland (66,000) and rabbit mammary gland (55,000, 77,000). In contrast to crosslinking studies, immunoblot analysis revealed the presence of a high M_r band for the rat liver PRL receptor, even in microsomes. Although the 84,000 form disappeared under reducing conditions, suggesting the existence of disulfide linkages in the subunit structure of the PRL receptor in accordance with other studies (Haeuptle et al., 1983; Bonifacino and Dufau, 1984), this hypothesis cannot yet be confirmed, since the monoclonal antibody used in these studies is sensitive to reducing agents, and the M_r 42,000 band was also markedly reduced. These results indicate that the larger M_r forms may represent oligomeric forms of smaller subunits or alternatively represent intrinsic receptors that easily degrade into smaller units. Moreover, the very large M_r obtained by non-dissociating conditions may be artifacts consisting of detergent micelles or other non-specific aggregation, or may represent holo-receptors containing two or more binding subunits or one binding subunit and other subunits that do not contribute to hormone binding.

Antireceptor antibodies have become a useful tool to help to understand the mechanism involved in the interaction of polypeptide hormones with cell surface receptors. These antibodies sometimes act paradoxically, that is, in addition to antagonistic effects, they may

also elicit agonistic actions. Antisera against partially purified PRL receptors have been produced and examined for their biological actions on casein and DNA synthesis in rabbit mammary gland (Shiu and Friesen, 1976a; Djiane et al., 1981), on corpora lutea of rat ovary (Bohnet et al., 1978), maintenance of PRL receptors in hepatocytes cultured in suspension (Rosa et al., 1982), mitogenic effect in Nb2 lymphoma cells (Shiu et al., 1983), and lactose and DNA synthesis in rat mammary tumor explants (Edery et al., 1983).

However, polyclonal antibodies present some limitations due to their heterogeneity and to binding to membrane components other than the PRL receptor, since the antigen used is a partially purified receptor preparation. For this reason, specific monoclonal antibodies directed against different epitopes of the rabbit mammary gland and rat liver PRL receptors were produced. Katoh et al., (1985) reported the production of 3 mAbs (M110, A82 and A917) against the rabbit mammary PRL receptor. Two of them (M110 and A82) were hormone binding site specific while the other (A917) bound to a different region. The mAbs M110 and A82 prevented the stimulating effect of PRL on casein synthesis and A917 mimicked the action of PRL on both casein and DNA synthesis (Djiane et al., 1985). Interestingly, only A917 was of the IgG_2 subclass and its PRL-like activity was lost when it was reduced to monovalent fragments (Dusanter-Fourt et al., 1984). Similarly it was observed that only the bivalent F(ab')2 fragments of an anti-PRL receptor polyclonal antibody were able to stimulate proliferation of Nb2 cells while the monovalent Fab' fragments were inactive (Shiu et al., 1983). These data suggest that aggregation of PRL receptors constitute an essential step of their

action and are in agreement with the proven aggregation of other receptors (see RTKs previously discussed). Contrary to the anti-PRL receptor polyclonal antibodies (Katoh et al., 1984; Shiu and Friesen, 1976a, 1976b) which showed cross-reactivity with all the species tested, these mAbs revealed strong species specificity, suggesting the existence of structural differences of the PRL receptor among species.

Six mAbs against the rat liver PRL receptor were also obtained (Katoh et al., 1987; Okamura et al., 1989b). Although directed against different epitopes of the receptor, three mAbs (E29, T1 and T2) are directed against the binding site region and the other three (E21, U5 and U6) were directed against domains distinct from the binding site. The mAb U5 showed significant binding to rabbit and pig mammary gland, and mouse liver demonstrating that there are some immunogenic domains of the receptor conserved between species. The anti-PRL receptor monoclonal antibodies have already been useful in immunoprecipitation and immunoblotting studies previously discussed and appear to be efficient tools for receptor purification, investigation of hormone binding characteristics, biological effects and physicochemical characterization of the PRL receptor.

3.6 Scope of the present investigation

As discussed above, a number of points concerning the structure and function of PRL receptor remains to be clarified. These include the number of PRL-R(s) mediating the multiple actions of PRL, the primary and subunit structures of the PRL-R, the mechanism of its regulation by hormones and by PRL itself, and the signal transduction pathway(s) used

by PRL.

In order to answer these questions, it is imperative to know more about the primary structure of the PRL receptor. However, until now it has not been possible to purify the PRL receptor to homogeneity. In the present investigation we wanted to circumvent this problem by using molecular biology techniques to obtain the primary structure of the receptor. In Chapter 4, we describe the first approach used to achieve the goal as described, which is based on the use of poly- and monoclonal antibodies, previously prepared in the laboratory. In Chapter 5, the successful purification, cloning and expression of the rat PRL receptor is described. With this new structural tool in hand, we examined the hormonal and developmental regulation of the PRL-R at both the protein and mRNA levels (Chapter 6). Finally, the human PRL-R was cloned by taking advantage of its homology to the rat PRL receptor.

These studies should lead to a better understanding of PRL-R structure and regulation and provide an experimental means to study the mechanism of action of prolactin.

PART II

EXPERIMENTAL SECTION

CHAPTER 4

USE OF POLYCLONAL AND MONOCLONAL ANTIBODIES FOR THE DETECTION OF PRL RECEPTOR mRNAs AND cDNAs

PREFACE

In Chapter 1, the different experimental approaches, including the most recent techniques used to clone low abundance cell surface proteins were presented. In the choice of a cloning strategy to isolate a PRL-R cDNA, two factors were important:

1) the source of PRL-R mRNA

2) the available tools to specifically probe the prolactin receptor or its mRNA.

Two tissues, the rabbit mammary gland and the rat liver, were considered good sources of PRL-R mRNA, since the receptor is relatively abundant and well-characterized in both tissues. However, no partial amino acid sequence of the receptor was available, therefore poly- and monoclonal antibodies constituted the best available tools at the time the study was undertaken. In this chapter we further characterize the anti-PRL-R antibodies with respect to their ability to recognize the PRL-R synthesized <u>in vitro</u> and <u>in vivo</u> and report their use to screen expression cDNA libraries.

SUMMARY

The identification of the primary structure of the prolactin receptor is central to a better understanding of the mechanism of action of prolactin. In the absence of partial sequence information of the receptor, its cDNA cloning will be difficult. We first tested the ability of polyclonal and monoclonal antibodies to the PRL receptor, developed in our laboratory, to specifically immunoprecipitate in vitro and in vivo translation products from crude rabbit and rat Poly(A)+ mRNAs. However, screening rat liver λ gt11 expression libraries with these antibodies resulted in only false positives. Next, we enriched rat liver mRNA for its content in PRL receptor mRNA by using the immunopurification of polysomes. We prepared a library in λ gt11 from this enriched poly(A)+ mRNA. Screening this library with polyclonal antibodies resulted in an approximately 500-fold increase in the number of positive clones when compared with those obtained from a non-selected library, thus confirming the enrichment of the mRNA. Screening this library with monoclonal antibodies resulted in 11 positive clones, all of which, however, appeared to be false positives. The limitations of the λ gt11 expression system for the cloning of the PRL receptor cDNA are discussed.

INTRODUCTION

Prolactin is the anterior pituitary hormone responsible for the establishment and maintenance of lactation in mammals. The initial action of PRL, as is true for other polypeptide hormones, involves an interaction with high affinity cell surface receptors. However, following hormone binding, the mechanism(s) by which these receptors transduce

information to various cellular compartments is not known (Houdebine et al., 1985). To approach this problem, our laboratory has focused on the characterization of the PRL receptor in both the rabbit mammary gland and the rat liver (Kelly et al., 1986). By developing anti-PRL receptor poly- and monoclonal antibodies and by using different biochemical approaches, such as crosslinking, immunoprecipitation or immunoblot analysis, the prolactin receptor has been shown to be a single subunit with a relative molecular mass (M_r) of approximately 40,000 (Katoh et al., 1984, 1985 and 1987). To determine the primary structure of this receptor, we decided to use recombinant DNA technology. Screening cDNA libraries with synthetic oligodeoxynucleotide probes prepared against known sequences was the technique successfully used for most of the cloned cell surface receptors, at the time this work was performed (Noda et al., 1982, 1983; Russell et al., 1983; Ullrich et al., 1984; Leonard et al., 1984; Nikaido et al., 1984; Cosman et al., 1984). However, partial amino acid sequence information of purified receptor is necessary to make oligonucleotide probes, but up to now, purification of the PRL receptor to homogeneity has not been obtained. Alternatively, expression cloning techniques were used to detect human EGF receptor cDNA clones with polyclonal antibodies (Lin et al., 1984). A more powerful expression system was devised by Young and Davis (1983a,b), using the bacteriophage expression vector λgt11, which allows the immunodetection of genes that are expressed at a low level. In the present study we report the use of our polyclonal and monoclonal antibodies to detect the PRL receptor from <u>in vitro</u> and <u>in vivo</u> translations and to screen rat liver λgt11 cDNA libraries.

RESULTS AND DISCUSSION

As a first step we wanted to test the ability of our poly and monoclonal antibodies to identify a protein, synthesized from genetic material, that would be compatible with the prolactin receptor. For this purpose we isolated poly(A)$^+$ mRNAs from estrogen-treated rat liver and pregnant rabbit mammary gland and translated them in vitro in reticulocyte lysates in the presence of ^{35}S-methionine. After immunoprecipitation with different antibodies, the proteins were separated by gel electrophoresis and an autoradiogram was obtained. Figures 1 and 2 show the results of such immunoprecipitations. In Fig. 1., estrogen-treated rat liver poly(A)$^+$ mRNA was translated in vitro. The polyclonal antibody no. 46 specifically immunoprecipitated a protein of 68,000 daltons, but no specific protein was recognized by the monoclonal antibody E21. A major band migrating at 68,000 was also seen in the total translation product. In Fig. 2., pregnant rabbit mammary gland mRNA was used and antiserum no. 46 specifically precipitated two proteins of 58 kDa and 125 kDa that seem to be minor proteins in this tissue. The monoclonal antibody M110 failed to precipitate any specific protein. In both cases, the protein recognized by the polyclonal antibody had a larger molecular weight than 40,000, expected for the prolactin receptor in both tissues (Borst and Sayare 1982; Haeuptle et al., 1983). This difference could be explained by a longer protein sequence present in the precursor form of the receptor that is cleaved during post-translational maturation in the cell. Alternatively, these specific bands could correspond to proteins completely unrelated to the PRL receptor, since the anti-prolactin receptor antisera were raised against partially purified ($\sim$1%) receptor

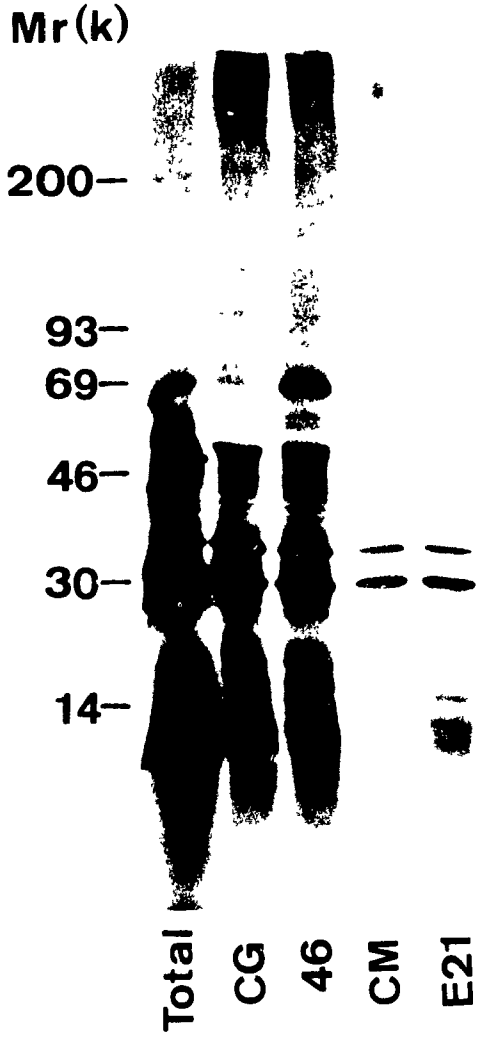

FIG. 1 Immunoprecipitation of the putative rat liver PRL receptor precursor synthesized in vitro.

Translation in reticulocyte lysates of estrogen-treated rat liver poly(A)$^+$ mRNA in the presence of ^{35}S-methionine. The translated products were incubated with one of the antibodies as indicated: CG, control goat serum; 46, goat anti-PRL receptor antiserum; CM, control mouse immunoglobulins and E21, anti-rat liver PRL receptor monoclonal γ-globulins. Following immunoprecipitation, the proteins were electrophoresed and fluorographed for 3 days. Total represents a sample of the total translated products. The position of molecular weight markers are indicated by numbers on the left.

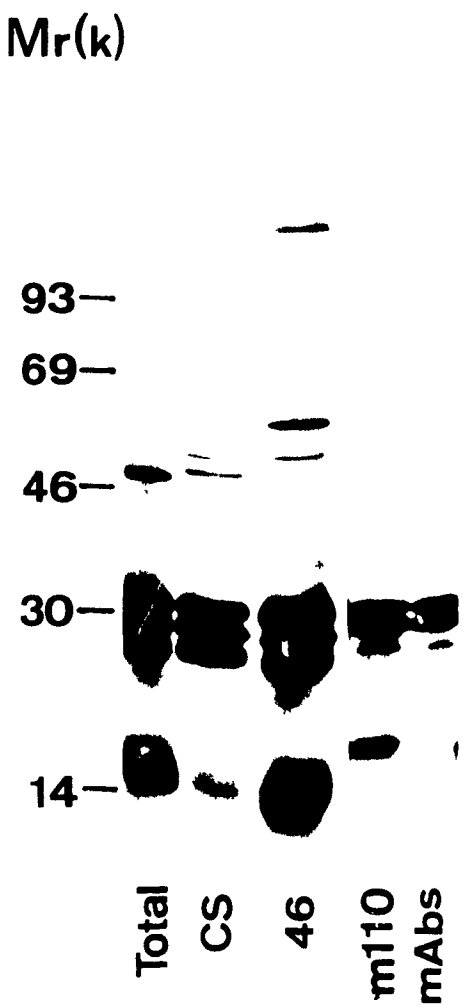

FIG. 2 Immunoprecipitation of the putative rabbit mammary gland PRL receptor precursor synthesized in vitro.

As in Fig. 1., except that pregnant rabbit mammary gland poly(A)$^+$ mRNA was used for in vitro translation. The antibodies used for immunoprecipitation were: CS, control goat serum; 46, goat anti-PRL receptor antiserum; mAbs, control mouse immunoglobulins and M110, anti-rabbit mammary gland PRL receptor monoclonal γ-globulins. The X-ray film was exposed for 1 day.

(Katoh et al., 1987). Moreover, the detection of a highly abundant protein supports the second hypothesis in the case of rat liver, since the prolactin receptor is expected to be a very low-abundance protein. The fact that the monoclonal antibodies did not immunoprecipitate any protein does not make the situation any clearer, since the epitope recognized by these antibodies could be masked by the different tertiary structure of the precursor. To circumvent this problem, we used an *in vivo* translation system that would allow maturation of the proteins. We injected estrogen-treated rat liver poly(A)$^+$ mRNA into Xenopus oocytes and used another polyclonal antibody (no. 201) to immunoprecipitate at least three specific proteins of 31, 41 and 68 kDa (Fig. 3.). Immunoprecipitation of the OCT protein (36 kDa) with a specific anti-OCT rabbit antiserum was used as a positive control (Argan et al., 1983). None of these bands were obtained with eggs injected with vehicle. The 41 kDa band is reminiscent of the M_r 40,000 form of the PRL receptor while the 68 kDa band could be the same as that found by *in vitro* translation using antiserum no. 46. The use of the monoclonal antibody E21 did not help to resolve this issue, since no protein was specifically precipitated. This could still be explained by a different processing of the receptor in the oocytes compared to the rat liver, resulting in a different conformation, thus modifying the antigenic determinants.

We next screened several commercially available female rat liver libraries in the expression vector λgt11, using as antisera those that gave specific immunoprecipitation. No positive signals were obtained. However, when we screened a non-amplified, estrogen-treated rat liver library with the anti-rat PRL receptor antiserum no. 203, we obtained 20

FIG. 3 Immunoprecipitation of the putative mature PRL receptor synthesized in Xenopus Laevis oocytes.

Xenopus Laevis oocytes were injected with either water (control) or estrogen-treated rat liver poly(A)$^+$ mRNA (RL mRNA) and incubated in the presence of ^{35}S-methionine. The in vivo translation products were mixed with one of the antibodies: NRS, normal rabbit serum; A-OCT, anti-ornithine carbamyl transferase monospecific antiserum; 201, anti-PRL receptor rabbit antiserum; NMab, normal mouse immunoglobulins and E21, anti-rat liver PRL receptor monoclonal γ-globulin. Following immunoprecipitation, the proteins were electrophoresed and fluoragraphed for 24 hours with an intensifying screen. Molecular weight markers are indicated by numbers on the left.

positive recombinants. Thirteen were confirmed with other specific antisera (no. 211 or 212) and cloned to homogeneity. When monoclonal antibodies were used, no positive signals were observed. On Southern blot analysis (not shown) we found that clones A1, A2, A6, A7 and A10 all cross-hybridized, as well as clones A8 and A9. In order to determine which of the cDNA clones encoded the PRL receptor we took advantage of our knowledge concerning PRL binding sites in rat liver. Indeed the rat liver PRL receptor is known to be a very low abundance protein (<0.001% of total protein) that is practically absent in hypophysectomized animals but is stimulated 5-10 fold by estrogen in normal female rats (Posner et al., 1974). Moreover, the tissue distribution of the receptor in rat is well-known (Posner and Khan 1983). By using the different positive clones as cDNA probes on Northern blot analysis of rat mRNAs, it is possible to examine the level of abundance of the signal, its response to estrogen and its specific tissue distribution (Fig. 4 and 5). None of the clones that were identified fulfilled the requirements necessary to be considered the rat liver PRL receptor cDNA. The clones could be divided into four classes according to their ability to hybridize with mRNA of liver from hypophysectomized and estrogen-treated rats (Fig. 4). In class A, (clone A3), the message is abundant, is present in hypophysectomized animals and is increased by estrogen. In class B, (clones A8 and A11), the message is of low abundance in hypophysectomized animals but responds well to estrogen. In class C (clone A12), the message is virtually absent after hypophysectomy but responds dramatically to estrogen treatment. Finally in class D, the message is more (clones A4 and A5) or less (clones A2 and A13) abundant and does not

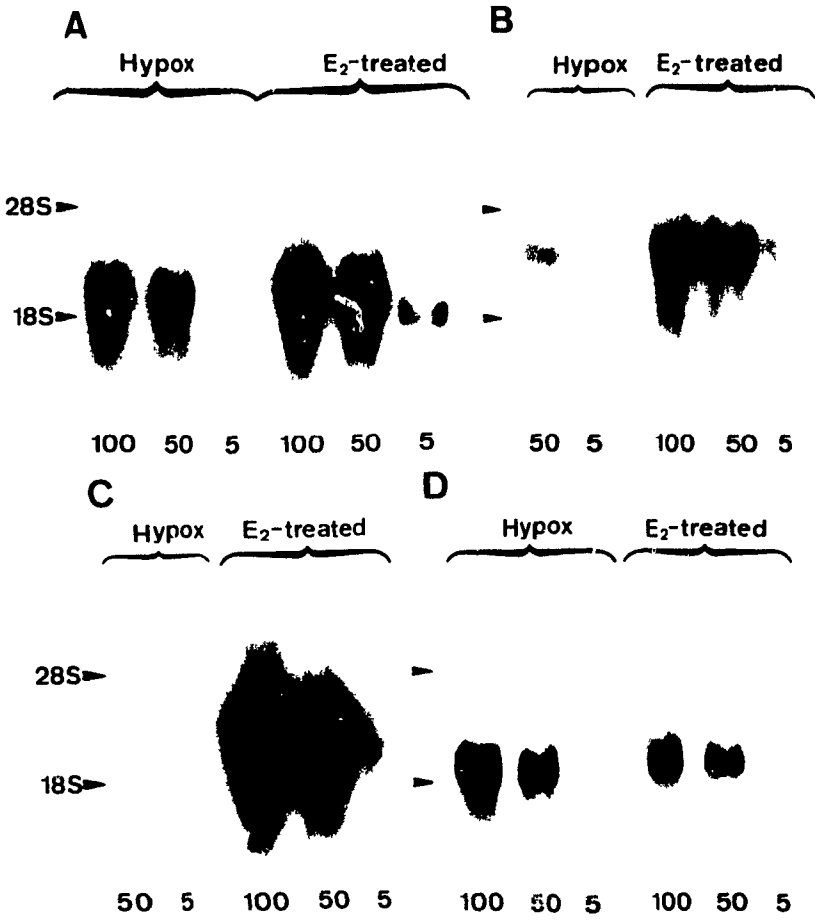

FIG. 4 Northern blot analysis of A3, A8, A12 and A2 mRNAs in rat liver from hypophysectomized or estrogen-treated animals.

One hundred, 50 and 5 μg of poly(A)$^+$ mRNA from hypophysectomized (Hypox) and estrogen-treated (E_2-treated) rats were loaded on the gel as indicated at the bottom of each blot. The blots were hybridized at high stringency with cDNA probes prepared from clones A3 in A, A8 in B, A12 in C and A2 in D. All the X-ray films were exposed overnight. Blot D was the only one for which an intensifying screen was used. The migration of 18S and 28S ribosomal RNAs is indicated by the arrows.

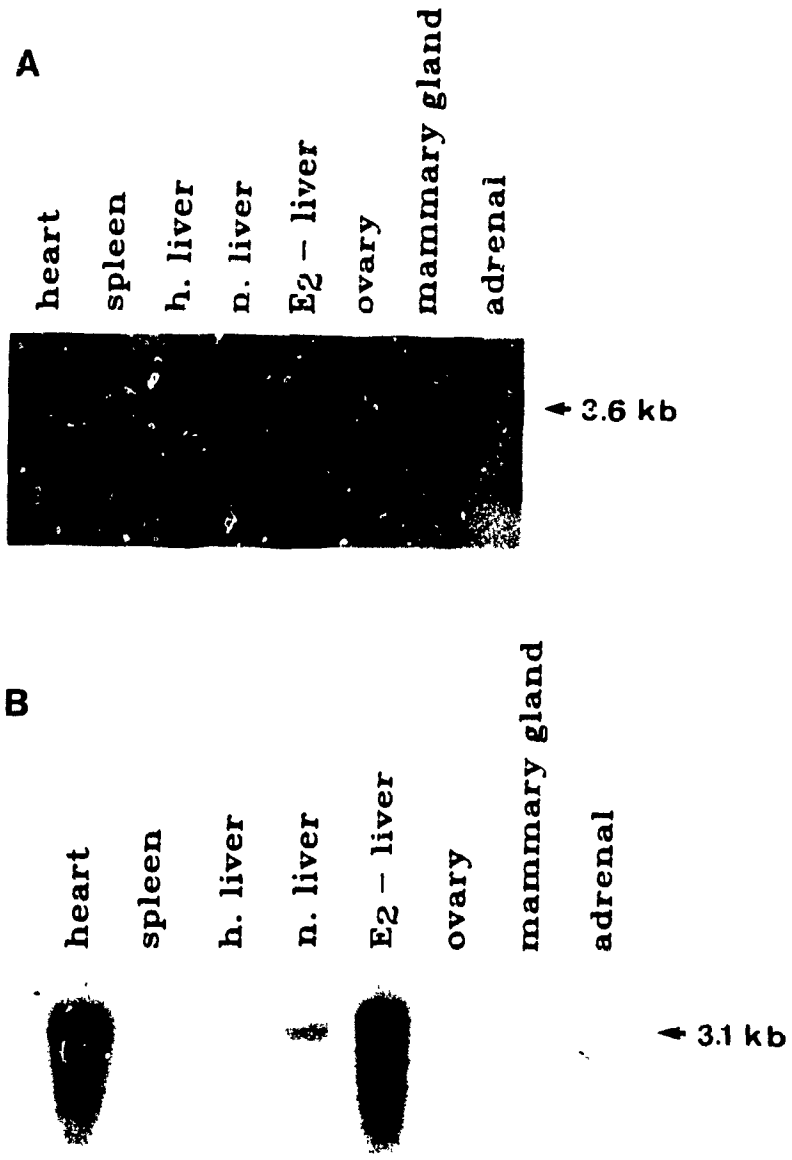

FIG. 5 Northern blot analysis of A8 and A12 mRNAs in different tissues of the rat.

Ten micrograms of poly(A)$^+$ mRNA was loaded in each well. The RNAs were extracted, as indicated above the blots, from the heart, spleen and liver from hypophysectomized rats, from the liver of normal female rat and from the liver, ovary, mammary gland and adrenal of estrogen-treated female rats. The blots were hybridized at high stringency with cDNA probes prepared from clones A8 in A and A12 in B. The X-ray films were exposed with an intensifying screen overnight in A and 36 hours in B. The size of the mRNA bands is indicated on the right of each blot.

increase at all in response to estrogen. From these findings we concluded that clones from class A and D were not compatible with the PRL receptor and tissue distribution of their mRNAs showed that they were present in the heart of female rats where PRL binding sites are absent (data not shown). Northern blot analysis using cDNAs of class B and C clones did not allow the elimination of these clones simply on the basis of response to hormones. However, the tissue distribution of Northern blot analysis of their mRNAs in rats did not match the pattern expected for the PRL receptor. Examples for these two classes are given in Fig. 5. When we used a cDNA probe for the clone A8 (Class B, Fig. 5A), we obtained a weak signal in the normal rat liver, that was increased by both hypophysectomy and estrogen treatment and the mRNA was absent in all the other tissues tested, including the ovary, the mammary gland and the adrenal where PRL receptor binding sites are found. A cDNA probe for clone A12 (class C, Fig. 5 B) gave the pattern expected for the PRL receptor in the liver of hypophysectomized, normal and estrogen-treated rats, but gave an even stronger signal in the heart of hypophysectomized rats where PRL receptor binding sites are absent. Since attempts to clone the PRL receptor failed and since its mRNA was of very low abundance, we decided to enrich the mRNA by using the technique of polysome immunopurification, which is considered the most powerful technique for this purpose (Kraus, 1985). In a typical experiment 11,400 OD_{260} units of polysomes were prepared from 970 g of estrogen-treated female rat liver and incubated with 110 mg of anti-rat hepatic PRL receptor - γglobulin. After purification on a protein-A agarose column, 34 OD_{260} units of polysomal material were eluted and 8 μg of poly(A)$^+$mRNA was obtained after

passage through an oligo-dT cellulose column. In this experiment the chromatography through the protein A agarose column provided a 335-fold purification. In general a 300 to 500-fold purification was obtained which corresponds to what has been reported for other hormone receptors (Schneider et al., 1983; Russell et al., 1983; Miesfeld et al., 1984). A typical sucrose gradient profile of the polysomes, before (A) and after (B) passage through the protein A agarose column, is illustrated in Fig. 6. The integrity of the polyribosomes, as demonstrated by these profiles, is central to the success of the technique. The amount of polysomes retained on the protein-A sepharose column is small, as shown by the minor change in the profile in Fig. 6. B.

Using such enriched poly(A)$^+$ mRNA, we performed translation in reticulocyte lysates and analyzed the synthesized products by immunoprecipitation and gel electrophoresis. The autoradiogram of Fig. 7. indicates that antiserum no. 212 precipitated three specific bands of 12, 32 and 46 kDa. This pattern is strikingly different from that obtained with crude poly(A)$^+$ mRNAs shown on this figure with the same antiserum or in Fig. 1. with the antiserum no. 46. The 32 kDa band was particularly promising, since we knew that treatment of partially purified receptor preparations with endoglycosidase F gives a band of 34-36 kDa (unpublished data). However, once again no specific band was precipitated with the monoclonal antibody E21.

The enriched mRNA was used as a template for synthesis of cDNAs and construction of a library in the expression vector λgt11. Screening of the entire library with polyclonal antibodies gave approximately 10,000 positive recombinants. This represented a 500-fold increase in the

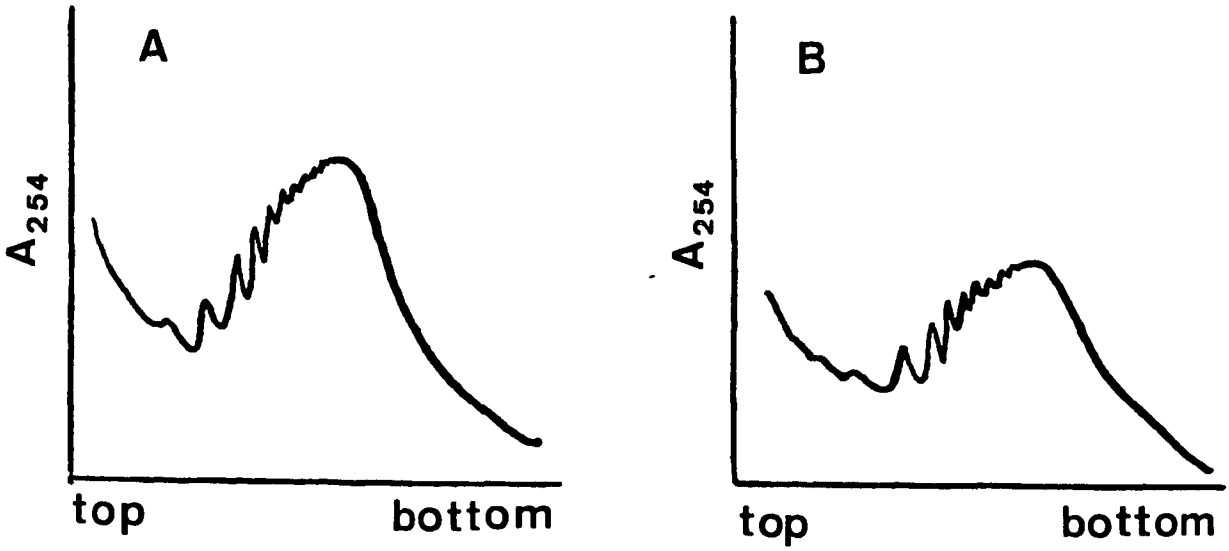

FIG. 6 Sedimentation profiles of polyribosomes.

0.5 OD_{260} units of rat liver polyribosomes were layered on a 5 ml 20-50% sucrose density gradient in buffer C (see Materials and Methods). After centrifugation the gradients were scanned at 254 nm with an ISCO density gradient fractionator and UV monitor. A, polyribosomes prior to antibody addition. B, non-adsorbed polyribosomes after passage of the antibody - polyribosome mixture through the protein-A agarose column.

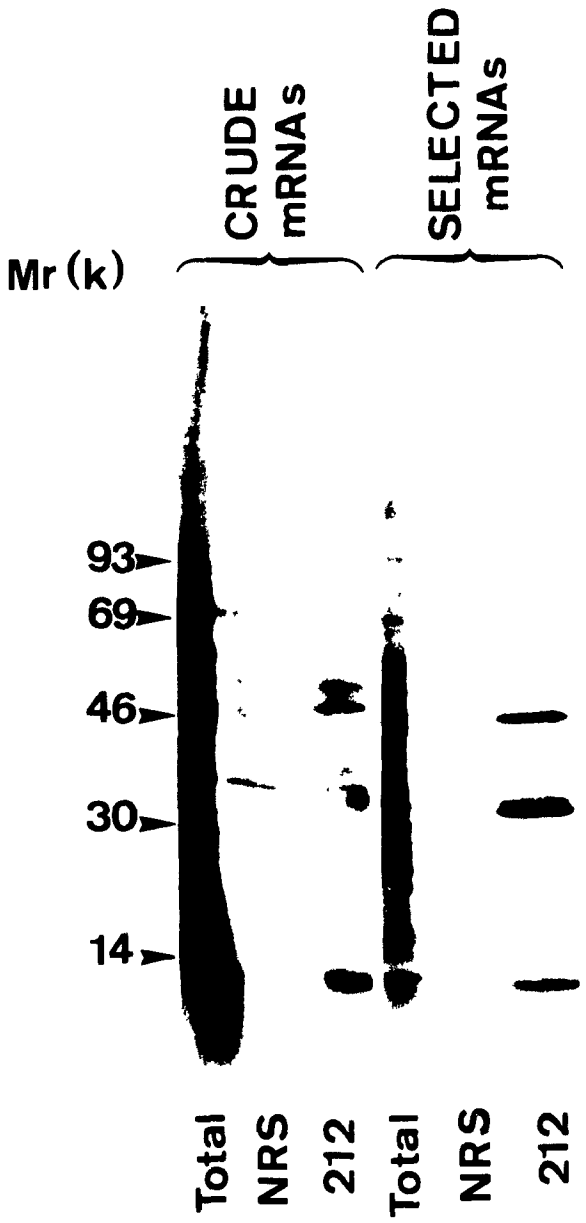

FIG. 7 Immunoprecipitation of the putative rat liver PRL receptor precursor synthesized in vitro from enriched mRNA.

As in Fig. 1., except that rat liver poly(A)+ mRNAs enriched by polysomes - immunopurification (selected mRNAs) as well as crude liver poly(A)+ mRNAs from estrogen-treated rats (crude mRNAs) were used for in vitro translation. The antibodies used for immunoprecipitation were: NRS, normal rabbit serum and 212, anti-rat liver PRL receptor rabbit antiserum. The X-Ray film was exposed for 11 days.

number of positives observed following screening of a classical, non-enriched library, and this increase is close to the calculated degree of enrichment of mRNA by the immunopurification of polysomes. Moreover, screening of this library with the anti-rat PRL receptor monoclonal antibodies E21 and F11 resulted in 11 positive recombinants (B1 to B11) which were purified to homogeneity. All these cDNA clones cross-hybridized on Southern blot (not shown) and probably represented the same cDNA. Strangely, positive signals were observed only with IgG prepared in mouse ascites but not with hybridoma culture medium. In addition, positive signals were also observed with nonspecific ascites (Sp20 and anti-CEA). This suggested that clones B1-B11 were all nonspecific. On Northern blot analysis (Fig. 8), B1 was used as a cDNA probe and recognized a message that was present in all the tissues examined independent of variation with hormonal manipulation. This 237 bp long cDNA probably encodes an abundant constitutive protein in the rat that is unrelated to the PRL receptor. We sequenced it but found no homology with any known genomic or protein sequences. It could become a useful control marker, similar to β-actin, for Northern blots, since its mRNA is found in every tissue and does not appear to be hormonally regulated.

At the time these studies were in progress, we were able to purify the rat liver PRL receptor to homogeneity and obtain amino acid sequences that were used to make synthetic oligonucleotide probes. The following chapter describes results obtained using these probes. With the successful cloning of rat liver PRL receptor using oligonucleotide probes, there was no need to try to identify among the 10,000 recombinants obtained in the enriched library screened with the polyclonal antibodies, the one

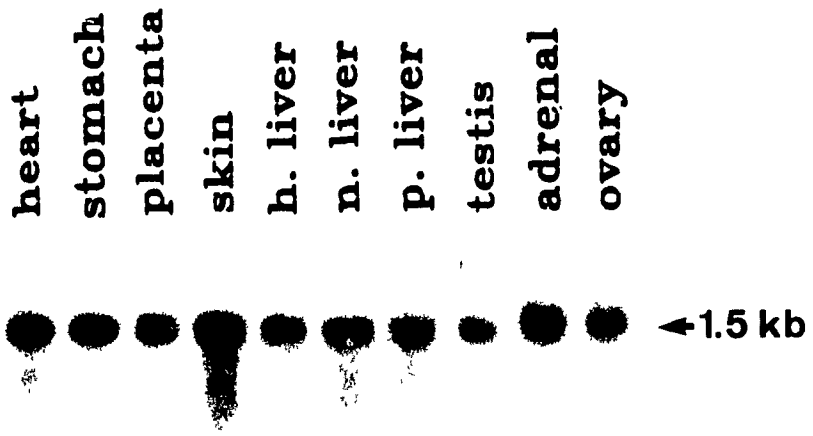

FIG. 8 Northern blot analysis of B1 mRNA in different tissues of the rat.

One hundred micrograms of poly(A)+ mRNA were loaded in each well. The RNAs were extracted, as indicated above the blot, from the heart, the stomach and the testis of normal male rats, the skin of newborn rats, the liver of hypophysectomized, normal or pregnant female rats, the placenta, the adrenal and ovary of pregnant rats. The blot was hybridized at high stringency with a B1 cDNA probe. The X-ray film was exposed one hour with an intensifying screen. The size of the B1 mRNA is indicated on the right.

encoding the PRL receptor. However, it would have been possible to design an approach to reach this goal. For instance we planned to isolate several clones, eliminate many of them on the basis of cross-hybridization with the false-positive clones obtained previously (A1-A13) and study the remaining ones by Northern blot analysis of their mRNA.

In conclusion, cloning of a cell surface receptor by screening λgt11 expression libraries is not a straightforward method. In the case of the PRL receptor, this can be explained by many factors: 1) the very low abundance of the mRNA and the need to prepare enriched mRNA to make a representative cDNA library; 2) the non-specificity of the antisera raised against a partially (~1%) purified receptor, resulting in identification of many false positive clones; 3) the difficulty of the monoclonal antibodies to recognize a fusion protein with β-galactosidase (Snyder and Davis 1985), where the antigenic site is masked or changed in conformation; and 4) the nonspecific clones obtained by screening with the monoclonal antibodies due to a common epitope shared by several proteins or by contaminating antibodies present in the mouse ascites preparations.

MATERIALS AND METHODS

Preparation of poly (A)$^+$ mRNAs

RNA for in vitro translation was extracted from livers of estrogen-treated female Sprague-Dawley rats and from mammary glands of lactating rabbits. A single subcutaneous injection of 1 mg of estradiol valerate (Squibb Canada Inc.) was used for estrogen treatment of female rats, and animals were killed 8 days later. RNA for Northern blot analyses was

extracted from different tissues of Sprague-Dawley adult rats: heart, spleen and liver from hypophysectomized rats; liver from normal female rats; liver, ovary, mammary gland and adrenal from estrogen-treated female rats; heart, stomach, testis from normal male rats, skin from newborn rats, and placenta, liver, adrenal and ovary from pregnant rats. Total RNA was purified using the guanidium thiocyanate/CsCl procedure (Chirgwin et al., 1979), and polyadenylated mRNA was purified by oligo-dT cellulose, chromatography (Aviv and Leder 1972).

Polysome isolation and immunopurification

The polysomes were isolated according to the procedure of Shapiro and Young (1981) with some modifications. Ribonuclease precautions were taken throughout the experiments and polysomes were always manipulated on ice. All solutions contained 20 mg heparine/100 ml and 2 mg cycloheximide/100 ml. Sixty grams of liver from estrogen-treated female rats were homogenized in 180 ml of buffer A (25 mM Tris-HCl, pH7.5, 300 mM NaCl, 10 mM $MgCl_2$, 0.4% Nonidet P-40, 250 mM sucrose and 800 µl of α-amylase/100 ml [Boehringer]) using a Tekmar homogenizer. The nuclei were pelleted in a Sorvall rotor SS-34 at 15,000 rpm for 15 min at 4 C. The supernatant was lavered over a cushion of 9 ml of 50% sucrose (BRL) in buffer B (25 mM Tris-HCl, pH 7.5, 300 mM NaCl and 10 mM $MgCl_2$) and centrifuged at 35,000 rpm in Sorvall rotor 50.38 for 2½ hours at 4 C. The pellets were rinsed and suspended in 1 ml of buffer C (25 mM Tris-HCl, pH7.5, 150 mM NaCl, 5 mM $MgCl_2$ and 0.1% Nonidet P-40), transferred to Eppendorf tubes and centrifuged 5 min at 4 C to remove aggregates. They were then pooled and stored at -70 C. One aliquot was kept to measure the absorption at

260 and 280 nm and another was used to obtain a sedimentation profile of polyribosomes through a sucrose density gradient as described previously (Jolicoeur et al., 1983).

Rabbit antisera (no. 211, and no. 212) against partially purified rat liver PRL receptor were used for immunopurification of the polysomes. They were produced as described previously, Katoh et al. (1987). Antibodies were isolated by affinity chromatography using the Affigel protein A MAPS II kit of Bio-Rad. After elution the antibodies were dialyzed against PBS buffer (20 mM sodium phosphate, pH7.6, 150 mM NaCl) and incubated overnight at 4 C with the rat liver polysomes at a ratio of 10 mg of γ-globulin for 1000 OD_{260} units of polysomes. The mixture was then applied twice to the protein A-Affigel column. After overnight washing with buffer C, mRNA in specifically bound polyribosomes was eluted from the column with buffer D (25 mM Tris-HCl, pH 7.5 and 20 mM EDTA). Finally, the eluate was incubated for 5 min at 65 C, placed on ice, made 0.5M NaCl, 10 mM in Tris-HCl, pH7.5, and 0.5% SDS, and passed over an oligo(dT)-cellulose column as previously described to obtain the enriched poly(A)$^+$ mRNA.

Cell-free translation and immunoprecipitation

Rabbit reticulocyte lysate was prepared according to Pelham and Jackson (1976). mRNA-dependent protein synthesis with either enriched or non-enriched poly(A)$^+$ mRNAs was performed in a micrococcal nuclease-treated lysate following the method of Merrick (1983) with some modifications. A typical assay contained 10 μl of master cocktail, 10 μl of reticulocyte lysate, 4 μl of ^{35}S-methionine (sp.act. 1200 Ci/mmol) and

0.1 to 2 μg of poly(A)$^+$ mRNA. The master cocktail (1 ml) is made of 63μl of 1 mM amino acid mixture (except methionine), 300 μl of 1 M KCH_3CO_2, pH7.5, 100 μl of 40 mM Mg $(CH_3CO_2)_2$, 66 μl of 37.5 mM ATP and 15 mM GTP, 50 μl of 1.25 M Hepes-KOH, pH7.5 and 100 mM DTT, 15 μl of 100 mM spermidine, 25 μl of 10 mg/ml CPK (creatine phosphokinase), 50 μl of 400 mM creatine phosphate, 5 μl of calf liver tRNA (5 mg/ml) and 321 μl of H_2O. Translation was for 60 min at 37 C. An aliquot (5 μl) was taken to measure TCA-precipitable radioactivity. Depending on the mRNA used, stimulation of 10- to 70-fold above background was obtained. The remaining reaction mixture was centrifuged at 100,000 xg for 30 min at 4 C and the supernatant was used for immunoprecipitation. Goat antiserum no. 46, recognizing both rabbit mammary gland and rat liver PRL receptors, rabbit antisera nos. 201, 203, 211 and 212 directed against rat liver PRL receptor, anti-rabbit PRL receptor monoclonal antibody M110 and anti-rat PRL receptor monoclonal antibodies E21 and E29, characterized in our laboratory (Katoh et al., 1984, 1985 and 1987) were used to immunoprecipitate the <u>in vitro</u> translation products. For this purpose 50 μl of the reaction mixture supernatant was mixed with 200 μl of 25 mM Tris-HCl, pH 7.5, 10 mM CHAPS, 1 mM PMSF and with the antiserum (1:100 dilution) or with the monoclonal antibody (1 to 10 μg) of interest, overnight at 4 C. 250 μl of MAPS binding buffer (Bio-Rad) plus 10 mM CHAPS with 50 μl of Pansorbin (Calbiochem) or 75 μl of Protein-A Affigel (Bio-RAD) were then added. After an incubation for 30 min at room temperature, the suspension was centrifuged for 1 min in a microcentrifuge and the pellet was washed twice with MAPS binding buffer, 10 mM CHAPS, once with MAPS binding buffer alone, resuspended in 50 μl of SDS-PAGE sample buffer

[2.5% (w/v) SDS, 10% (w/v) glycerol, 5% (v/v) 2-mercaptoethanol, 65.2 mM Tris-HCl, pH 6.8] boiled for 3 minutes and after centrifugation, the supernatant was electrophoresed on a 5-15% SDS-polyacrylamide gel as described by Laemmli (1976). The gel was then fixed in 40% methanol/10% acetic acid, treated with EN^{3}HANCE (New England Nuclear), and fluorographed at -70 C on a Kodak O-Mat XAR film.

Oocyte microinjection and labeling

Fragments of ovary were surgically removed from an anaesthetized Xenopus laevis frog (Xenopus One, Ann Arbor, Mi.). Individual mature oocytes were isolated manually, rinsed well, and stored overnight at 19 C in modified Barth's saline, (Colman, 1984). Using a Leitz micromanipulator and borosilicate micropipettes of 0.75 mm internal and 1.5 mm outer diameters (FHC, Brunswick, ME), 50 nl of a 1 mg/ml solution of poly(A)$^+$ mRNA was injected into the vegetal pole of 50 to 100 healthy oocytes. They were then incubated by batches of 10 oocytes in 54 μl of modified Barth's saline with 6 μl of ^{35}S-methionine (sp.act. 1200 Ci/mmol) into an Eppendorf tube for 40 hours at 19 C. Afterwards the medium was removed and the oocytes were quickly frozen in liquid nitrogen and stored at -70 C until further procedure. Homogenization and immunoprecipitation were performed according to the method of Colman (1984) and the immunoprecipitates were electrophoresed and radioautographed as described above.

Construction of cDNA libraries and immunoscreening

Several female rat liver libraries prepared in Lambda gt 11 were obtained from Clontech (La Jolla, CA). Another λgt11 library was made by

Clontech from estrogen-treated female rat liver poly(A)$^+$ mRNA prepared in our laboratory. This unamplified library had a titer of 1.2×10^6/ml with 87% of clear plaques for a total of 7.3×10^5 independent clones and the mean insert size of 0.9 kb. The enriched library was prepared from selected poly(A)$^+$ mRNAs obtained through immunopurification of polysomes as described above. The polysomes were passed twice through an oligo-dT cellulose column and 100 μl fractions collected after the second passage were used directly for random-primed cDNA synthesis (Gubler and Hoffman 1983; Kraus et al., 1986). The double-stranded cDNA was blunt-ended, ligated to 8-mer EcoRI linkers (Pharmacia), and assembled in the EcoRI site of λgt11 phage DNA (Stratagene). The library contained 9×10^5 independent recombinants.

The immunoscreening method of Clontech was used to screen these libraries with both polyclonal (nos. 201, 211 and 212) and monoclonal antibodies (E21 and F11). However, we used the preformed streptavidin-peroxidase complex of Amersham as a sensitive detection method. The working dilution of the first antibody (1:200) was determined by detection of a fixed amount of partially purified receptor applied to a nitrocellulose filter.

Northern and Southern blot analysis

Poly(A)$^+$ mRNA used for Northern blot analysis was denatured for 60 min at 50 C in 1 M glyoxal, 50% DMSO, 10 mM phosphate buffer pH 6.5, before separation by electrophoresis on a 1.5% agarose gel in 10 mM phosphate buffer pH 6.5. RNA was transferred to a nylon membrane (Genescreen, NEN/Dupont) by capillary action in 1 M NaCl, baked at 90 C

and hybridized. The cDNAs used to make the probes were the EcoRI inserts of the purified phage DNAs. The λgt11 phages were isolated according to the method of Yamamoto et al. (1970) with some modifications: the CsCl gradient was composed of only two layers of 5 M and 3M. The phage DNA was extracted with 26 μl of 1M Tris-HCl, pH 8.0, 13 μl of 0.2 M EDTA, 150 μl of formamide and 800 μl of absolute ethanol, then it was phenol extracted.

DNA probes were labelled using the random-primed labelling method of Feinberg and Vogelstein (1983). Hybridization was performed overnight at 42 C in 50% formamide, 50 mM phosphate buffer (pH 6.5), 0.8 M NaCl, 0.5% SDS, 1mM EDTA. Filters were washed at 55 C in 12.5 mM NaCl, 0.1% SDS, and films were exposed from 1 to 36 hours with or without intensifying screens.

Southern blots were prepared as described by Maniatis et al., (1982) and hybridization and washing were as described for Northern blot except that 10% Dextran sulfate was used during hybridization.

REFERENCES

Argan C, Lusty CN, Shore GC 1983 J Biol Chem 258:6667-6670

Aviv H, Leder P 1972 Proc Natl Acad Sci (USA) 69:1408-1412

Borst DW, Sayare M 1982 Biochem Biophys Res Comm 105:194-201

Chirgwin JM, Przybyla AE, MacDonald RJ, Rutter WJ 1979 Biochemistry 18:5294-5299

Colman A 1984 In: Hames BD, Higgins SJ eds. Transcription and Translation -A Practical Approach, Oxford, IRL Press. pp 271-302

Cosman D, Cerretti DP, Larsen A, Park L, March C, Dower S, Gillis S, Urdal D 1984 Nature 312:768-771

Feinberg AP, Vogelstein B 1983 Anal Biochem 132:6-13

Gubler U, Hoffman BJ 1983 Gene 25:263-269

Haeuptle MT, Aubert ML, Djiane J, Kraehenbuhl JP 1983 J Biol Chem 258:305-314

Houdebine LM, Djiane J, Dusanter-Fourt I, Martel P, Kelly PA, Devinoy E, Severly JL 1985 J Dairy Sci 68:489-500

Jolicoeur C, Brakier-Gingras L 1983 Can J Biochem Cell Biol 61:1-7

Katoh M, Djiane J, Leblanc G, Kelly PA 1984 Mol Cell Endocrinol 34:191-200

Katoh M, Djiane J, Kelly PA 1985 J Biol Chem 260:11422-11429

Katoh M, Raguet S, Zachwieja J, Djiane J, Kelly PA 1987 Endocrinology 120:739-749

Kelly PA, Katoh M, Djiane J, Ferland LH 1986 In: Conn PM ed. "The Receptor", Academic Press (London), pp 355-391

Kraus JP 1985 In: Springer TA ed. Medicine, ed. Hybridoma Technology, in the Biosciences and Medicine, Plenum, New York, pp 407-417

Kraus JP, Williamson CL, Firgaira FA, Yang-Feng T, Munke M, Francke U, Rosenberg LE 1986 Proc Natl Acad Sci (USA) 83:2047-2051

Laemmli UK 1970 Nature 227:680-685

Leonard WJ, Depper JM, Crabtree GR, Rudikoff S, Dumphrey J, Robb RJ, Krönke M, Svetlik PB, Peffer NJ, Waldmann TA, Greene WC 1984 Nature 311:626-631

Lin CR, Chen WS, Kruiger W, Stolarsky LS, Weber W, Evans RM, Verma IM, Gill GN, Rosenfeld MG 1984 Nature 224:843-848

Maniatis T, Fritsch EF, Sambrook J 1982 Molecular Cloning: A Laboratory Manual, Cold Spring Harbor, New York: Cold Spring Harbor Laboratory

Merrick WC 1983 In: (ed) Methods in Enzymology vol 101, Academic Press Inc. pp 606-615

Miesfeld R, Okret S, Wikström A-C, Wrange O, Gustafsson J-A, Yamamoto KR 1984 Nature 312:779-781

Nikaido T, Shimizu A, Ishida N, Sabe H, Teshigawarak K, Maeda M, Uchiyama T, Yodoi J and Honjo T 1984 Nature 311:631-635

Noda M, Takahashi H, Tanabe T, Toyosato M, Furutami Y, Hirosi T, Asai M, Inayama S, Miyata T, Numa S. 1982 Nature 299:793-797

Noda M, Takahashi H, Tanabe T, Toyosato M, Kikyotami S, Hirosi T, Asai M, Takashima H, Inayama S, Miyata T, Numa S. 1983 Nature 301:251-255

Pelham HRB, Jackson RJ 1976 Eur J Biochem 67:247-256

Posner BI, Kelly PA, Friesen HG 1974 Proc Natl Acad Sci (USA) 71:2407-2410

Posner BI, Khan MN 1983 In: Tolis G et al. ed. Prolactin and Prolactinomas, Raven Press, NY, pp 9-18

Russell DW, Yamamoto T, Schneider J, Slaughter CJ, Brown MS, Goldstein JL 1983 Proc Natl Acad Sci (USA) 80:7501-7505

Schneider C, Kurkinen M, Greaves M 1983 EMBO J 2:2259-2263

Shapiro SZ,, Young JR 1981 J Biol Chem 256:1495-1498

Snyder M, Davis RW 1985 In: Springer TA ed. Hybridoma Technology, in the Biosciences and Medicine, Plenum, New York, pp 397-406

Ullrich A, Coussens L, Hayflick JS, Dull TJ, Gray A, Tam AW, Lee J, Yarden Y, Libermann TA, Schlessinger J, Downward J, Mayer ELV, Whittle N,

Waterfield MD, Seeburg PH 1984 Nature 309:418-425

Yamamoto KR, Alberts BM, Benzinger R, Lawhorne L, Treiber G 1970 Virology 40:734-

Young RA, Davis RW 1983a Proc Natl Acad Sci (USA) 80:1194-1198

Young RA, Davis RW 1983b Science 222:778-782

CHAPTER 5

CLONING AND EXPRESSION OF THE RAT PROLACTIN RECEPTOR, A MEMBER OF THE GROWTH HORMONE PROLACTIN RECEPTOR FAMILY

CLONING AND EXPRESSION OF THE RAT PROLACTIN RECEPTOR, A MEMBER OF THE GROWTH HORMONE/PROLACTIN RECEPTOR GENE FAMILY

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PREFACE

As mentioned in the Discussion of the last chapter, the use of immunoaffinity chromatography was a turning point in our investigation since it permitted the production of purer PRL-R preparations in greater yield. With the availability of amino acid sequence of tryptic fragments of receptor, oligonucleotide probes were prepared. As described in Chapter 1, such probes are, by far, the most effective tools for screening cDNA libraries. In this Chapter we report their use in the successful cloning of the rat prolactin receptor.

SUMMARY

The primary structure of the rat liver prolactin receptor has been deduced from a single complementary DNA clone. The sequence begins with a putative 19 amino acid signal peptide followed by the 291 amino acid receptor that includes a single 24 amino acid transmembrane segment. In spite of the fact that the prolactin receptor has a much shorter cytoplasmic region than the growth hormone receptor, there is strong localized sequence identity between these two receptors in both the extracellular and cytoplasmic domains, suggesting the two receptors originated from a common ancestor.

INTRODUCTION

The anterior pituitary hormone prolactin (PRL) is encoded by a member of the growth hormone/prolactin/placental lactogen gene family. In mammals, it is primarily responsible for the development of the mammary gland and lactation. Prolactin stimulates the expression of milk protein genes by increasing both gene transcription and mRNA half-life (Matusik and Rosen, 1980; Houdebine, 1985). In addition to the classical effects in the mammary gland, prolactin has been shown to have a number of other actions, all of which are initiated by an interaction with specific high affinity receptors located on the plasma membrane and widely distributed in a number of tissues (Posner et al., 1974a; Djiane et al., 1977). Different biochemical approaches (crosslinking, immunoprecipitation or immunoblot with monoclonal antibodies) have shown that the prolactin receptor has a relative molecular mass (M_r) of ~40,000

and is apparently not linked by disulfide bonds to itself or to other subunits (Haeuptle et al., 1983; Katoh et al., 1984, 1985, 1987; Kelly et al., 1984; Sakai et al., 1984).

Prolactin receptor levels are differentially regulated depending on the tissue studied. In rat liver, one of the tissues with the highest prolactin binding, receptor levels vary during the estrous cycle (Kelly et al., 1974), increase during pregnancy (Kelly et al., 1975) and are markedly stimulated by estrogens (Posner et al., 1974b). Prolactin plays a major role in the regulation of its own receptor, inducing both up- and down-regulation depending on the concentration and duration of exposure to prolactin (Posner et al., 1975; Manni et al., 1978; Djiane et al., 1979). As is true for the growth hormone (GH), no means of signal transduction has been identified for prolactin. There are no clear effects of prolactin on cyclic AMP, cyclic GMP, inositol phospholipids, phosphorylation, calcium ions, or ion channels (Matusik and Rosen, 1980; Kelly et al., 1984; Houdebine et al., 1985). Neither the GH nor the PRL receptor appear to be a tyrosine kinase. A better understanding of prolactin receptor structure, regions involved in hormone binding, signal transduction and possible homology with other hormone receptors should help shed some light on the mechanism by which prolactin induces its various actions.

Recently, the amino acid sequences of the rabbit and human GH receptors were deduced from their respective cDNA sequences (Leung et al., 1987). The authors found no sequence homology of the GH receptor with any other reported protein.

Here we report the complete amino acid sequence of the rat prolactin receptor deduced from liver cDNA clones, revealing the structural extracellular, single membrane spanning and cytoplasmic domains. A comparison of sequences demonstrates regions of identity between the rat prolactin receptor and mammalian growth hormone receptors.

RESULTS

Purification and protein sequencing

Prolactin receptors were purified from solubilized estrogen-treated female rat liver membrane preparations (Fig. 1 and Katoh et al., 1987). Approximately 6 mg of partially purified receptor were prepared by immunoaffinity chromatography using E21, a monoclonal antibody specific to rat prolactin receptor. Crosslinking of the prolactin receptor reveals a specific M_r 61,000 band corresponding to the hormone-receptor complex (Fig. 1a). When this partially purified preparation was run on a polyacrylamide gel and analyzed by Western immunoblot, the receptor had an apparent M_r of 41,000 (Fig. 1b). Preparative amounts of receptor were purified by polyacrylamide gel electrophoresis after reduction and alkylation and the protein in the 38-43 kDa region was electroeluted. Fig. 1c shows the electroeluted material rerun on a Phast SDS gel (Pharmacia).

The homogeneous prolactin receptor was digested exhaustively with trypsin and the resulting peptides purified on reverse phase HPLC. Amino acid sequences were obtained by gas phase sequence analysis. Ten readable sequences were identified. From the first few sequences obtained, three synthetic oligonucleotide probes were prepared. These

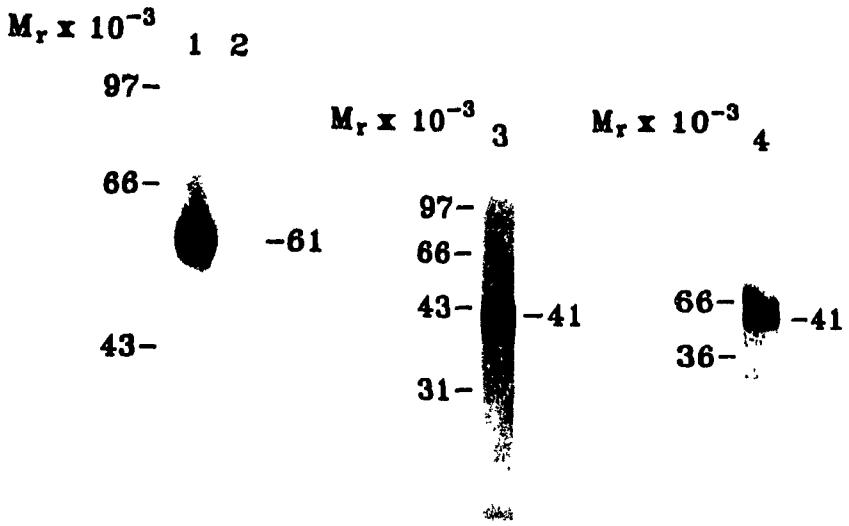

FIG. 1 Prolactin Receptor Purification: SDS-polyacrylamide Gel Electrophoresis (SDS PAGE)

a) Crosslinked [^{125}I]oPRL to membrane bound (microsomal) PRL receptor from rat liver in the absence (lane 1) and presence (lane 2) of an excess of unlabelled oPRL. The PRL-receptor complex migrates with an apparent M_r of 61,000. b) Partially purified receptor revealed by Western immunoblot analysis using a monoclonal antibody (E21) against the rat liver PRL receptor (lane 3). c) Receptor purified by electroelution and detected by silver staining (lane 4). The purified receptor has a M_r of ~41,000 as detected either by immunoblot or silver staining.

corresponded to residues 173-180, 203-210 and 173-187 (see Fig. 3). The first two, a 23-mer and a 24-mer were partial mixtures of nucleotides and a third, a 45-mer was derived from the best predicted sequence from mammalian codon usage.

Cloning of PRL receptor cDNA

Initially, we attempted to isolate a PRL receptor cDNA by screening λgt11 expression libraries prepared from normal and estrogen-treated female rat liver mRNA with both polyclonal and monoclonal antibodies isolated in our laboratory (Katoh et al., 1987). All positive signals were considered to be false positive recombinants by Northern blot analysis of mRNA from various rat tissues, based on mRNA abundance and the pattern of receptor distribution. Since the abundance of the receptor protein is very low (0.0002%), we decided to enrich PRL receptor mRNA by immunopurification of polysomes (Shapiro and Young, 1981). A λgt11 cDNA library was prepared using enriched (500-fold) mRNA from estrogen-treated female rat liver. Screening with monoclonal antibodies to the rat PRL receptor again yielded only false positives.

Initial screening of 40,000 clones with the 23-mer oligonucleotide PRLR-2A

$$5'\text{-}GT^{A}_{G}AA^{A}_{G}TG^{A}_{G}AT^{T}_{C}TCCCA^{T}_{C}TC^{T}_{C}TC\text{-}3'$$

detected two positive recombinant phages in the polysome-enriched cDNA library, clones E1 and E2. Insert E1 (93 bp), contains the entire sequence corresponding to peptide T21.1 used to prepare the PRLR-2A probe, plus regions corresponding to two flanking tryptic fragments (T25.1 and T27). Insert E2 was shorter and included within the sequence

of E1. A longer cDNA clone (F3) was subsequently isolated from a library prepared using size-selected mRNA from estrogen-treated liver with clone E1 as a RNA probe (2.5×10^5 recombinants screened). A restriction map of clone F3 is shown in Fig 2. Figure 3 illustrates the entire sequence of the EcoRI insert of clone F3. The hepatic PRL receptor is encoded by a messenger of approximately 2.2 kb in length (see Fig. 5), of which 1635 bp are included in the insert F3, containing a complete open reading frame that corresponds to a protein of 310 amino acids. The sequence surrounding the initiation codon (AACATGC) does not exactly match the consensus sequence proposed by Kozak (1986). A potential polyadenylation signal (AATAAA) is found at position 1414. An unusual feature of clone F3 nucleotide sequence is a 50 bp long repetitive TC sequence beginning at position 1284 in the 3' non-translated region, the potential significance of which is not known.

The initial methionine is followed by a 19 hydrophobic amino acid stretch (Fig. 4)indicative of a signal peptide sequence (Gascuel and Danchin, 1986). Two attempts to obtain the N-terminal sequence were unsuccessful, which suggests that the Gln at position 20 may be present as a pyro-Glu residue. The mature receptor would thus correspond to a 291 amino acid protein with a theoretical M_r of 33,368. Differences between the predicted M_r of the F3-encoded protein and the M_r of the hepatic PRL receptor detected by Western blot analysis (41,000) might be accounted for by glycosylation, since treatment of highly purified receptor with endoglycosidase F gives a band at ~36,000 (data not shown). All ten tryptic fragments previously identified are located within the protein sequence deduced from F3 without any mismatches. The deduced amino acid sequence allows determination of the hydropathy

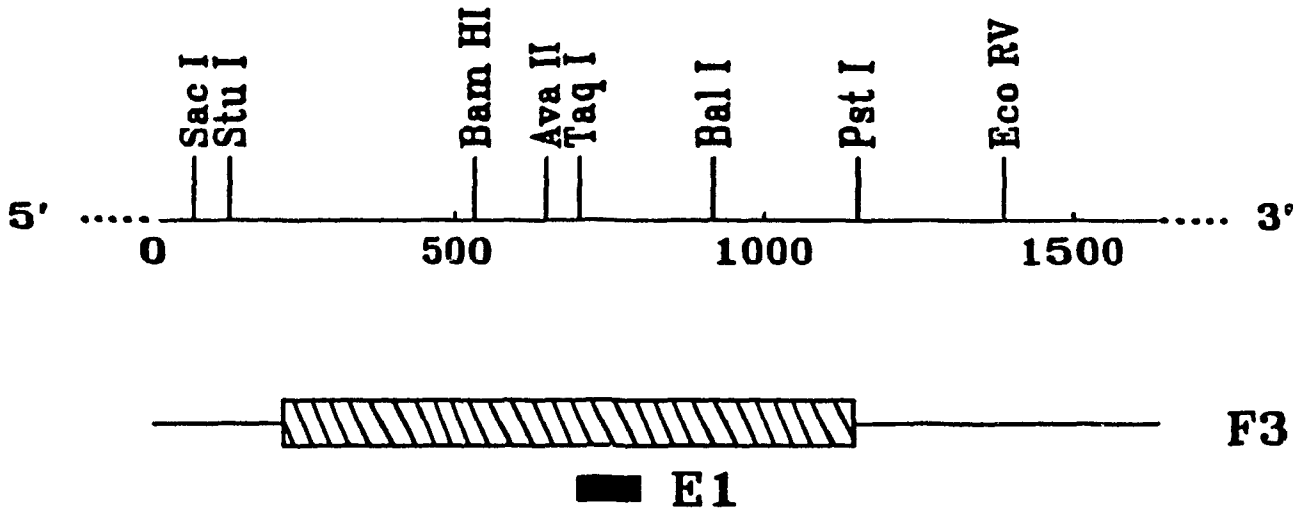

FIG. 2 Prolactin Receptor cDNA Restriction Map

The hatched box indicates the predicted coding region of clone F3. Unique restriction sites are indicated, as well as the localization of clone E1.

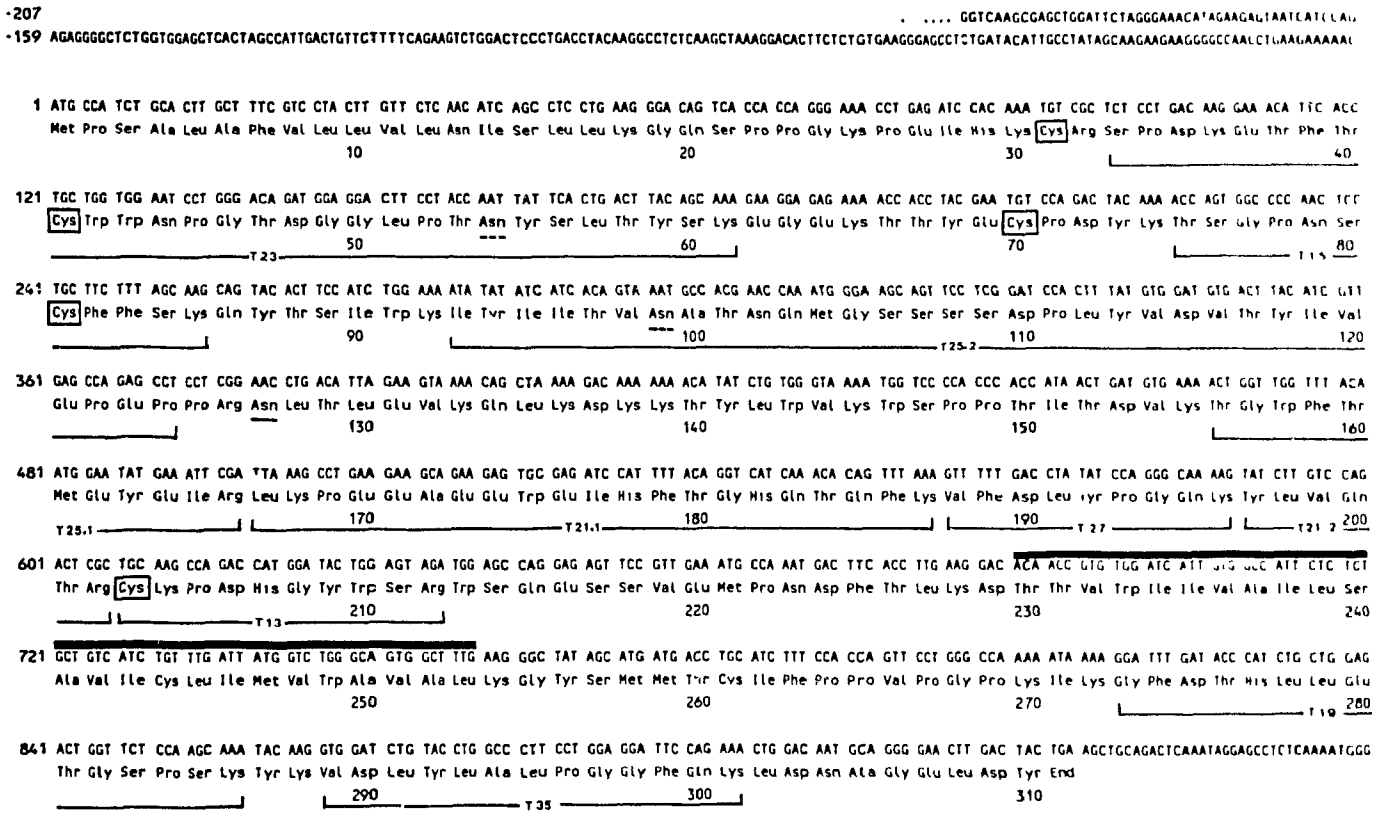

FIG. 3 Nucleic Acid and Predicted Amino Acid Sequence of the Prolactin Receptor

Nucleotides are numbered at left and amino acids below the sequence, starting with the initiation codon and the corresponding methionine, respectively. Sequences of tryptic fragments (T) obtained from purified receptor are underlined and numbered according to their order of elution. The black bar above the nucleotides and the corresponding amino acids represents the putative transmembrane domain. Extracellular cysteines are boxed and potential Asn-linked glycosylation sites are underlined with a dashed line.

profile (Fig. 4), which reveals, besides the signal peptide, a single strongly hydrophobic region of 24 amino acids (230-253). This presumably corresponds to the transmembrane region of the PRL receptor, separating the N-terminal, extracellular PRL binding region from the very short C-terminal cytoplasmic region, likely involved in signal transduction. Five Cys (residues 31, 41, 70, 81 and 203) are present in the extracellular domain of the PRL receptor, as well as three potential Asn glycosylation sites (amino acids 54, 99 and 127), the first two of which were shown to be glycosylated by sequence analysis, while the third was not analyzed.

Northern Blot Analysis

Using a RNA probe derived from the cDNA E1, the tissue distribution of mRNA was analyzed in 17 rat tissues (Fig. 5a). Northern blot analysis shows that mRNAs hybridizing to this probe are present only in tissues which have previously been shown to contain PRL receptors, and absent in all other tissues thus far examined. Large amounts of poly $(A)^+$ mRNA (100 μg) were used to clearly demonstrate this fact.

The relative abundance of the mRNA correlates well with specific binding of prolactin in these tissues (Posner et al., 1974a; Kelly et al., 1984). Indeed, the strongest signal is found in liver, followed by ovary and prostate and a weak signal is present in the other tissues expressing the PRL receptor, including the mammary gland. Moreover, the level of expression is hormone dependent in liver, since the signal is weak in hypophysectomized and normal males, higher in normal females, and markedly increased in estrogen-treated female rats (Posner et al., 1974b,

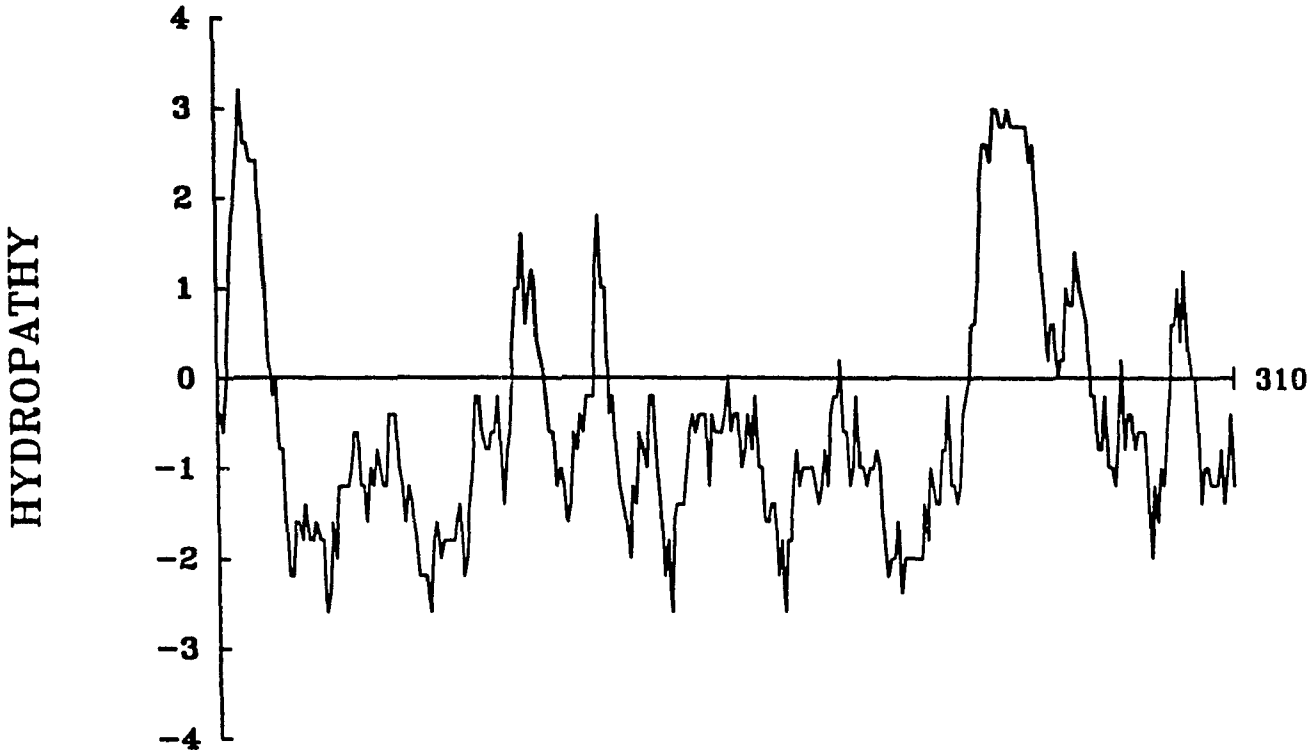

FIG. 4 Prolactin Receptor Hydropathy Profile

The values are calculated using the method of Kyte and Doolittle (1982), with a window of 11 residues; positive values indicate hydrophobicity.

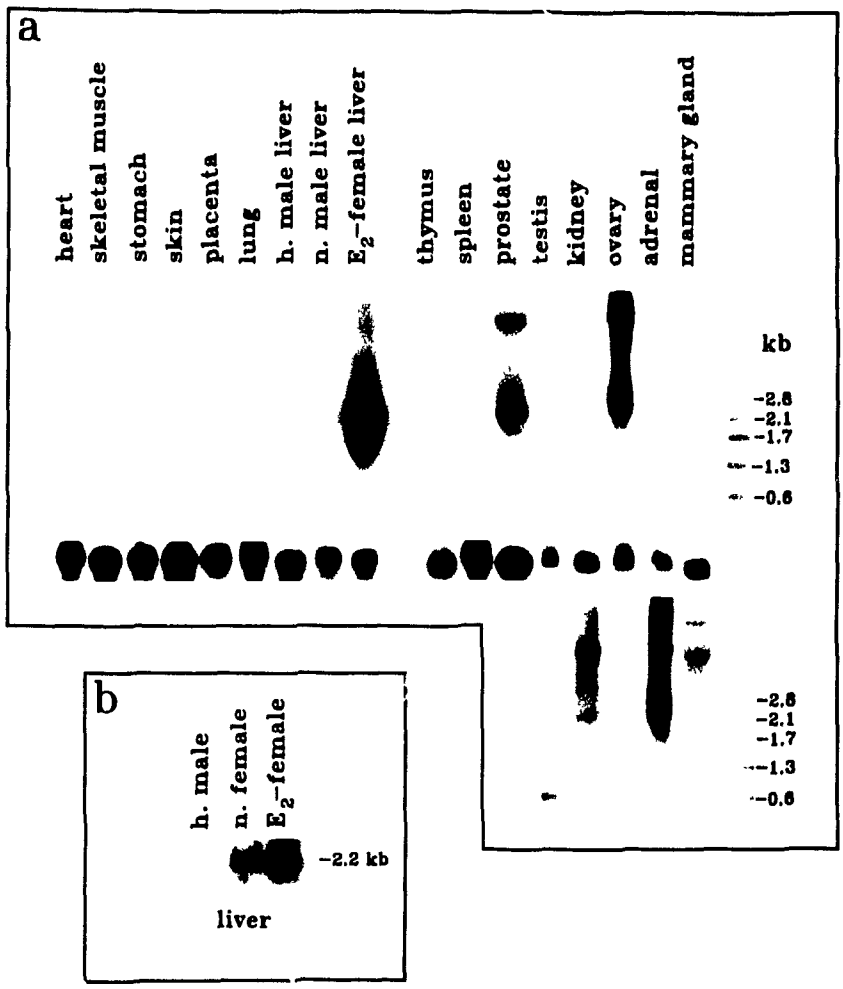

FIG. 5 Northern Blot Analysis of Prolactin Receptor mRNA Expression

a) Northern blot of 17 rat tissues analyzed with a RNA probe complementary to clone E1 (93 bp). One hundred μg of polyadenylated mRNA were used for each lane. In the upper part of the figure, the X-ray film was exposed 16 h without intensifying screen. The same blot was autoradiographed 36 h with an intensifying screen, in order to detect low-abundance mRNA in testis, kidney, adrenal and mammary gland, shown in the right bottom part of the figure. Size of RNA molecular weight markers is indicated on the right. The middle panel illustrates hybridization of the same blot with a DNA probe complementary to clone B1 (see Materials and Methods). b) Northern blot of rat liver corresponding to different hormonal status, analyzed with a DNA probe complementary to clone F3 (1635 bp). Fifty μg of polyadenylated mRNA were used. X-ray film was exposed 2 h without an intensifying screen.

1975; Djiane et al., 1979). This progression is illustrated in Fig 5b.

As is the case for other hormone receptors, multiple forms of messenger RNAs exist (Ebina et al., 1984; Hollenberg et al., 1984; Ullrich et al., 1984; Conneely et al., 1986). The 2.2 kb band is the only one found in the liver, and is clearly a major species in the prostate and ovary. A slightly larger band is seen in the adrenal. Another major mRNA form is the ~4 kb band, which is the predominant form in the kidney and mammary gland. Larger forms are also seen in the ovary and adrenal. In the testis, the signal is very weak, although faint bands are seen at 2.2 and 4 kb. In addition, a 0.6 kb mRNA is detected only in this tissue.

Expression

In order to determine whether the clone F3 would direct the synthesis of functional rat PRL receptor in a heterologous cell system, F3 was inserted into different vectors and expressed in various cell types. First, we used the Bluescript vector with its transcription promoter T3 to synthesize F3 mRNA and microinjected this in vitro transcript in Xenopus oocytes. The expression of the PRL receptor at the cell surface is shown by significant binding of [^{125}I]ovine PRL (oPRL) and specific displacement with unlabelled oPRL (Fig. 6).

In addition, the PRL receptor was expressed both transiently and stably in mammalian cells (Fig. 7 a-d) by using vectors containing the promoter region, origin of replication and polyadenylation signal of the SV 40 genome. Transient transfections were performed in COS-7 monkey kidney cells using the pECE/F3 construct while stable CHO cell lines were

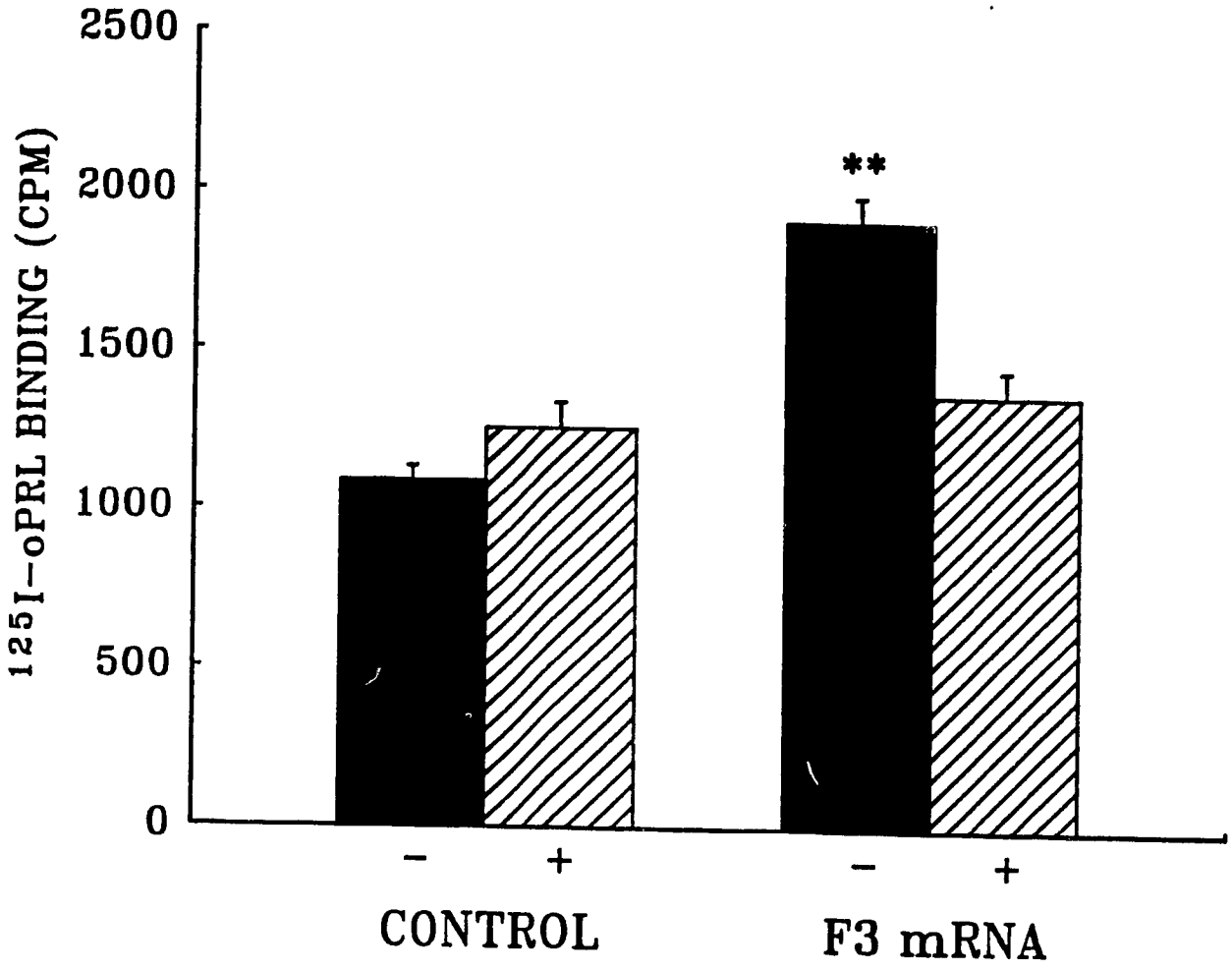

FIG. 6 **Expression of the Rat Liver PRLR/F3 mRNA in Xenopus Laevis Oocytes**

Binding of [^{125}I]oPRL by oocytes injected with water (control) or with F3 mRNA in the absence (-) or presence (+) of an excess of unlabelled oPRL. Each value represents the mean ± SEM of 20 oocytes. ** indicates $p < 0.01$.

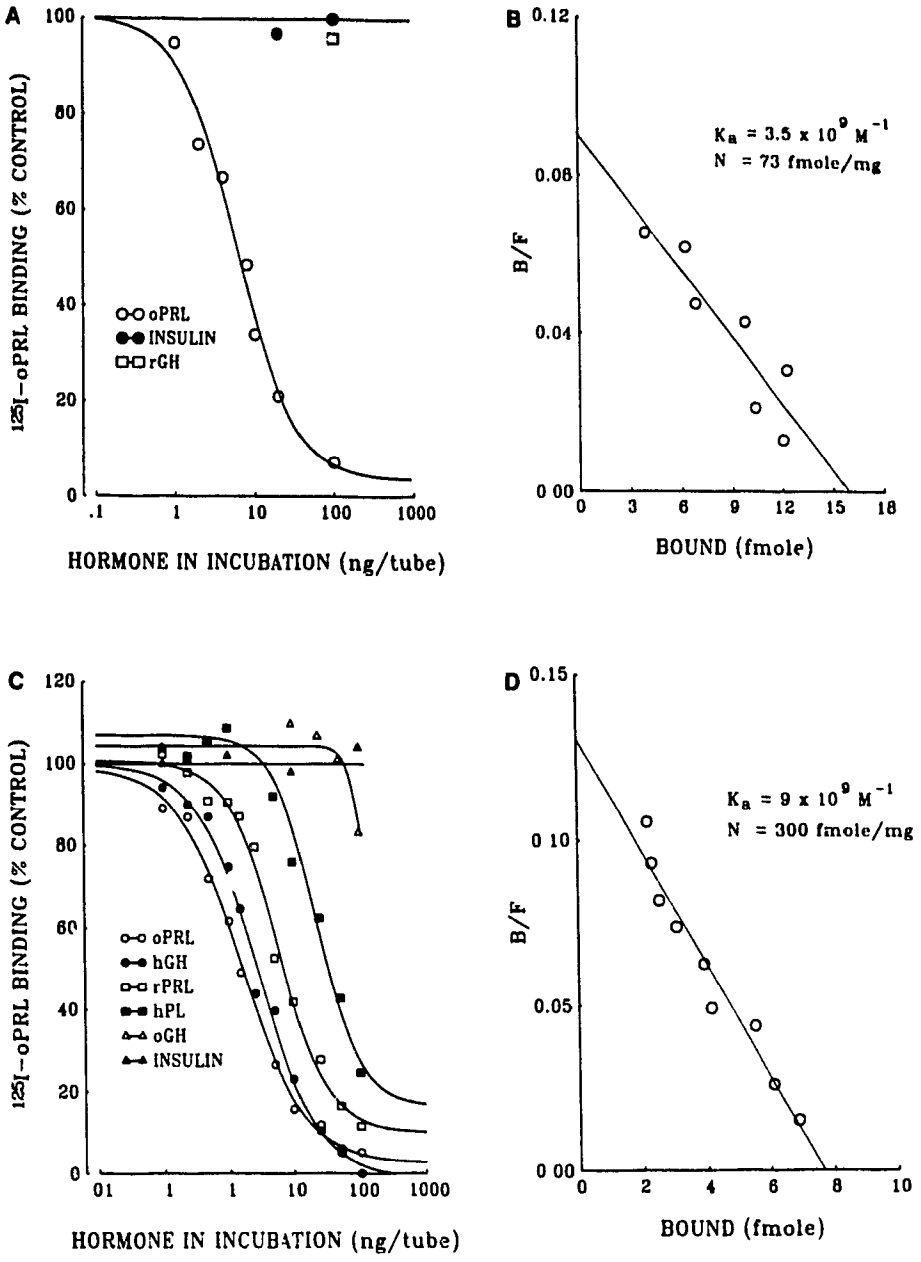

FIG. 7 Expression of the Rat Liver PRLR/F3 cDNA clone in Mammalian Cells

(a) Competition assay with unlabelled oPRL (○), bovine insulin (●) and rat GH (□) of membranes from COS-7 cells transfected transiently with pECE/F3. Values are expressed as a % of specific binding calculated in the absence of unlabelled hormone (4930 ± 80 cpm/200 μg protein). Non-specific binding is 4520 ± 130 cpm. b) Scatchard plot of PRL binding data in COS-7 cells. c) Competition assay with unlabelled oPRL (○), human GH (●), rat PRL (□), human PL (■), ovine GH (△), bovine insulin (▲) of membranes from CHO cells stably transfected with pKCR2/F3. Values are expressed as a % of specific binding calculated in the absence of any unlabelled hormone (4500 ± 90 cpm/25 μg protein). Non-specific binding is 1120 ± 40 cpm. d) Scatchard plot of PRL binding data in CHO cells.

established by co-transfection of the pKCR2/F3 construct with the plasmid pSV2 neo conferring resistance to the neomycin analog G418. Scatchard analysis demonstrated that membrane extracts from transfected COS-7 cells bind [^{125}I]oPRL with an affinity (K_a = 3.5 nM^{-1}) similar to that reported for the rat liver PRL receptor (Posner et al., 1974b; Kelly et al., 1983), with a receptor number of 73 fmol/mg protein (Fig 7b). For the stably transfected CHO cells, binding affinity is slightly higher (K_a = 9 nM^{-1}) but within the normal range, with a total receptor number of 300 fmol/mg protein (Fig 7 d). Binding specificity studies with different hormones (oPRL, rat PRL, human GH, rat GH, ovine GH, human placental lactogen and bovine insulin), confirm that the clone F3 encodes the PRL receptor (Fig 7 a,c). The transiently transfected COS-7 cells contained $\sim$ 30,000 receptors per cell. Although receptor number varied a great deal in the different stably transfected CHO cell lines, a value of $\sim$ 10,000 receptors per cell was observed. At the concentration of membrane protein used in the assay, no binding was detected with control cells nor with those transfected with pKCR2, pECE or with the antisense construct pKCR2/3F.

DISCUSSION

The cloning and expression studies described here clearly demonstrate that the PRL receptor is a small M_r protein ($\sim$40,000) that does not require association with another protein for hormone binding. This confirms our previous studies as well as those of others (Liscia and Vonderhaar, 1982; Haeuptle et al., 1983; Necessary et al., 1984). Larger M_r forms of the PRL receptor have been reported for detergent solubilized receptors from rat ovary and liver (Bonifacino and Dufau,

1984; Haldosén and Gustafsson, 1987). Although we have confirmed this interesting observation (Kelly et al., 1988), specific conditions are necessary for the larger M_r form to be seen. Whether the M_r 80,000 band identified in these studies is formed by two M_r 40,000 receptor molecules crosslinked together or a M_r 40,000 receptor linked to some other protein remains to be determined. Functional studies of transfected PRL receptor cDNA coupled with structural analysis should help clarify this point.

Northern blot analysis was an important tool in identifying the PRL receptor cDNA. The clone E1 was the first positive recombinant for which the RNA profile corresponded to the tissue distribution of the PRL receptor. Interestingly, multiple mRNA forms in various tissues are observed. These different transcripts could be derived from different genes or from the same gene either by use of different initiation (Leonard et al, 1985) or polyadenylation (Tosi et al., 1981) sites or by alternative splicing of a common primary transcript (Rosenfeld et al., 1984; Schejter et al., 1986).

As recently reported for the GH receptor (Leung et al., 1987), the prolactin receptor shows no sequence similarity to any other reported protein. However, a comparison of the sequence of these two receptors with each other shows regions of striking similarity. There is ~30% overall sequence identity between the two proteins, when the last 293 amino acids of the cytoplasmic domain of the GH receptor are excluded. As shown in Fig. 8, there are five regions of increased homology between the rat PRL receptor and both the rabbit and human GH receptors. The residues between the first and second Cys and the third and fourth Cys are highly conserved (> 67% identity), followed by two regions of lower

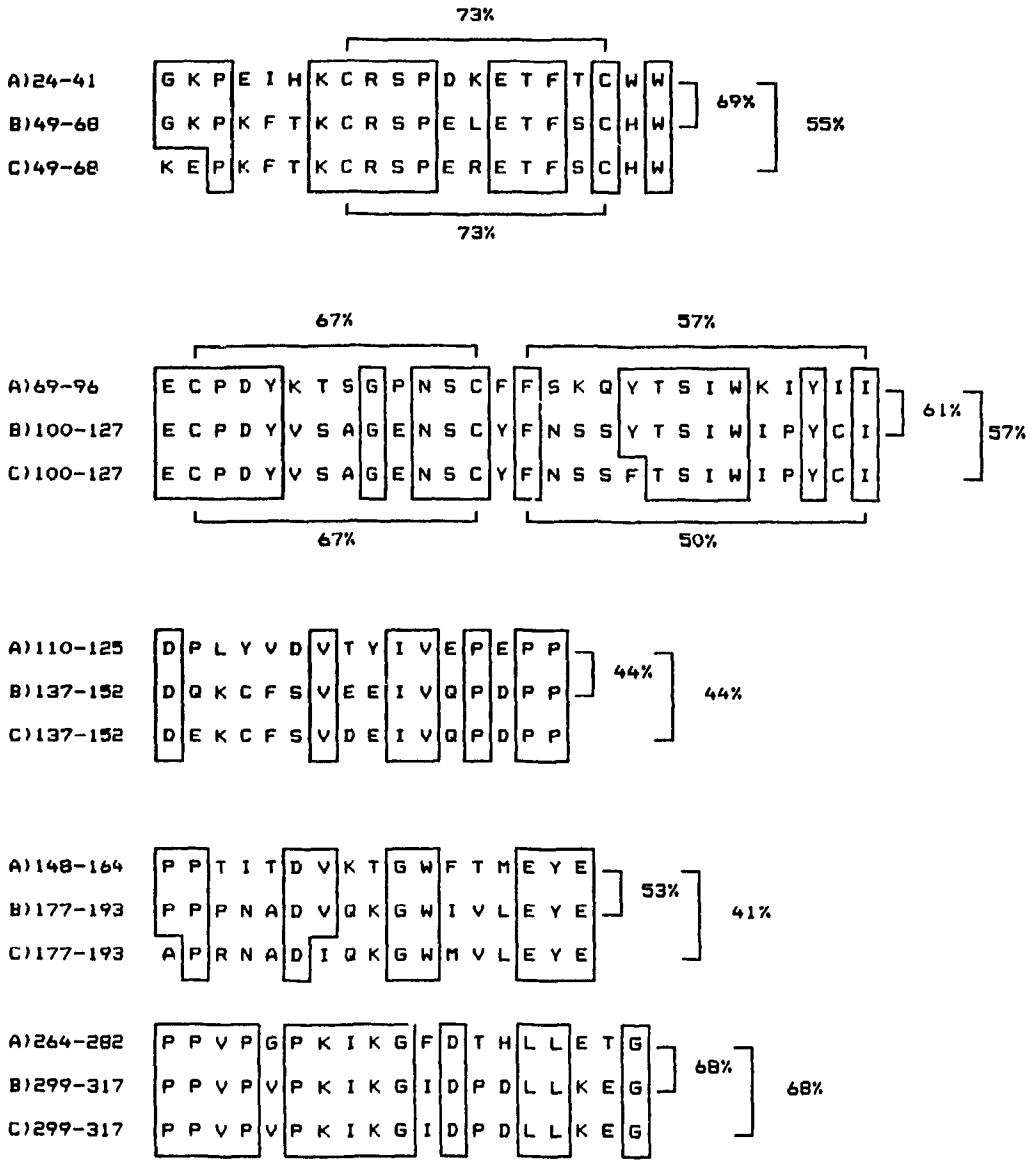

FIG. 8 Prolactin and Growth Hormone Receptor Sequence Comparison

Extracellular and cytoplasmic sequences of PRL receptor are compared with corresponding segments of rabbit and human GH receptors (Leung et al., 1987). Matching residues are boxed. A indicates rat PRL receptor, B and C, rabbit and human GH receptors, respectively. Numbers to the left of the sequences indicate the amino acids at the beginning and end of the respective homologous regions, numbered from the initial methionine. Percent identity between various regions is indicated.

homology (40-60%). In addition, a series of 19 residues in the cytoplasmic domain of both receptors is highly conserved (68% identity). When conservative amino acid substitutions are considered, the similarity in the cysteine regions increases to 75-100%, with similarity in the other regions also increasing proportionally. Figure 9 shows that the linear arrangement of these homologous segments is also conserved. Interestingly, despite a marked difference in the size of the cytoplasmic domains of the PRL and GH receptors, the identical location of a highly conserved sequence suggests its involvement in signal transduction for both hormones and may be of potential interest in elucidating their mechanisms of action.

When the overall structure of the prolactin receptor is considered, it has a large extracellular region involved in hormone binding, a single transmembrane segment and a very short cytoplasmic domain. Both the GH and PRL receptors are thus a part of the single membrane-spanning class of receptors. However, they do not possess tyrosine kinase activity, or a potential phosphorylation site in their cytoplasmic domain, as is true for many growth factor receptors (Hunter, 1987). In addition, the short cytoplasmic segment of the PRL receptor is reminiscent of the structural arrangement of the transferin (Schneider et al., 1984), LDL (Yamamoto et al., 1984) and IGF-II/M6P (Morgan et al., 1987) receptors, which act primarily as transporters for transferin, cholesterol and mannose 6-phosphate, respectively. In fact, prolactin has been detected in milk, cerebral spinal fluid and semen, suggesting that in the mammary gland, choroid plexus and testis where prolactin receptors have been localized, these molecules may, in addition to their normal function of signal transduction, also act as transport proteins, translocating the hormone

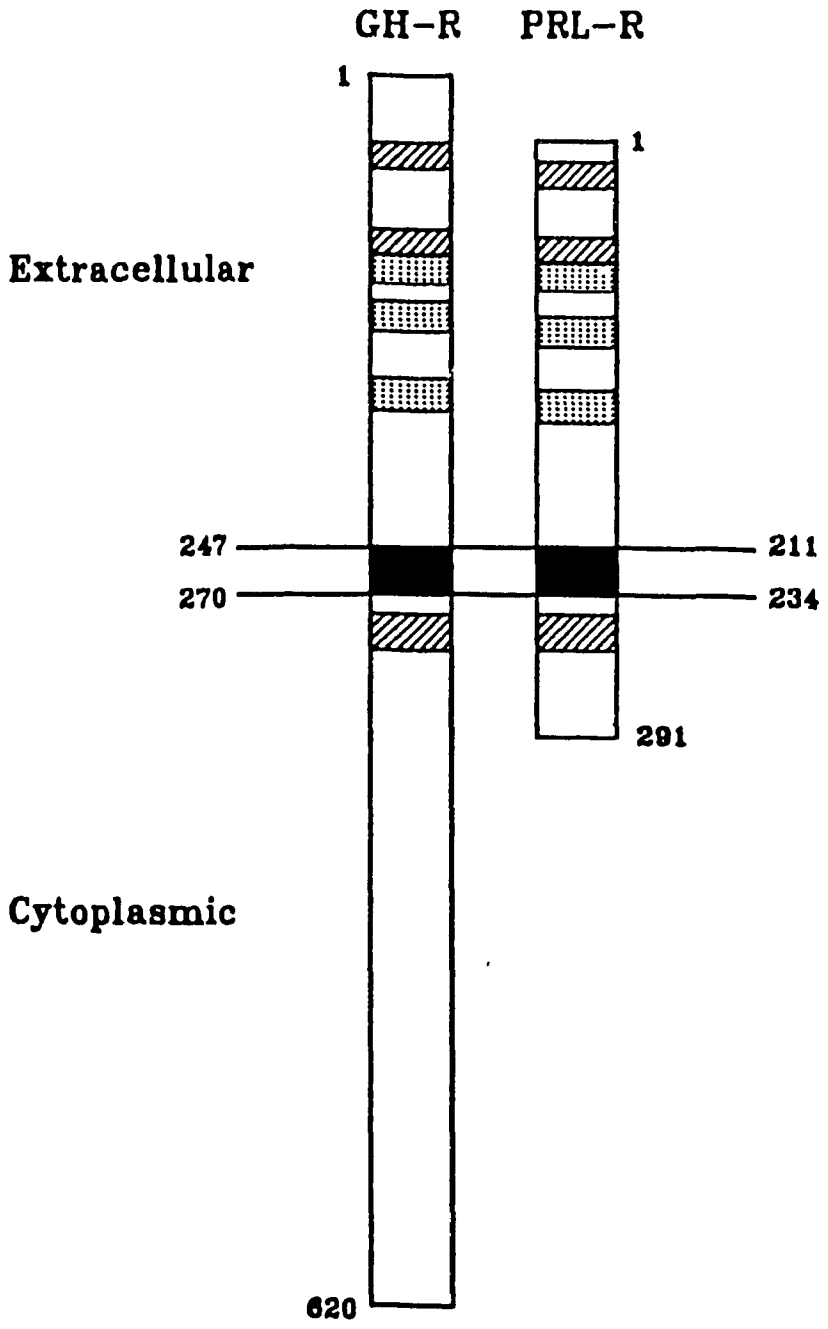

FIG. 9 Schematic Comparison of GH and PRL Receptor Structures

Numbers indicate the putative position of the first amino acid, the transmembrane region (black) and the last amino acid of the mature receptor. Regions of high similarity (>67%) are crosshatched and moderate similarity (40-60%) are stippled.

from one compartment to another.

The localized region of high homology with the GH receptor in the cytoplasmic domain suggests some important role. Whether this short segment alone, or in association with some other membrane component is responsible for the transfer of the hormonal message must await mutagenesis studies in a functional system.

MATERIALS AND METHODS

Receptor Crosslinking, Immunoblot Analysis and Purification

For crosslinking, 300 μg of rat liver microsomes were incubated with 2 x 10^5 cpm (specific activity = 80 μCi/μg) of [^{125}I]oPRL for 16 h in the absence or presence of 1 μg of unlabelled oPRL in a total volume of 500 μl in 25 mM Tris, 10 mM $MgCl_2$, 0.1% BSA, pH 7.4, after which disuccinimidyl suberate (0.5 mM) was added and the tubes incubated 15 min at 4°C (Katoh et al., 1985). An aliquot was analyzed in a 7.5% SDS PAGE. For Western immunoblot, 10 μg of partially purified (2% pure) receptor was run on a 5-15% SDS PAGE and transferred to nitrocellulose membrane. Following incubation with monoclonal antibody E21 (Katoh et al., 1987) the band was detected and visualized with colloidal immunogold and silver. The prolactin receptor was purified initially by immunoaffinity chromatography using E21 monoclonal antibody, according to the techniques described for hormone affinity chromatography (Katoh et al., 1987), reduced and alkylated and subsequently repurified by preparative SDS PAGE. The band corresponding to M_r of 38,000 to 43,000 was cut out and electroeluted (Jacobs and Clad, 1986) using an Elutrap (Schleicher and Schuell). Analytical SDS PAGE was performed on a Phast

system (Pharmacia) using a 10-15% gradient gel and stained with silver according to manufacturer's instructions.

Protein Sequence Analysis

Peptides of the homogeneous, electroeluted PRL receptor were prepared by digesting ~ 1 nmole of reduced and alkylated protein with trypsin (25:1) in 25 mM Tris pH 7.4 at 37°C overnight. The peptides were purified by high-pressure liquid chromatography (HPLC) on a Hypersil ODS 5 μm (100 x 2.1 mm) column in 0.1% trifluoroacetic acid with a gradient of acetonitrile. Chromatography was performed with a Hewlett Packard system model 1090 equipped with a diode array detector. Fractions were collected by hand and when necessary, further purified on the same HPLC system with a shallower gradient. Sequences were determined by gas-phase analysis on an Applied Biosystems model 470A equipped with an on-line system for PTH amino acid detection.

Construction of Rat Liver cDNA Libraries

Polysomes (11,400 $O.D._{260}$ units) were prepared from estrogen-treated female rat liver (970 g), and immunopurified by incubation overnight with rabbit anti-rat hepatic PRL receptor γglobulin (110 mg) and passage through a protein-A agarose column as described by Shapiro and Young, 1981. After elution of the polysomal material (34 $O.D._{260}$ units), poly $(A)^+$ mRNA was purified twice by chromatography on oligo-dT cellulose (Aviv and Leder, 1972). Eight μg of poly $(A)^+$ mRNA were obtained after the first passage and 100 μl fractions collected after the second passage were used directly for random-primed cDNA synthesis (Gubler and Hoffman,

1983; Kraus et al., 1986). The double-stranded cDNA was blunt-ended, ligated to 8-mer EcoRI linkers (Pharmacia), and assembled in the EcoRI site of λgt11 phage DNA (Stratagene). The library contained 9 x 10^5 independent recombinants. Clone F3 was isolated from a λgt11 library, custom prepared by Clontech Laboratories, Inc. using size-selected poly (A^+) mRNA from estrogen-treated female rat liver. This library contained 7.3 x 10^5 independent recombinants.

Nucleic Acid Hybridization Screening

Synthetic oligonucleotides were ^{32}P-labelled by 5'-end phosphorylation (Maniatis et al., 1982). Hybridizations with oligonucleotide probes were performed overnight at 37°C in 5X SSPE, 1X Dernhardt, 2mM Na-pyrophosphate, and 10% dextran sulfate. Probe concentration was 1 pmole/ml. Filters were washed in 3M TMAC (Wood et al., 1985) at 50-55°C and autoradiographed 48 h with an intensifying screen. The RNA probe E1 was synthesized using Bluescript transcription system (Stratagene). Hybridizations with RNA probe were performed overnight at 42°C in 50% formamide, 50 mM Na_2HPO_4 pH 6.5, 0.8M NaCl, 0.5% SDS, 1mM EDTA, and 10% dextran sulfate. Filters were washed at 60°C in 12.5 mM NaCl, 0.1% SDS, and films were exposed overnight with intensifying screens. The nucleotide sequence of both clones E1 and F3 were determined by the dideoxy chain termination method (Sanger et al., 1977).

Northern Blot Analysis

RNA was extracted from different tissues; except where specified, adult animals were used: heart, skeletal muscle, stomach, liver, lung, thymus, spleen, prostate and testis from normal male; kidney, ovary and adrenal from normal female; skin from newborn; placenta and mammary gland from 18-day pregnant female; liver from hypophysectomized male and estrogen-treated female. Total RNA was purified using the guanidium thiocyanate/CsCl procedure (Chirgwin et al., 1979), and polyadenylated mRNA was purified by oligo-dT cellulose chromatography (Aviv and Leder, 1972). Poly $(A)^+$ mRNA samples were treated 60 min at 50°C in 1M glyoxal, 50% DMSO, 10 mM Na_2HPO_4 pH 6.5, and run through a 1.5% agarose gel in 10 mM Na_2HPO_4. RNA was transferred to a nylon membrane (GeneScreen, NEN/Dupont), baked, and hybridized to either E1 RNA probe or F3 DNA probe. The hybridized material was subsequently removed by boiling 30' in 0.1X SSC, 1% SDS and the membrane hybridized with a DNA probe complementary to clone B1 and autoradiographed three hours without intensifying screen. Clone B1 is a cDNA previously obtained from antibody screening, which codes for a 1.5 kb mRNA of similar relative abundance in all tissues studied, as revealed by comparison with an actin probe (not shown). RNA molecular weight markers were constructed by linearizing the pGEM vector (Promega BIO/CAN) at different positions; the RNA fragments subsequently transcribed (555 bp, 1311 bp, 1653 bp, 2095 bp and 2835 bp) were treated as described for the samples and revealed by hybridization to a DNA probe complementary to pGEM. Hybridization and membrane washing were performed as described

previously except that the membrane was washed at 55°C for the DNA probes and at 60°C for the RNA probe. RNA probe was synthesized as described previously and DNA probes were labelled using the random-primed labelling method of Feinberg and Vogelstein, 1983.

Translation of mRNA in Xenopus Laevis Oocytes

The entire prolactin receptor F3 cDNA was subcloned into Bluescript vector at the EcoRI site. RNA transcripts were obtained after linearization and transcription in vitro with T_3 polymerase as described by Melton et al. (1984). Fifty nl of this synthetic RNA (1 $\mu g/\mu l$) was injected into individual Xenopus oocytes as described by Colman (1984). The oocytes were then incubated at 18°C for 2 days in modified Barth's medium. During the last 18 hours of incubation, 1 x 10^6 cpm of [^{125}I]oPRL per 70 μl of medium was added and [^{14}C]sucrose was used as an impermeant tracer according to Unkeless et al. (1985). After washing, ^{125}I and ^{14}C counts were measured in individual oocytes.

Transient and Stable Expression in Eukaryotic Cells

Expression vectors were constructed by inserting the prolactin receptor F3 cDNA at the EcoRI site of both pECE (Ellis et al., 1986) and pKCR2 (Breathnach and Harris, 1983) vectors. Fifty % confluent COS-7 and CHO cells were transfected by the calcium phosphate method (Southern and Berg, 1982) with 10 μg of pECE/F3 or pKCR2/F3, respectively. Transient assays were conducted in COS-7 cells which were maintained 3 days in DMEM with 10% fetal bovine serum, then scraped with 1 ml of 25 mM Tris-HCL pH7.5, 10 mM $MgCl_2$ and lysed in Eppendorf tubes by two freeze-thaw cycles. Membranes were prepared by centrifugation for 5 min and the

pellet resuspended in Tris-HCL 25 mM pH 7.5, $MgCl_2$ 10 mM and PMSF 1mM. Two hundred μg of suspension and 80,000 cpm of [^{125}I]oPRL were used per tube for binding studies which were performed as previously described (Posner et al., 1974a). Stable CHO cell lines were established by co-transfection of rat PRL receptor pKCR2/F3 with 2 μg of pSV2 neo. Cells were maintained in Hams' F12 medium supplemented with Hepes 20 mM, pyruvate 0.2 mM and 10% fetal bovine serum. Selection was made with 200 μg/ml of active G418, the eukaryotic neomycin analogue. Two other series of clones were obtained with the vector pKCR2 itself and the antisense construct pKCR2/3F and were used as control transfected cells. Membrane preparations and binding studies were carried out as described for the transient assays, except that each point was assayed in duplicate with 25 μg of membrane suspension and 50,000 cpm of [^{125}I]oPRL. In control CHO cells, although no binding is observed under the conditions utilized, a very small amount of [^{125}I]oPRL binding (500 cpm/200 μg protein) is detected when membrane protein is increased 8-fold. Ovine PRL (NIADDK oPRL-16; 30.5 IU/mg), rat PRL (NIADDK rPRL-I-5; 30 IU/mg), ovine GH (NIADDK oGH-S-11), rat GH (NIADDK rGH-I-5; 2.0 IU/mg) and human GH (NIH hGH AFP-5180A; 2.2 IU/mg) were kindly supplied by the National Hormone and Pituitary Program. Bovine insulin was obtained from Schwartz/Mann (Spring Valley, NY) and human placental lactogen from ICN Pharmaceuticals, Inc. (Cleveland).

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REFERENCES

Aviv, H. and Leder, P. (1972) Purification of biologically active globin messenger RNA by chromatography on oligothymidylic acid-cellulose. Proc. Natl. Acad. Sci. (USA) 69, 1408-1412.

Bonifacino, J. S. and Dufau, M. L. (1984) Structure of the ovarian lactogen receptors: analysis with bifunctional cross-linking reagents. J. Biol. Chem. 259, 4542-4549.

Breathnach, R. and Harris, B. A. (1983) Plasmids for the cloning and expression of full-length double-stranded cDNAs under control of the SV40 early or late gene promoter. Nucleic Acid Res. 11, 7119-7136.

Chirgwin, J. M., Przybyla, A. E., MacDonald, R. J. and Rutter, W. J. (1979) Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. Biochemistry 18, 5294-5299.

Colman, A. (1984) Translation of eukaryotic messenger RNA in Xenopus Oocytes. Transcription and Translation - A Practical Approach, B. D. Hames and S. J. Higgins eds. (Oxford: IRL Press), pp. 271-302.

Conneely, O. M., Sullivan, W. P., Toft, D. O., Birnbaumer, M., Cook, R. G., Maxwell, B. L., Zarucki-Schulz, T., Greene, G. L., Schrader, W. T. and O'Malley, B. W. (1986) Molecular cloning of the chicken progesterone receptor. Science 233, 767-770.

Djiane, J., Durand, P. and Kelly, P. A. (1977) Evolution of prolactin receptors in rabbit mammary gland during pregnancy and lactation. Endocrinology 100, 1348-1356.

Djiane, J., Clauser, H. and Kelly, P. A. (1979) Rapid down-regulation of prolactin receptors in mammary gland and liver. Biochem. Biophys. Res. Comm. 90, 1371-1378.

Ebina, Y., Ellis, L., Jarnagin, K., Edery, M., Graf, L., Clauser, E., Ou, J. H., Masiarz, F., Kan, Y. W., Goldfine, I. D., Roth, R. A. and Rutter, W. (1985) The human insulin receptor cDNA: the structural basis for hormonal-activated transmembrane signalling. Cell 40, 747-758.

Ellis, L., Clauser, E., Morgan, D. O., Edery, M., Roth, R. A. and Rutter, W. J. (1986) Replacement of insulin receptor tyrosine residues 1162 and 1163 compromises insulin-stimulated kinase activity and uptake of 2-deoxyglucose. Cell 45, 721-732.

Feinberg, A. P. and Vogelstein, B. (1983) A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal. Biochem. 132, 6-13.

Gascuel, O. and Danchin, A. (1986) Protein export in prokaryotes and eukaryotes: indications of a difference in the mechanism of exportation. J. Mol. Evol. 24, 130-142.

Gubler, U. and Hoffman, B. J. (1983) A simple and very efficient method for generating cDNA libraries. Gene 25, 263-269.

Haeuptle, M. T., Aubert, M. L., Djiane, J. and Kraehenbuhl, J. P. (1983) Binding sites for lactogenic and somatogenic hormones from rabbit mammary gland and liver. J. Biol. Chem. 258, 305-314.

Haldosén, L.-A. and Gustafsson, J.-A. (1987) Characterization of hepatic lactogen receptor: subunit composition and hydrodynamic properties. J. Biol. Chem. 262, 7404-7411.

Hollenberg, S. M., Weinberger, C., Ong, E. S., Cerelli, G., Oro, A., Lebo, R., Thompson., E. B., Rosenfeld, M. G. and Evans, R. M. (1985) Primary structure and expression of a functional human glucocorticoid receptor cDNA. Nature 318, 635-641.

Houdebine, L. M., Djiane, J., Dusanter-Fourt, I., Martel, P., Kelly, P. A., Devinoy, E. and Servely, J. L. (1985) Hormonal action controlling mammary activity. J. Dairy Science **68**, 489-500.

Hunter, T. (1987) A thousand and one protein kinases. Cell **50**, 823-829.

Jacobs, E. and Clad, A. (1986) Electroelution of fixed and stained membrane proteins from preparative sodium dodecyl sulfate-polyacrylamide gels into a membrane trap. Anal. Biochem. **154**, 583-589.

Katoh, M., Djiane, J., Leblanc, G. and Kelly, P. A. (1984) Characterization of antisera to a partially purified prolactin receptor: effect on binding in different target tissues. Mol. Cell. Endocrinol. **34**, 191-200.

Katoh, M., Djiane, J. and Kelly, P. A. (1985) Prolactin binding components in rabbit mammary gland: characterization by partial purification and affinity labelling. Endocrinology **116**, 2612-2620.

Katoh, M., Raguet, S., Zachwieja, J., Djiane, J. and Kelly, P.A. (1987) Hepatic prolactin receptors in the rat: characterization using monoclonal antireceptor antibody. Endocrinology **120**, 739-749.

Kelly, P. A., Posner, B. I., Tsushima, T. and Friesen, H. G. (1974) Studies of insulin, growth hormone and prolactin binding: ontogenesis, effects of sex and pregnancy. Endocrinology **96**: 532-539.

Kelly, P. A., Posner, B.I. and Friesen, H. G. (1975) Effects of hypophysectomy, ovariectomy,and cycloheximide on specific binding sites for lactogenic hormones in rat liver. Endocrinology **97**, 1408-1415.

Kelly, P. A., Djiane, J. and Leblanc, G. (1983) Preferential establishment of a slowly dissociable component in plasma membrane compared to intracellular prolactin receptors. Proc. Soc. Exp. Biol. Med. **172**, 219-224.

Kelly, P. A., Djiane, J., Katoh, M., Ferland, L. H., Houdebine, L. M., Teyssot, B. and Dusanter-Fourt, I. (1984) The interaction of prolactin with its receptors in target tissues and its mechanism of action. Recent Prog. in Horm. Res.40, 379-436.

Kelly, P. A., Okamura, H., Zachwieja, J., Raguet, S., Gould, D., Boutin, J.-M., Jolicoeur, C., Mélançon, R. and Djiane, J. (1988) Development and uses of monoclonal antibodies to the prolacin receptor. In Proceedings of the 3rd International Congress on Hormones and Cancer, Hamburg. (Raven Press: New York), in press.

Kozak, M. (1986) Point mutations define a sequence flanking the AUG initiator codon that modulates translation by eukaryotic ribosomes. Cell 44, 283-292.

Kraus, J. P., Williamson, C. L., Firgaira, F. A., Yang-Feng, T., Munke, M., Francke, U. and Rosenberg, L. E. (1986) Cloning and screening with nanogram amounts of immunopurified mRNAs: cDNA cloning and chromosomal mapping of cystathionine @-synthase and the @ subunit of propionyl-CoA carboxylase. Proc. Natl. Acad. Sci. (USA) 83, 2047-2051.

Kyte, J. and Doolittle, R. F. (1982) A simple method for displaying the hydropathic character of a protein. J. Molec. Biol. **157**, 105-132.

Leonard, W. J., Depper, J. M., Kanehisa, M., Kronke, M., Peffer, N. J., Svetlik, P. B., Sullivan, M. and Greene, W. C. (1985) Structure of the human interleukin-2 receptor gene. Science **230**, 633-639.

Leung, D. W., Spencer, S. A., Cachianes, G., Hammonds, R. G., Collins, C., Henzel, W. J., Barnard, R., Waters, M. J. and Wood, W. I. (1987) Growth hormone receptor and serum binding protein: purification, cloning and expression. Nature **330**, 537-543.

Liscia, D. S. and Vonderhaar, B. K. (1982) Purification of a prolactin receptor. Proc. Natl. Acad. Sci. (USA) **79**, 5930-5934.

Maniatis, T., Frisch, E. F., and Sambrook, J. (1982) Molecular Cloning: A Laboratory Manual (Cold Spring Harbor, New York: Cold Spring Harbor Laboratory).

Manni, A., Chambers, M. J. and Pearson, O. M. (1978) Prolactin induces its own receptors ir rat liver. Endocrinology 103, 2168-2171.

Matusik, R. J. and Rosen, J. M. (1980) Prolactin regulation of casein gene expression: possible mediators. Endocrinology 106, 252-259.

Melton, D. A., Krieg, P. A., Rebagliati, M. R., Maniatis, T., Zinn, K. and Green, M. R. (1984) Efficient in vitro synthesis of biologically active RNA and RNA hybridization probes from plasmids containing a bacteriophage SP6 promoter. Nucl. Acids. Res. 12, 7035-7056.

Morgan, D. O., Edman, J. C., Standring, FD. N., Fried, V. A., Smith, M. C., Roth, R. A. and Rutter, W. J. (1987) Insulin-like growth factor II receptor as a multifunctional binding protein. Nature 329, 301-307.

Necessary, P. C., Humphrey, P. A., Mahajan, P. B. and Ebner, K. E. (1984) Purification of rabbit mammary prolactin receptor by acidic elution from a prolactin affinity column. J. Biol. Chem. 259, 6942-6946.

Posner, B. I., Kelly, P. A., Shiu, R. P. C. and Friesen, H. G. (1974a) Studies of insulin, growth hormone and prolactin binding: tissue distribution, species variation and characterization. Endocrinology 96, 521-531.

Posner, B. I., Kelly, P. A. and Friesen, H. G. (1974b) Induction of a lactogenic receptor in rat liver: influence of estrogen and the pituitary. Proc. Natl. Acad. Sci. (USA) 71, 2407-2410.

Posner, B. I., Kelly, P. A., and Friesen, H. G. (1975) Prolactin receptors in rat liver: possible induction by prolactin. Science 188, 57-59.

Rosenfeld, M. G., Amara, S. G. and Evans, R. M. (1984) Alternative RNA processing: determining neuronal phenotype. Science 225, 1315-1320.

Sakai, S., Katoh, M., Berthon, P. and Kelly, P. A. (1984) Characterization of prolactin receptors in porcine mammary gland. Biochem. J. 224, 911-922.

Sanger, F., Nicklen, S. and Coulson, A. R. (1977) DNA sequencing with chain terminating inhibitors. Proc. Natl. Acad. Sci. (USA) 74, 5463-5467.

Schejter, E. D., Segal, D., Glazer, L. and Shilo, B. Z. (1986) Alternative 5' exons and tissue-specific expression of the drosophila EGF receptor homolog transcripts. Cell 46, 1091-1101.

Schneider, C., Owen, M. J., Banville, D. and Williams, J. G. (1984) Primary structure of human transferrin receptor deduced from the mRNA sequence. Nature 311, 675-678.

Shapiro, S. Z. and Young, J. R. (1981) An immunochemical method for mRNA: application to messenger RNA encoding trypanosome variable surface antigen. J. Biol. Chem. 256, 1495-1498.

Southern, P. J. and Berg, P. J. (1982) Transformation of mammalian cells to antibiotic resistance with a bacterial gene under control of the SV40 early region promoter. Mol. Appl. Genet. 1, 327-341.

Tosi, M., Young, R. A., Hagenbuchle, O. and Schibler, W. (1981) Multiple polyadenylation sites in a mouse alpha-amylase gene. Nucleic Acids Res. 9, 2313-2323.

Ullrich, A., Coussens, L., Hayflick, J. S., Dull, T. J., Gray, A., Tam, A. W., Lee, J., Yarden, Y., Libermann, T. A., Schlessinger, J., Downward, J., Mayes, E. L. V., Whittle, N., Waterfield, M. D. and Seeburg, P. H. (1984) Human epidermal growth factor receptor cDNA sequence and aberrant expression of the amplified gene in A431 epidermal carcinoma cells. Nature 309, 418-425.

Unkeless, J. C., Portnoy, D. A., Luster, A. D. and Pure, E. (1985) Expression of foreign proteins on the surface of Xenopus laevis oocytes. In Hybridoma Technology in the Biosciences and Medicine, T. A. Springer, ed. (New York: Plenum), pp. 427-438.

Wood, W. I., Gitschier, J., Lasky, L. A. and Lawn, R. M. (1985) Base composition-independent hybridization in tetramethylammonium chloride: a method for oligonucleotide screening of highly complex gene libraries. Proc. Natl. Acad. Sci. (USA) 82, 1585-1588.

Yamamoto, T., Davis, C. G., Brown, M. S., Schneider, W. J., Casey, M. L., Goldstein, J. L. and Russell, D. W. (1984) The human LDL receptor: a cysteine-rich protein with multiple alu sequences in its mRNA. Cell 39, 27-38.

CHAPTER 6

MULTIPLE REGULATION OF PROLACTIN RECEPTOR GENE EXPRESSION IN RAT LIVER

MULTIPLE REGULATION OF PROLACTIN RECEPTOR GENE EXPRESSION IN RAT LIVER

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Running title: Regulation of PRL receptor gene expression.

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PREFACE

With the availability of the cDNA for the rat liver PRL-R, we had, for the first time, a tool that allowed us to study the regulation of the PRL receptor at the gene level. This was an important step because all the previous studies examining the hormonal and developmental regulation of the PRL-R were based only on the measurement of the number of binding sites. As discussed in Chapter 3, the regulation of the PRL-R is complex and probably involves the interaction of many factors.In the present chapter, we try to shed some light on the estrogen and developmental control of the rat PRL-R, at both the mRNA and protein levels.

SUMMARY

Sex steroids are major regulators of prolactin receptor expression in rat liver. Using a probe encoding the rat prolactin receptor we have studied receptor mRNA level in female rat liver during ontogeny, and in response to estrogen treatment. Steady-state mRNA levels were determined by Northern blot and densitometric analysis. Messenger RNA levels have been compared to the number of binding sites, which were assessed by Scatchard analysis of [^{125}I] oPRL binding in membrane preparations. Our results show that steady-state mRNA and binding levels of prolactin receptors are both regulated by development and estrogens, but that binding does not exactly parallel mRNA levels. From the developmental stages of prepuberty to adult, receptor numbers increase 8-fold, whereas mRNA levels increase 3-fold. Estrogen treatment stimulates receptor levels 6-fold but mRNA levels are only increased 3-fold. These results suggest that prolactin receptor gene expression in rat liver is regulated at the transcriptional or post- transcriptional level, as well as at the translational level .

INTRODUCTION

Prolactin (PRL) is encoded by a member of the growth hormone/prolactin/placental lactogen gene family. Recently, receptors for growth hormone (1) and prolactin (2) have been cloned and have been shown to form a new receptor gene family. The prolactin receptor (PRL-R) is a small protein (41 kDa) (2-6) that is widely distributed in a number of

tissues (7). The liver is the tissue with the highest PRL binding in the rat. Although specific effects on ornithine decarboxylase, somatomedin, synlactin, IGF-I, and a liver lactogenic factor have been reported (8-12), the precise role of PRL in liver remains to be determined.

Rat hepatic PRL-R is hormonally regulated. Growth hormone has been shown to positively regulate PRL-R level (13-15). Prolactin itself exerts a control on its receptor, inducing up- or down-regulation, depending on the concentration and duration of exposure to PRL (15-19). Gonadal steroids are also major regulators of PRL-R expression. The number of receptors is very low in livers of young animals, but increases 7-fold in females during sexual maturation, while levels are low or undetectable in males during the corresponding period (20,21). In females, receptor levels fluctuate during the estrous cycle (22), decrease following ovariectomy (22,23),and increase during pregnancy (20). In males, castration results in an increase of the number of hepatic PRL binding sites (24). In both sexes, PRL-R levels are strongly stimulated by estrogens (25) and reduced by androgens (13,26).

The variations in PRL-R expression could be regulated at the transcriptional, post-transcriptional or translational level. Few studies have been completed on regulation of receptor gene expression. Coordinate regulation of distinct insulin receptor mRNA species by glucocorticoid has been reported in rat Fao hepatoma cells (27). Multiple mRNA forms encoding the human progesterone receptor are synchronously regulated by estrogen and progesterone in breast cancer cells (28). Down-regulation of the estrogen receptor and the glucocorticoid receptor is associated with a reduction in mRNA level (29,30). In the current study, we present the steady-state PRL-R mRNA levels in female

rat liver as a function of age, and in response to estrogen administration and compare them with the number of prolactin receptors. These results suggest that there are multiple levels of regulation of prolactin receptor synthesis in rat liver.

RESULTS

Ontogenesis of PRL Receptor Expression in Female Rat Liver

The number of prolactin receptors and receptor mRNA levels have been determined in rat liver at various ages: in fetus, newborn, and in 21, 40, and 70 day-old females. At least four independent samples were measured at each stage. Each sample was divided for PRL-R mRNA and hormone binding measurements. Figure 1 shows the Northern blot analysis of PRL-R mRNA in female rat liver as a function of age, performed by using an RNA probe prepared from a cDNA clone encoding the PRL-R (2). The upper band corresponds to PRL-R mRNA, while the lower band represents a control mRNA which hybridized to B1 cDNA probe and whose abundance is stable at all ages studied. As previously observed (2), the 2.2 kb band is by far the major PRL-R mRNA apparent in rat liver PRL-R mRNA is undetectable in livers from fetal and newborn animals, even with long-term (3 days) film exposure (not shown). At day 21, the relative abundance of PRL-R mRNA is low, but is markedly increased after puberty in livers from 40 day-old animals. No further significant change occurs from days 40 to 70. Figure 2 shows the Scatchard analysis of [^{125}I] oPRL binding data at the different ages studied. Note the change in the scale of the absissa. No significant variation of binding affinity was observed.

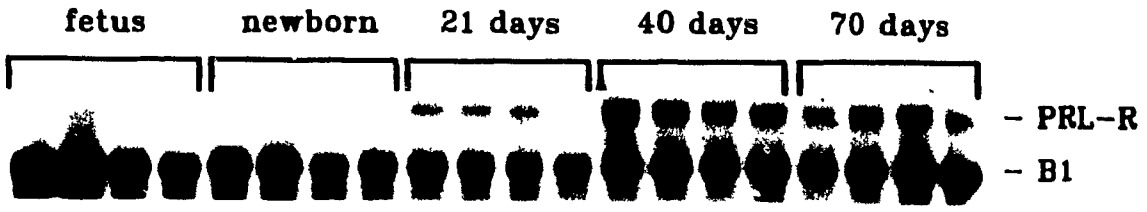

FIG. 1 Northern blot analysis of PRL-R mRNA in female rat liver at different ages.

Each lane represents mRNA from individual rats. Ten micrograms of poly (A)$^+$ RNA was loaded in each well. The blot was hybridized at high stringency (see Materials and Methods) with a complete 1.6 kb F3 RNA probe and a 300 bp B1 RNA probe. The X-ray film was exposed 16 h without an intensifying screen. The length of PRL-R mRNA is 2.2 kb and that of B1 mRNA is 1.5 kb.

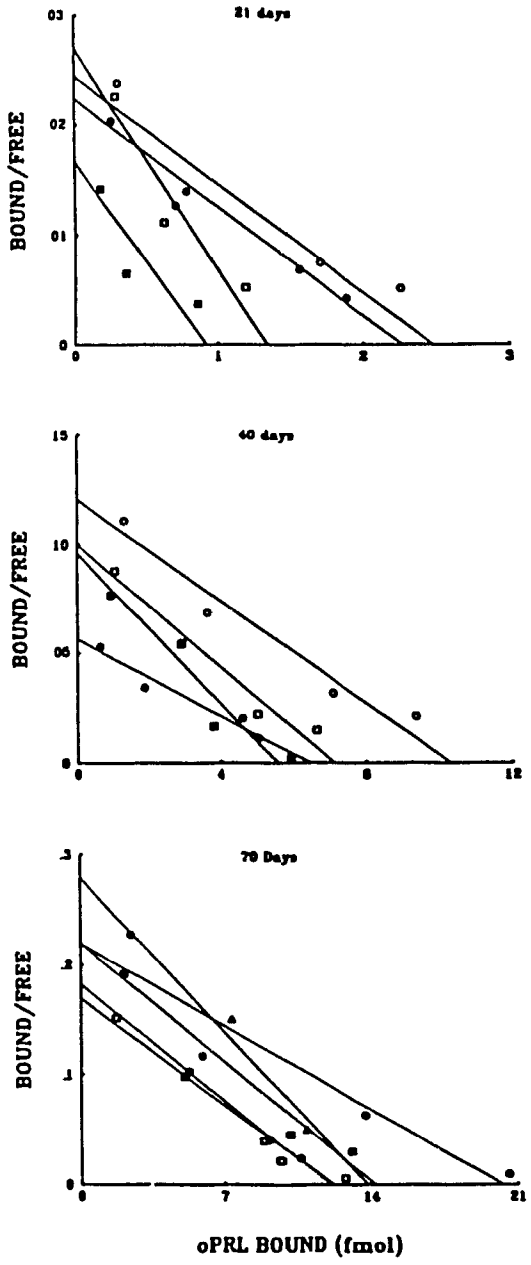

FIG. 2 Scatchard analyses of [^{125}I]oPRL binding to rat liver microsomes at 21,40 and 70 days of age.

Individual rats are represented by different symbols and accompanying straight lines.

Band intensities on the Northern (Fig. 1) were quantified by densitometric scanning. The area under the scan of each band was integrated, and to normalize for sample concentration and/or variations from lane to lane during Northern blot transfer, PRL-R mRNA levels were expressed as the ratio of the intensities of the PRL-R band and the control (B1) band. Different times of exposure were performed in order to obtain a linear film response for both mRNAs. A comparison of the relative PRL-R mRNA abundance and binding levels as a function of age is presented on Figure 3. Free receptor levels were calculated from the Scatchard analyses shown in Fig. 2. Free and total receptor levels, measured following treatment of membranes with 4M $MgCl_2$ (31), were not significantly different, therefore only free receptor levels are reported. Both mRNA and binding are undetectable in fetus and newborn. Interestingly, the increase in the number of PRL binding sites is not paralleled by steady-state mRNA levels after 40 days of age, when receptor numbers increase while the mRNA levels remain relatively stable.

PRL Receptor Expression in Estrogen-Treated Female Rat Liver

The effect of the administration of a long-acting estrogen on hepatic PRL-R mRNA and receptor numbers were studied in adult female rat liver. Estradiol valerate was injected subcutaneously at day 0, and animals were sacrificed 1, 3, 5 and 7 days after initiation of treatment. Poly A^+ mRNA and microsomes were prepared from livers of 4-8 animals at each time point. Figure 4 shows Northern blot analysis of PRL-R mRNA in estrogen-treated rat liver, while Figure 5 illustrates the

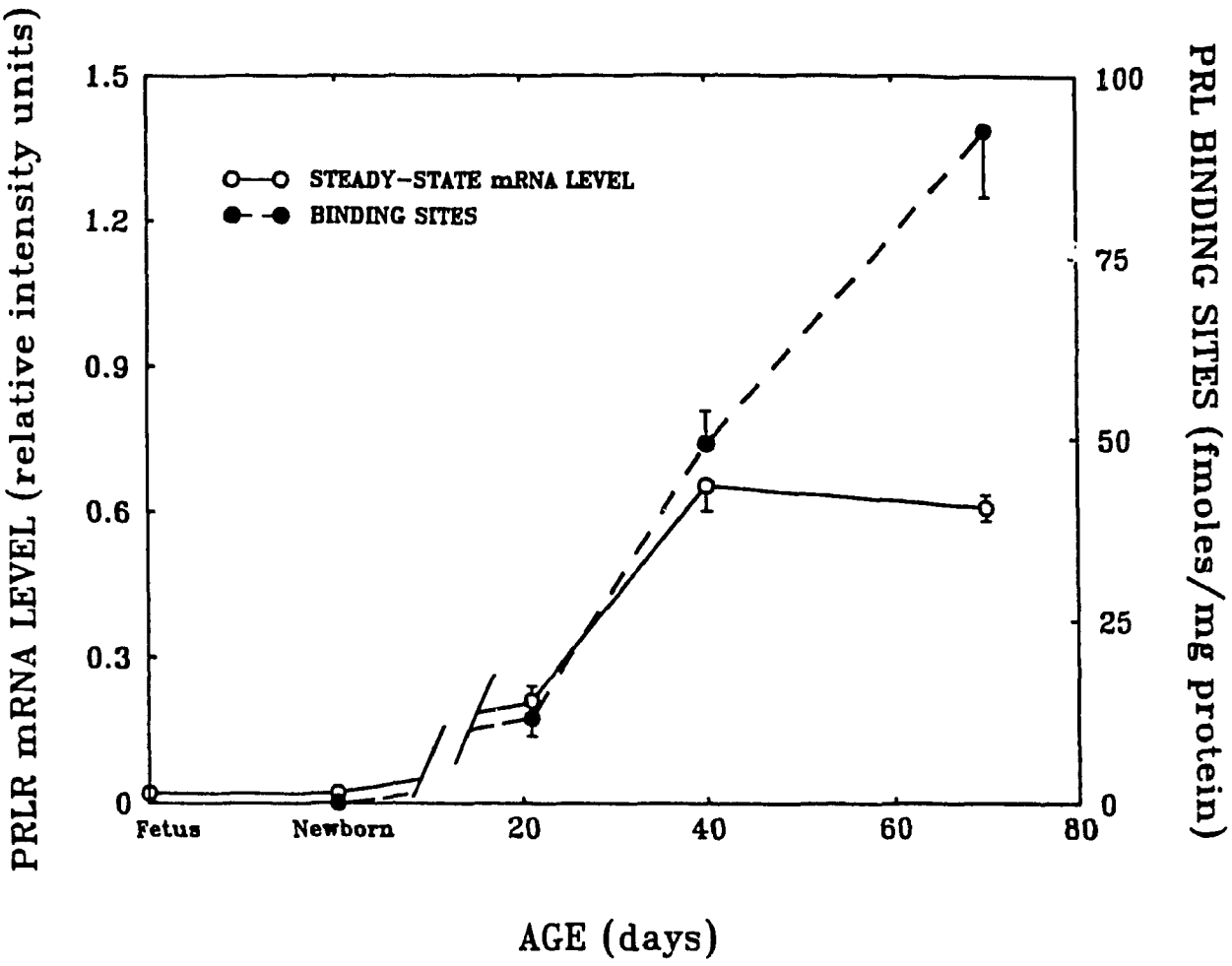

FIG. 3 Ontogenesis of PRL-R expression in female rat liver: comparison of steady-state PRL-R mRNA and binding levels.

PRL-R mRNA level is expressed as a ratio of the intensities of the PRL-R mRNA and the control B1 mRNA band. Values are expressed as mean ± SEM. Statistical significance determined by the multiple range test (48): for mRNA, days 21 vs 40, $p < 0.01$; for binding sites, days 21 vs 40, and 40 vs 70, $p < 0.01$.

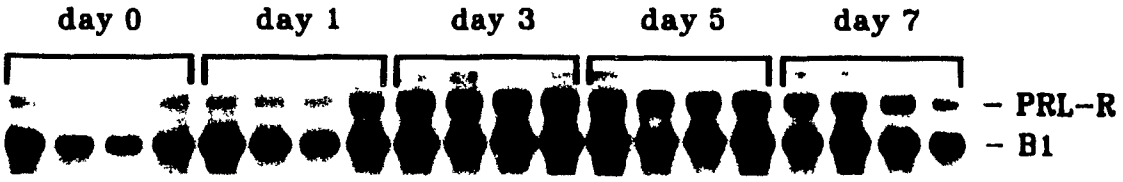

FIG. 4 Northern blot analysis of PRL-R mRNA in estrogen-treated female rat liver.

Each lane represents mRNA from individual rats. Ten micrograms of poly $(A)^+$ RNA was loaded in each well. The blot was hybridized at moderate stringency (see Materials and Methods) with a 245 bp F3/EcoRV RNA probe and a 300 bp B1 RNA probe. The X-ray film was exposed 60 min without an intensifying screen.

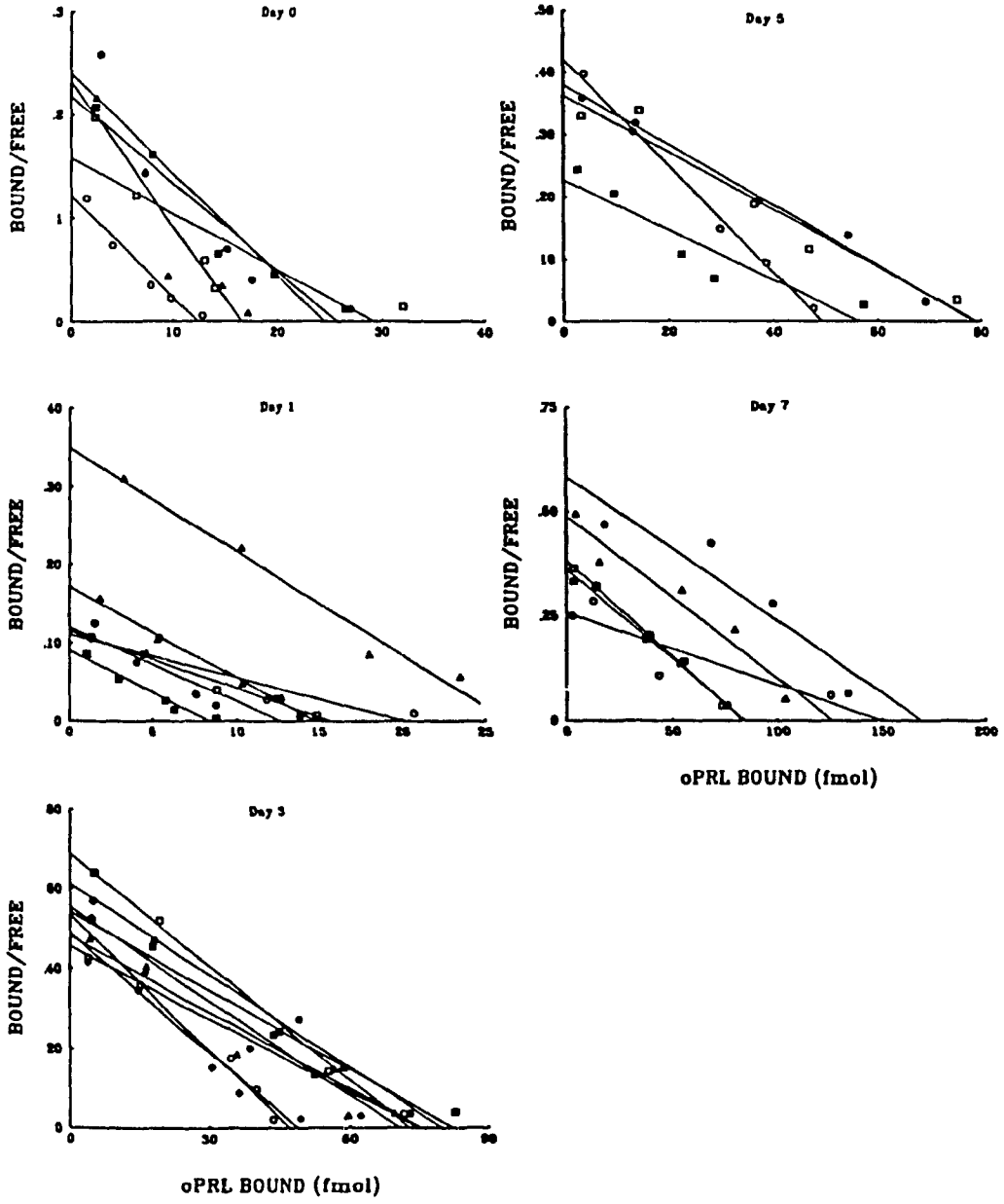

FIG. 5 Scatchard analyses of [^{125}I]oPRL binding in rat liver microsomes before (day 0) and after (day 1,3,5 and 7) treatment with estradiol valerate.

Individual rats are represented by different symbols and accompanying straight lines.

Scatchard analysis of binding at the corresponding days of treatment. In Fig. 6, the quantitation of mRNA levels as well as the number of PRL binding sites are shown. Relative abundance of PRL-R mRNA is low in non-treated animals (day 0) and no significant change is visible on day 1. There is a marked increase in PRL-R mRNA abundance between days 1-3, after which levels remain elevated until the end of the study. As was true for the developmental studies, the induction of receptor number did not directly parallel steady-state mRNA levels. Although there is a similar lag period, a greater increase occurs in the number of prolactin binding sites than in mRNA levels from days 1 to 3. Steady-state mRNA levels stabilized between days 3 to 7 while receptor numbers increased between days 1 to 7.

DISCUSSION

The effects of sex steroids on PRL binding sites in liver are well established (20-22,25,26,32). In this report we present for the first time a comparison between the PRL-R mRNA level and the number of prolactin receptors at various ages, and in response to estrogen treatment.

Prolactin receptor mRNA in liver was studied by Northern blot analysis and the number of PRL-R in the corresponding samples was determined by Scatchard analysis. It should be clear that comparisons are not made between absolute values at each time point, but rather between relative steady-state mRNA and binding levels.

The number of PRL binding sites does not exactly parallel PRL-R mRNA levels during ontogeny in normal female rat liver. The divergence between binding and mRNA levels became apparent at 21 days of age. At day 40, after puberty occurred, relative mRNA levels increased 3-fold,

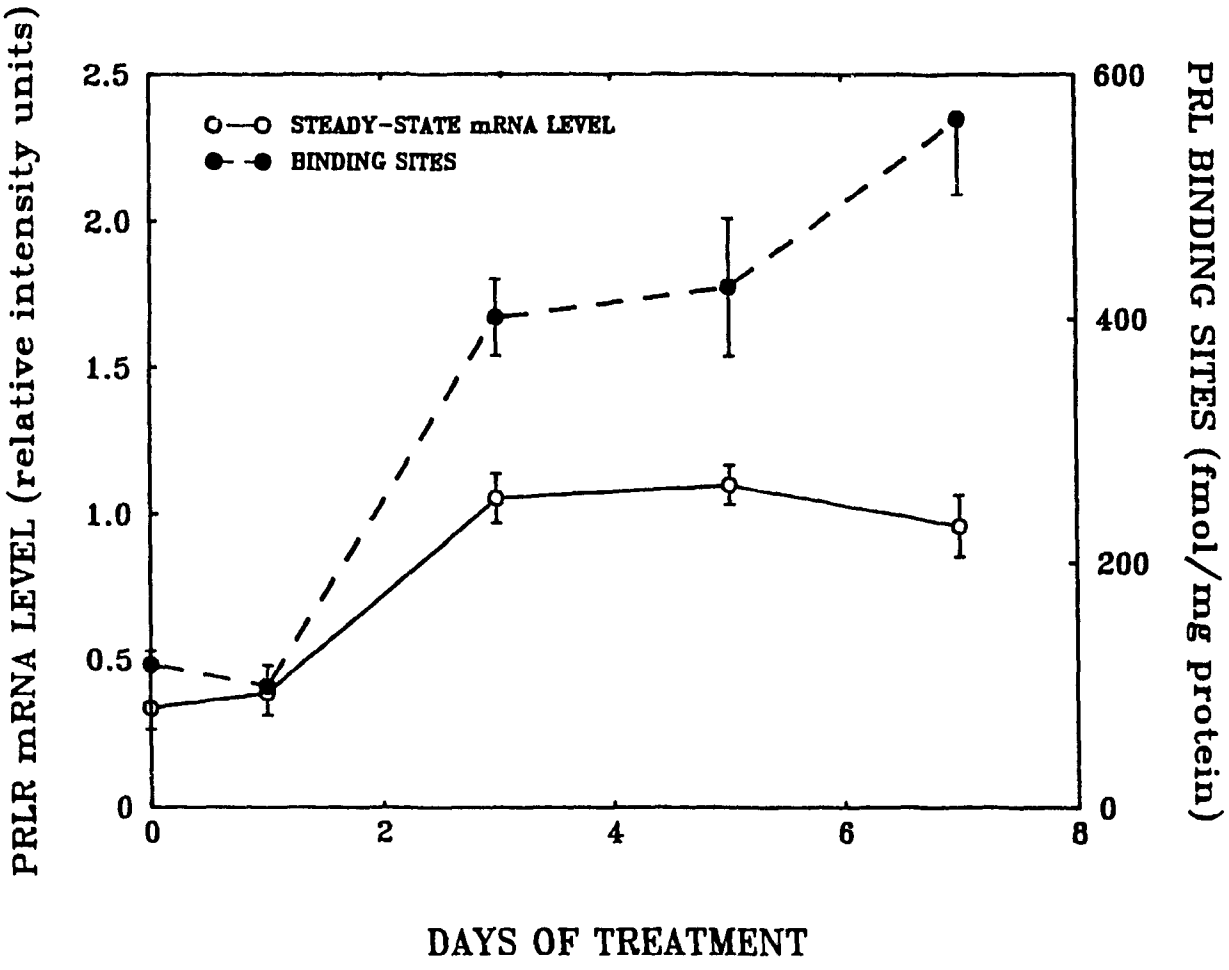

FIG. 6 PRL-R expression in estrogen-treated female rat liver: comparison of steady-state PRL-R mRNA and binding levels.

Values are expressed as mean ± SEM. Statistical significance determined by the multiple range test (48): for mRNA, days 1 vs 3; $p < 0.01$, for binding sites, days 1 vs 3 and 5 vs 7, $p < 0.01$.

while the number of PRL binding sites increased 4-fold. This difference is even more striking from days 40 to 70 when the number of binding sites doubled and mRNA levels remained stable. This results in an overall 3-fold increase of prolactin receptor mRNA and an 8-fold increase in prolactin receptor levels from day 21 to 70, consistent with values reported previously (20,21). A similar divergence occurred in the study with estrogen-treated female rats. From days 1 to 3 of treatment, a less than 3-fold increase in relative mRNA levels was seen, while the rise in the number of PRL binding sites was nearly 4-fold. Messenger RNA levels stabilized after day 3 but the number of receptors continued to increase until the end of the study when the final number was approximately 6 times the number of receptors present prior to estrogen stimulation (day 0). The increase in PRL receptor numbers following estrogen treatment was identical to results obtained previously (25).

These results indicate that hepatic PRL receptor induction during puberty or in females following estradiol administration is the consequence of at least two phenomena: an increase in steady-state PRL-R mRNA levels and an increase in the number of binding sites in the liver cell that cannot be completely accounted for by mRNA changes. The increase in the steady-state mRNA level could result either from a stimulation of transcription or a stabilization of the messenger. Estrogen regulates vitellogenin synthesis by increasing both gene transcription rate and messenger stability (33). Hormone action on receptor mRNA steady-state level has been reported for the insulin receptor (27), estrogen receptor (29), progesterone receptor (28), and glucocorticoid receptor (30). Regulation of progesterone receptor mRNAs level by progesterone in T47D cells appears to be mainly mediated at the transcriptional level, while

progesterone agonists and antagonists seem to act also on messenger stability (28). It has been proposed that glucocorticoid would regulate glucocorticoid receptor expression at the transcriptional level by a direct interaction with the receptor gene (30). Prolactin-regulated genes represent a classical system of control of gene expression at both the transcriptional and post-transcriptional level. In mammary gland organ culture, the binding of prolactin to its receptor is followed by stimulation of casein gene transcription, but the subsequent increase in casein mRNA level results mainly from its stabilization (34). In T47D cells, prolactinpost-transcriptionally regulates prolactin-induced protein (PIP) mRNA level (35). Whether the estrogen-induced increase in the steady-state PRL-R mRNA level results from a stimulation of transcription, a stabilization of mRNA or both, remains to be determined.

To explain the disproportionate increase in binding versus mRNA, the main hypothesis to be considered is that there is a translational control of PRL-R expression. However, alternative mechanisms, such as post-translational modification of the receptor protein (e.g. phosphorylation) cannot be excluded. If such a modification does occur, it does not appear to affect receptor affinity, since there were no significant differences in K_a values in any of the various samples. There are various examples of regulation of gene expression in which effects of transient biological signals are mediated through reversible translational regulation, including the heat-shock proteins (36,37), the housekeeping enzymes ornithine decarboxylase (38) and ornithine aminotransferase (39), ferritin (40), and ovalbumin, reported to be positively regulated by progesterone and estrogen in the chick oviduct (41). In many of these cases, translational control of gene expression has been shown to depend

on specific features of the primary and/or secondary structure of the mRNA. The presence of a small open reading frame upstream of the main translation initiation codon (38,42), and regions of dyad symmetry, allowing formation of stem-loop structures (39,42-44), are believed to account for regulation at the level of translation. From sequences thus far obtained in the 5' or 3' untranslated region of the PRL receptor cDNA (2), it is not known as yet if such a mechanism can be implicated.

Although estrogen receptors are present in rat liver, estrogen is thought to induce hepatic PRL-R via an increased pituitary secretion of PRL and GH, since both hormones are able to up-regulate the PRL receptor (15), and since estrogen is not able to induce PRL-R in hypophysectomized rats (25). A direct in vivo effect of estrogens on the induction of hepatic PRL receptors along with the requirement for a pituitary factor (PRL, GH) cannot be ruled out, although direct addition of estradiol to hepatocytes in primary culture had no effect above that of GH or PRL alone (15).

Estrogens have both a dose- and time-related effect on the induction of hepatic PRL-R in the rat (25). The present results confirm the stimulatory effect of estrogen on the number of prolactin receptors and demonstrate that regulation by estrogen occurs in part at the mRNA level.

MATERIALS AND METHODS

Estrogen Treatment

Estradiol valerate (Squibb Canada Inc.) was injected subcutaneously (1 mg in 0.1 ml) to adult female rats (70 day-old, 200-225 g). Non-treated controls were sacrificed the same day, and treated controls were sacrificed on days 1, 3, 5 and 7 following injection. Whole livers

were removed, separated into two equal parts for preparation of RNA and membranes and frozen immediately in liquid nitrogen for storage at -70° C.

Developmental Studies

Livers from 18-day fetuses and newborn rats were removed, pooled (male-female) and then divided for preparation of RNA and membranes before storage at -70°. For 21, 40 and 70 day-old female rats, whole individual livers were removed and frozen.

Northern Blot Analysis

Total RNA was prepared using the guanidium thiocyanate/CsCl purification procedure of Chirgwin et al (45). Poly (A) mRNA was purified by oligo-dT cellulose (Type 7, Pharmacia) affinity chromatography (46) and denatured for 60 min at 50° C in 1 M glyoxal, 50% DMSO, 10 mM Na_2HPO_4 pH 6.5, before separating by electrophoresis on a 1.5% agarose gel in 10 mM Na_2HPO_4 pH 6.5. RNA was transferred to a nylon membrane (GeneScreen, NEN/Dupont) by capillary action in 1M NaCl, baked at 90°C and hybridized. RNA samples were analyzed with different probes. Prolactin receptor mRNA was identified using clone F3, a 1.6 kb probe containing the entire open reading frame of the PRL receptor plus 5' and 3' nucleotides (2). Hybridizations were performed either with the complete 1.6 kb F3 RNA probe for the developmental studies or a 245 bp F3/EcoRV fragment RNA probe for the estrogen study. The long cRNA probe was used to increase the PRL-R mRNA signal in order to obtain a workable ratio with B1. Clone B1-encoded mRNA was used as a control to normalize results for variations in sample concentration and/or Northern blot transfer, since it does not

appear to be hormonally regulated. Clone B1 is a cDNA not related to the PRL-R and previously obtained in our laboratory (2). B1 mRNA was identified by hybridization with a 300 bp B1 RNA probe. RNA probes were labelled with [^{32}P]UTP by using the Bluescript T3/T7 transcription system by modifications of conditions described by Melton et al (47). When using the complete F3 and B1 RNA probes, hybridizations were performed 16 h at 65°C in 50% formamide, 50 mM Na_2HPO_4 pH6.5, 0.8 M Nacl, 0.5% SDS, 1 mM EDTA, and filters were washed at 70 - 75° C. When using the F3/EcoRV and B1 RNA probes, hybridization conditions were the same except that the temperature was 55° C and filters were washed at 65-70° C. Northern blot autoradiograms were analyzed by densitometric analysis using a Helena Laboratories (Texas) Quick Scan Junior scanner. The area under the curves were calculated using a a program (GS-350H) developed by Hoefer Scientific (California). In each lane, relative levels of PRL-R mRNA were determined by calculating the ratio between the intensity of the PRL-R mRNA band and the intensity of the B1 mRNA band.

Determination of the Number of PRL Binding Sites

Rat liver membranes (microsomes) were prepared as previously described (25). Scatchard analysis of binding was determined by competition with 0.5 to 100 ng/tube of unlabelled PRL. One hundred and fifty micrograms of suspension and 40,000 cpm of [^{125}I] oPRL were used per tube, and each point was assayed in duplicate. The specific activity of [^{125}I] oPRL was 80 μCi/μg. Ovine PRL (NIADDK oPRL-16; 30.5 I.U./mg) was kindly supplied by the National Hormone and Pituitary Program.

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REFERENCES

1. Leung, D. W., Spencer, S. A., Cachianes, G., Hammonds, R.G., Collins, C., Henzel, W. J., Barnard, R., Waters, J., and Wood, W. I. (1987) Growth hormone receptor and serum binding protein: purification, cloning and expression. Nature 330:537-543

2. Boutin JM, Jolicoeur C, Okamura H, Gagnon J, Edery M, Shirota M, Banville D, Dusanter-Fourt I, Djiane J, Kelly PA 1988 Cloning and expression of the rat prolactin receptor, a member of the growth hormone/prolactin receptor gene family. Cell 53:69-77

3. Liscia DS, Vonderhaar BK 1982 Purification of a prolactin receptor. Proc Natl Acad Sci (USA) 79:5930-5934

4. Haeuptle MT, Aubert ML, Djiane J, Kraehenbuhl JP 1983 Binding sites for lactogenic and somatogenic hormones from rabbit mammary gland and liver. J Biol Chem 258:305-314

5. Necessary PC, Humphrey PA, Mahajan PB, Ebner KE 1984 Purification of rabbit mammary prolactin receptor by acidic elution from a prolactin affinity column J Biol Chem 259:6942-6946

6. Katoh M, Raguet S, Zachwieja J, Djiane J, Kelly PA 1987 Hepatic prolactin receptors in the rat: characterization using monoclonal antireceptor antibody. Endocrinology 120:739-749

7. Hughes JP, Elsholtz HP, Friesen HG 1985 Growth hormone and prolactin receptors. In: Posner BI (ed) Receptors. Marcel Decker, Inc, New York, p. 157-199

8. Francis MJO, Hill DJ 1975 Prolactin-stimulated production of somatomedin by rat liver. Nature 255:167-168

9. Richards JF 1975 Ornithine decarboxylase activity in tissues of prolactin treated rats. Biochem Biophys Res Commun 63:292-299

10. Mick CCW, Nicoll CS 1985 Prolactin directly stimulates the liver in vivo to secrete a factor (synlactin) which acts synergistically with the hormone. Endocrinology 116:2049-2053

11. Murphy LJ, Tachibana K, Friesen HG 1988 Stimulation of hepatic in sulin-like growth factor-I gene expression by ovine prolactin: Evidence for intrinsic somatogenic activity in the rat. Endocrinol ogy 122:2027-2033

12. Hoeffler JP, Frawley LS 1987 Liver tissue produces a potent lactogenic factor that partially mimics the actions of prolactin. Endocrinology 120:1679-1681

13. Norstedt G, Mode A, Enerotr P, Gustafsson JA 1981 Induction of prolactin receptors in rat liver after the administration of growth hormone. Endocrinology 108:1855-1861

14. Baxter RC, Zaltsman Z 1984 Induction of hepatic receptors for growth hormone (GH) and prolactin by GH infusion is sex independent. Endocrinology 115: 2009-2014

15. Barash I, Cromlish W, Posner BI 1988 Prolactin (PRL) receptor induction in cultured rat hepatocytes: dual regulation by PRL and growth hormone. Endocrinology 122:1151-1158

16. Costlow M, Buschow R, McGuire W 1975 Prolactin stimulation of prolactin receptors in rat liver. Life Sci 17:1457-1466

17. Manni A, Chambers M, Pearson O 1978 Prolactin induces its own receptor in rat liver. Endocrinology 103:2168-2171

18. Djiane J, Clauser H, Kelly PA 1979 Rapid down-regulation of prolactin receptors in mammary gland and liver. Biochem Biophys Res Comm 90:1371-1378

19. Kelly PA, Djiane J, De Léan A 1980 Prolactin receptor dissociation and down-regulation. Prog Reprod Biol 6:124-136

20. Kelly PA, Posner BI, Tsushima T, Friesen HG 1974 Studies of insulin, growth hormone and prolactin binding: ontogenesis, effects of sex and pregnancy. Endocrinology 96:532-539

21. Maes M, De Hertogh R, Watrin-Granger P, Ketelslegers JM 1983 Ontogeny of liver somatotropic and lactogenic binding sites in male and female rats. Endocrinology 113: 1325-1332

22. Kelly PA, Posner BI, Friesen HG 1975 Effects of hypophysectomy, ovariectomy, and cycloheximide on specific binding sites for lactogenic hormones in rat liver. Endocrinology 97:1408-1415

23. Gelato M, Marshall S, Boudreau M, Bruni J, Campbell GA, Meites J 1975 Effects of thyroid and ovaries on prolactin binding activity in rat liver. Endocrinology 96:1292-1296

24. Aragona C, Bohnet HG, Friesen HG 1976 Prolactin binding sites in the male rat liver following castration. Endocrinology 99: 1017-1022

25. Posner BI, Kelly PA, Friesen HG 1974 Induction of a lactogenic receptor in rat liver: influence of estrogen and the pituitary. Proc Natl Acad Sci USA 71:2407-2410

26. Norstedt G, Mode A 1982 On the primary site of action of estrogens and androgens in the regulation of hepatic prolactin receptors. Endocrinology 111:645-649

27. Goldstein BJ, Muller-Wieland D, Kahn RC 1987 Variation in insulin receptor messenger ribonucleic acid expression in human and rodent tissues. Mol Endocrinol 1:759-766

28. Wei LL, Krett NL, Francis MD, Gordon DF, Wood WM, O'Malley BW, Horwitz KB 1988 Multiple human progesterone receptor messenger ribonucleic acids and their autoregulation by progestin agonists and antagonists in breast cancer cells. Mol Endocrinol 2:62-72

29. Maxwell BL, McDonnell DP, Conneely DM, Schulz TZ, Greene GL, O'Malley BW 1987 Structural organization and regulation of the chicken estrogen receptor. Mol Endocrinol 1:25-34

30. Okret S, Poellinger L, Dong Y, Gustafsson JA 1986 Down-regulation of glucocorticoid receptor mRNA by glucocorticoid hormones and recognition by the receptor of a specific binding sequence with a receptor cDNA clone. Proc Natl Acad Sci USA 83:5899-5903

31. Kelly PA, Leblanc G, Djiane J 1979 Estimation of total prolactin - binding sites after in vitro desaturation. Endocrinology 104:1631-1-638

32. Kelly PA, Ferland L, Labrie F 1978 Endocrine control of prolactin receptors. In: Robyn C, Harter M (eds) Progress in Prolactin Physiology and Pathology. Elsevier/North-Holland, Amsterdam, p.59

33. Schapiro DJ, Brock ML 1985 Messenger RNA stabilization and gene transcription in the estrogen induction of vitellogenin mRNA. In: Litwack G (ed) Biochemical Actions of Hormones, Academic Press Inc, Orlando, FL, pp 139-172.

34. Guyette WA, Matusik RJ, Rosen JM 1979 Prolactin-mediated transcriptional and post-transcriptional control of casein gene expression. Cell 17:1013-1023.

35. Murphy LC, Tsuyuki D, Myal Y, Shiu RPC 1987 Isolation and sequencing of a cDNA clone for a prolactin-inducible protein (PIP). J Biol Chem 262:15236-15241.

36. Klemenz R, Hultmark D, Gehring WJ 1985 Selective translation of heat shock mRNA in Drosophila melanogaster depends on sequence information in the leader. EMBO J 4:2053-2060

37. McGarry TJ, Lindquist S 1985 The preferential translation of Drosophila hsp 70 mRNA requires sequences in the untranslated leader. Cell 42:903-911

38. Kahana C, Nathans D 1985 Translational regulation of mammalian ornithine decarboxylase by polyamines. J Biol Chem 260:15390-15393

39. Mueckler MM, Merrill MJ, Pitot HC 1983 Translational and pretranslational control of ornithine aminotransferase synthesis in rat liver. J Biol Chem 258:6109-6114

40. Hentze MW, Rouault TA, Caughman SW, Dancis A, Harford JB, Klausner RD 1987 A cis-acting element is necessary and sufficient for translational regulation of human ferritin expression in response to iron. Proc Natl Acad Sci USA 84:6730-6734

41. Pennequin P, Robins DM, Schimke RT 1978 Regulation of translation of ovalbumin messenger RNA by estrogens and progesterone in oviduct of withdrawn chicks. Eur J Biochem 90:51-58

42. Geballe AP, Spaete RR, Mocarski ES 1986 A cis-acting element within the 5' leader of cytomegalovirus @ transcript determines kinetic class. Cell 46:865-872

43. Kozak M 1986 Influences of mRNA secondary structure on initiation by eukaryotic ribosomes. Proc Natl Acad Sci USA 83:2850-2854

44. Casey JL, Hentze MW, Koeller DM, Caughman LSW, Rouault TA, Klausner RD, Harford JB 1988 Iron responsive elements: regulatory RNA sequences that control mRNA levels and translation Science 240:924-928

45. Chirgwin JM, Przybyla AE, MacDonald RJ, Rutter WJ 1979 Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. Biochemistry 18:5294-5299

46. Aviv H, Leder P 1972 Purification of biologically active globin messenger RNA by chromatography on oligothymidylic acid-cellulose. Proc Natl Acad Sci (USA) 69:1408-1412

47. Melton DA, Krieg PA, Rebagliati MR, Maniatis T, Zinn K, Green MR 1984 Efficient in vitro synthesis of biologically active RNA and RNA hybridization probes from plasmids containing a bacteriophage SP6 promoter. Nucl Acids Res 12:7035-7056

48. Kramer CY 1956 Extension of multiple range tests to group means with unequal numbers of replications. Biometrics 12:307-310.

CHAPTER 7

IDENTIFICATION OF A cDNA ENCODING A LONG FORM OF PRL RECEPTOR IN HUMAN HEPATOMA AND BREAST CANCER CELLS

IDENTIFICATION OF A cDNA ENCODING A LONG FORM OF PRL RECEPTOR IN HUMAN HEPATOMA AND BREAST CANCER CELLS

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Running title: Cloning of the human PRL receptor

Key words: Prolactin, receptor, breast cancer, cDNA, GH/PRL receptor gene

PREFACE

Technical problems have been a major limitation in studying and understanding the role of PRL in humans. Indeed there is the problem related to thepresence of hGH, which is a lactogenic hormone, the effect of which can be confounded with that of PRL. In addition, there is the problem of low tissue content of the PRL-R which, combined with the limited availability of human tissues, that can be supplied, renders the specific evaluation of PRL effects extremely difficult. In this context, it was a logical step to use the rat liver PRL-R cDNA as a probe to clone the human PRL-R cDNA. In this Chapter we describe the isolation of this receptor cDNA and the interesting discovery of a second form of PRL-R which will certainly become an important tool in examining the specific effects of PRL in humans.

SUMMARY

Human prolactin receptor cDNA clones from hepatoma (Hep G2) and breast cancer (T-47D) libraries were isolated by using a rat PRL receptor cDNA probe. The nucleotide sequence predicts a mature protein of 598 amino acids with a much longer cytoplasmic domain than the rat liver PRL receptor. Although this extended region has additional segments of localized sequence identity with the human growth hormone receptor, there is no identity with any consensus sequences known to be involved in hormonal signal transduction. This cDNA will be a valuable tool to better understand the role of prolactin in the development and growth of human breast cancer.

INTRODUCTION

Prolactin (PRL) has an extremely wide spectrum of activities in different species (1). In the human, the physiological effects of PRL on mammary development and lactation and the pathological effects of hyperprolactinemia on reproduction, are well-known (2). However, the fact that PRL is secreted by tissues other than the pituitary (3-6) and that PRL receptors are found in many tissues (7) suggests that PRL may have multiple functions. Indeed, there is evidence to suggest that this hormone plays a role in water transport by fetal membranes (8), steroid production in ovaries (9) and immunomodulation (10). There is no doubt that PRL directly affects the incidence and growth of mammary tumors in rodents, but its role in human breast cancer is less clear (11).

Although there have been numerous clinical reports attempting to correlate levels of circulating prolactin with the development or progression of breast cancer, the results are often contradictory (11). The fact that in the human, both PRL and GH are lactogenic and are secreted cyclically and episodically have complicated the interpretation of some studies. Moreover, estimation of PRL levels by radioimmunoassay does not necessarily reflect the biological activity of the hormone, especially under certain pathological conditions (12).

In human breast cancer cells in culture, a stimulatory effect of prolactin on cell growth has been suggested (13,14). Since this action is initiated at the level of a cell surface receptor, several studies have concentrated on fluctuations in receptor levels. In human breast cancer cells and biopsies, PRL receptor levels have been shown to be correlated to estrogen receptor levels and relapse-free survival (15-16). However, the prognostic value of PRL receptor measurements is limited by the difficulty in accurately quantifying receptor binding, and by their low level (17). The development of a sensitive and specific method to measure PRL receptors is essential to any further progression in this field. This necessitates a detailed characterization of the receptor, which has thus far proven extremely difficult to purify. Molecular cloning offers a suitable alternative to determine receptor primary structure.

Recently we deduced the primary structure of the rat liver prolactin receptor from a single cDNA clone (F3) (18). We showed that it is a member of the growth hormone/PRL receptor gene family and it encodes a mature protein of 291 amino acids (aa) that binds PRL when expressed at

the cell surface. Moreover, we subsequently identified a second form of PRL receptor in the rabbit mammary gland (19). Here we report the cloning and expression of the human prolactin receptor. This protein consists of a mature protein of 598 amino acids with a much longer cytoplasmic domain than that of the rat liver PRL receptor, but similar to that of the rabbit receptor.

RESULTS

A size-selected human hepatoma (Hep G2) cDNA library was screened at low stringency with the rat liver clone F3 used as a cDNA probe. Screening of 6 x 10^5 clones resulted in only one positive clone (H1) of 2 kb which was used to rescreen 10^6 clones of the same library at high stringency. Five other overlapping clones were identified. Two of the clones (H1 and H2) resulted in a single long open-reading frame (Fig. 1a). A third clone, H5, adds 500 bp to the 3' untranslated region, but contains a stretch of 70 bp at its 5' extremity, within the putative coding region, which is completely unrelated to the other sequences. These nucleotides may be the product of alternative splicing or may represent a cloning artifact (Fig. 1a). A partial restriction map of the full length clone ($\sim$ 3 kb) is shown in Fig. 1a. The complete open-reading frame corresponds to a protein of 622 amino acids, which includes a putative signal peptide of 24 aa. By homology with the rat PRL receptor, the first Gln residue was considered as the N-terminus of the mature protein. Therefore, the mature non-glycosylated protein (598 aa) has a theoretical molecular weight of 66,905.

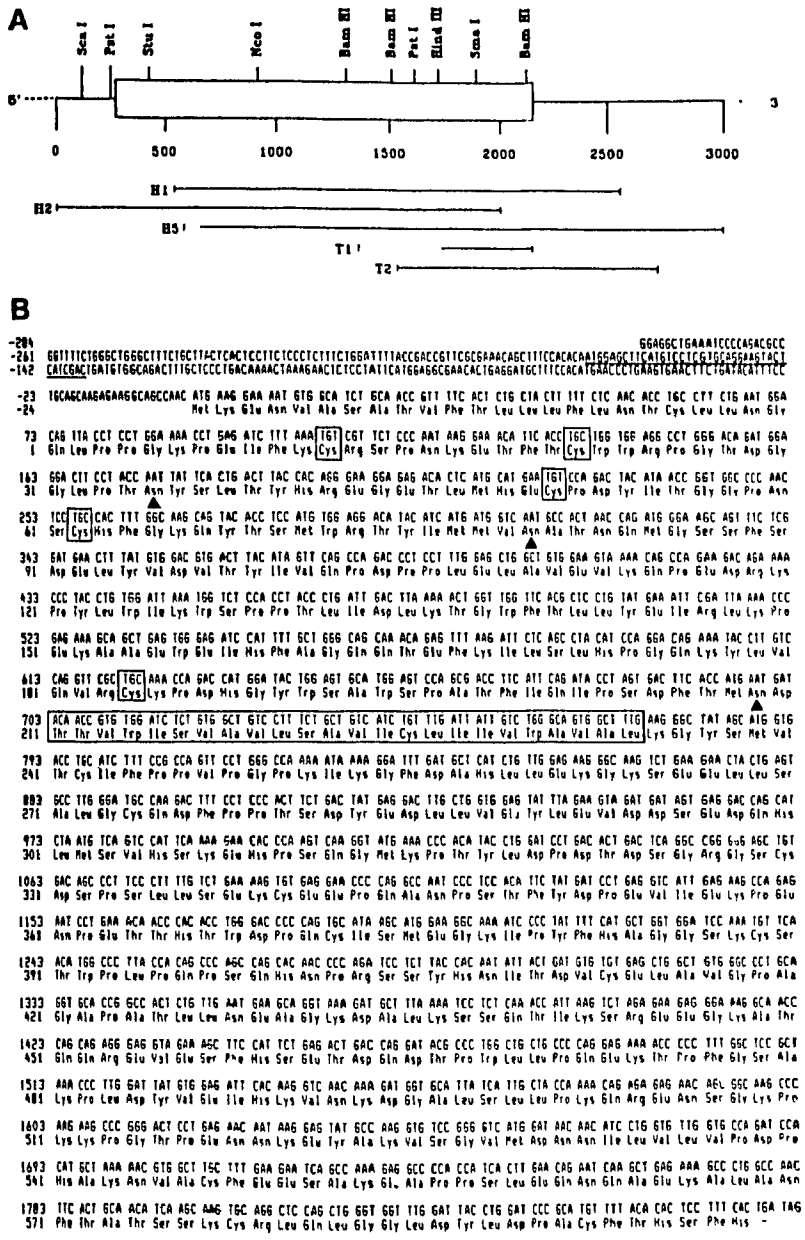

FIG. 1 Restriction map, nucleic acid and predicted amino acid sequence of the human PRL receptor.

(a) The open box indicates the predicted coding region. Some restriction sites are indicated as well as the localization of the hepatoma (H1, H2, H5) and T-47D (T1, T2) cDNA clones. Divergent sequences are indicated by dotted lines. **(b)** Amino acids are numbered from the NH_2 terminal amino acid of the mature protein; nucleotides are numbered from the first base of the codon for the initiation methionine. The underlined sequence in the 5' untranslated region corresponds to a putative minicistron. Extracellular cysteines and their codons are boxed and potential Asn-linked glycosylation sites are marked by triangles. The transmembrane region is surrounded by a rectangle.

The initiation codon at position 1 is flanked by some nucleotides matching the consensus sequence propcsed by Kozak (20). The sequence upstream to this ATG, contains 6 other ATG codons (positions -174, -164, -133, -86, -67, -56), the most upstream of which (position -174) is flanked by nucleotides which have a closer homology to Kozak's consensus sequence than the putative initiation codon (Fig. 1b). This ATG is followed, 36 nucleotides downstream, by a TGA stop codon. Interestingly, a similar sequence (72% identity) is found in the rabbit PRL receptor cDNA (19). Such a minicistron, preceding the major coding sequence, occurs in a small subset of cellular messenger RNAs, including many oncogene and steroid receptor mRNAs, as well as mRNAs for other genes requiring precisely regulated expression (21). The function of such motifs remains speculative.

As has been reported for the rat liver and rabbit mammary gland PRL receptors, the extracellular domain of the human receptor contains five cysteine residues (aa 12, 21, 51, 62 and 194) and three potential N-linked glycosylation sites (aa 35, 80 and 209) although the last is located so close to the putative site of insertion within the membrane that it may not be glycosylated. There is a single strongly hydrophobic region of 24 amino acids (211-234) which corresponds to the trans-membrane region of the PRL receptor (Fig. 1b). This sequence is highly conserved between the rat, rabbit and the human (18, 19). Using the H1 RNA probe, we screened 5 x 10^4 clones of a size-selected T-47D (human breast cancer cell line) cDNA library and found two positive clones. One (T2) extends between bases 1244 and 2439 of the hepatoma clone and is identical to it except at position 180 where a thymidine is replaced by a

cytidine. This substitution results in an Arg residue in place of a Cys residue at position 578. The functional importance of this difference is not known.

Human breast cancer biopsies or cells in culture (T-47D) and a membrane of the human placenta, the chorion laeve are the two tissues in which human PRL receptors have been best characterized (22-24). Northern blot analysis of human chorion laeve and T-47D cells revealed three distinct bands of mRNA of 2.8, 3.5 and 7.3 kb (Fig. 2). This pattern is strikingly similar to that found in the rabbit, where three bands were detected in all tissues examined (19). The low molecular weight band present in most rat tissues (~2 kb) was not found in human tissues. No signal was detected in Hep G2 cells. This is probably due to the very low abundance of the PRL receptor message in these cells since only 6 positive clones were obtained by screening 1.6 x 10^6 clones of the Hep G2 library. Moreover, there was no specific binding of prolactin to Hep G2 cells (data not shown), although prolactin receptors have been detected by immunocytochemistry in human hepatoma (25).

In order to show that the cDNA cloned from the hepatoma library could direct the synthesis of a protein able to specifically bind human PRL in mammalian cells, we prepared a cDNA containing the entire coding region (H1/H2), inserted it into the pECE vector and stably transfected Chinese hamster ovary (CHO) cells. Binding experiments using [^{125}I] labelled hGH (a hormone with lactogenic and growth promoting activity) show that the expressed membrane receptor has a binding specificity confirming that the H1/H2 cDNA encodes a human PRL receptor (Fig. 3a). Scatchard analysis (Fig. 3b.) of the hGH binding curve revealed an

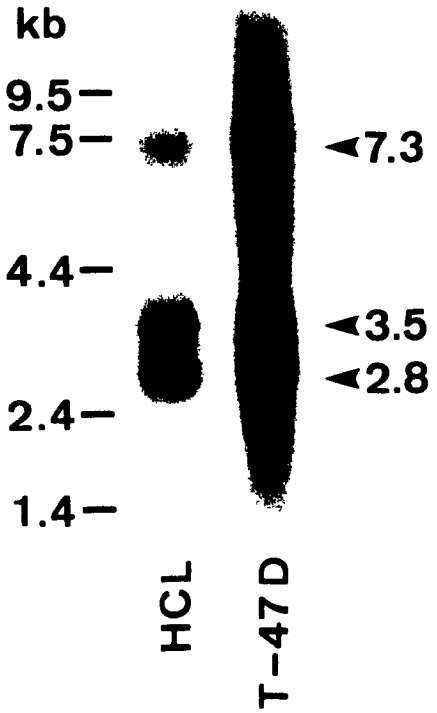

FIG. 2 Northern blot analysis of human PRL receptor mRNA expression.

Migration of an RNA ladder (BRL) is indicated on the left for size markers and the size of the PRL receptor mRNAs on the right. HCL:human chorion laeve.

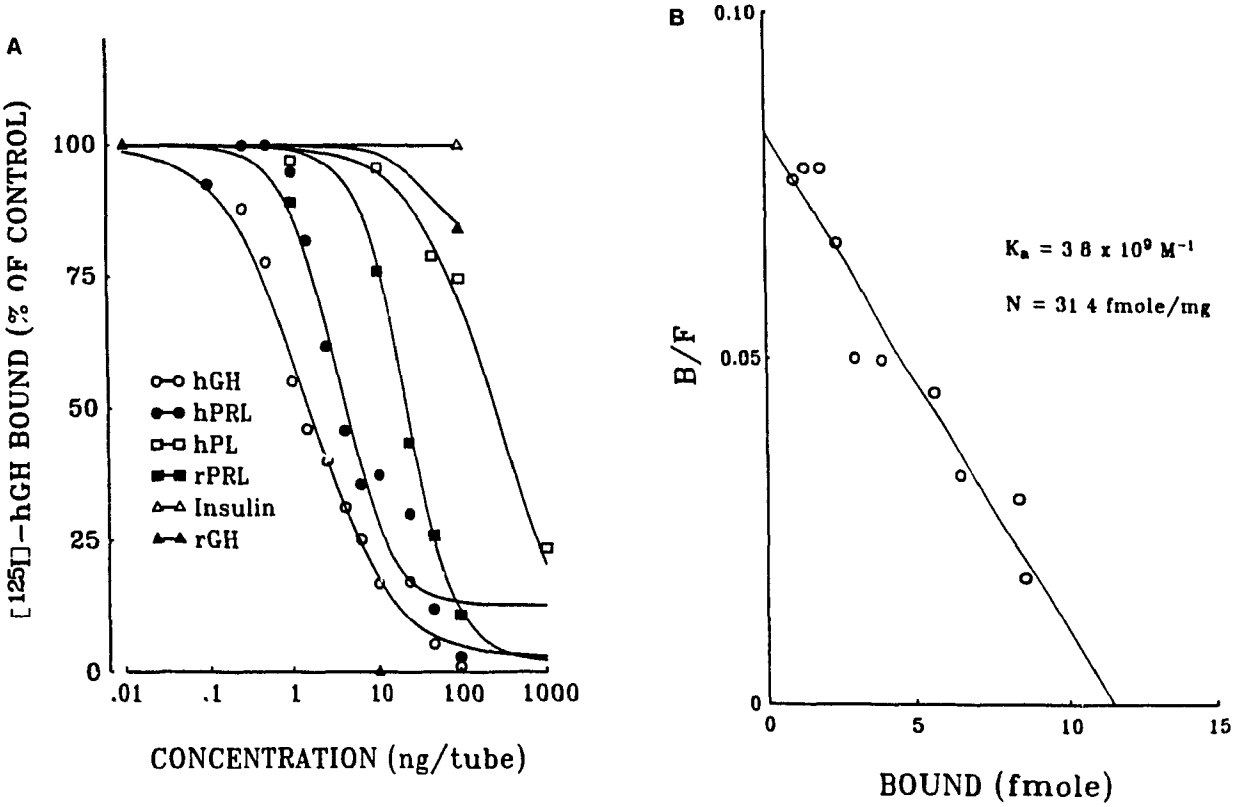

FIG. 3 Expression of the human hepatoma PRL receptor cDNA clone in CHO cells.

(a) Competition assay with unlabelled human GH (o), human PRL (●), rat PRL (▪), human PL (□), rat GH (▲) and bovine insulin (△) of membranes from the transfected CHO cells (22). Values are expressed as a percentage of specific binding calculated in the absence of any unlabelled hormone (3100 ± 50 cpm/350 μg protein) for 30,000 cpm added. Nonspecific binding is 1200 ± 100 cpm. (b) Scatchard plot of human GH binding data in transfected CHO cells.

affinity (K_a = 3.9 x 10^9 M^{-1}) similar to values reported for the human PRL receptor (22). A nearly identical value was obtained for the competition curve using hPRL (K_a = 4.5 x 10^9 M^{-1}).

DISCUSSION

The strong degree of sequence identity between the human PRL receptor sequence and that of the rat and rabbit is illustrated in Fig. 4a. The long cytoplasmic region of the human PRL receptor constitutes an obvious difference with the rat liver PRL receptor. The overall amino acid identity is 67% when the last 307 amino acids of the human receptor are excluded. However, similarity between the two receptors abruptly ends at residue 261 of the rat PRL receptor, so that a sequence comparison only to this point results in an identity of 75%, or 84% based on conservative amino acid substitutions. As seen in Fig. 4b, there is also strong identity (75%) between the human and rabbit receptors, which increases to 80% when conservative substitutions are considered. There are two gaps in the sequence identity in the cytoplasmic domain of the two receptors (Fig. 4b): the first (2 spaces) is inserted after aa 365 of the human receptor, and the second (8 spaces) after aa 420 of the rabbit receptor. Fig. 5 shows a schematic representation of the three PRL receptors (rat, rabbit and human) in comparison with the human/rabbit GH receptors (26). As expected, the human PRL receptor shares regions of striking similarity previously recognized with the GH receptor (18). The highest degree of sequence identity is found between the first two pairs of Cys residues in the extracellular domain and in the intracellular region flanking the transmembrane segment. The extended cytoplasmic domain of the human PRL

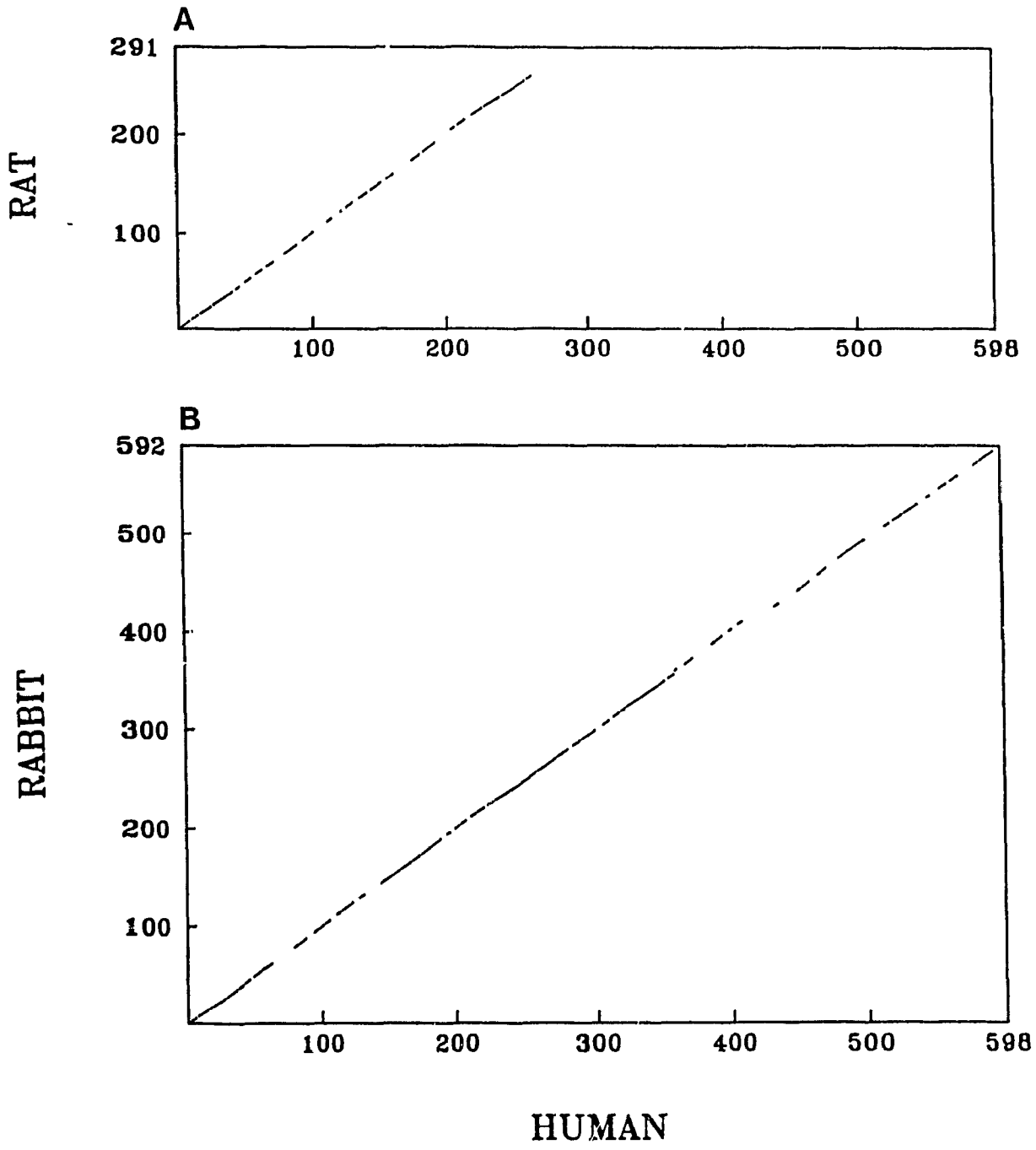

FIG. 4 Comparison of prolactin and GH receptor structures.

Dot matrix plot of the regions of amino acid sequence identity comparing the human PRL receptor with the rat (a) and the rabbit (b) PRL receptors.

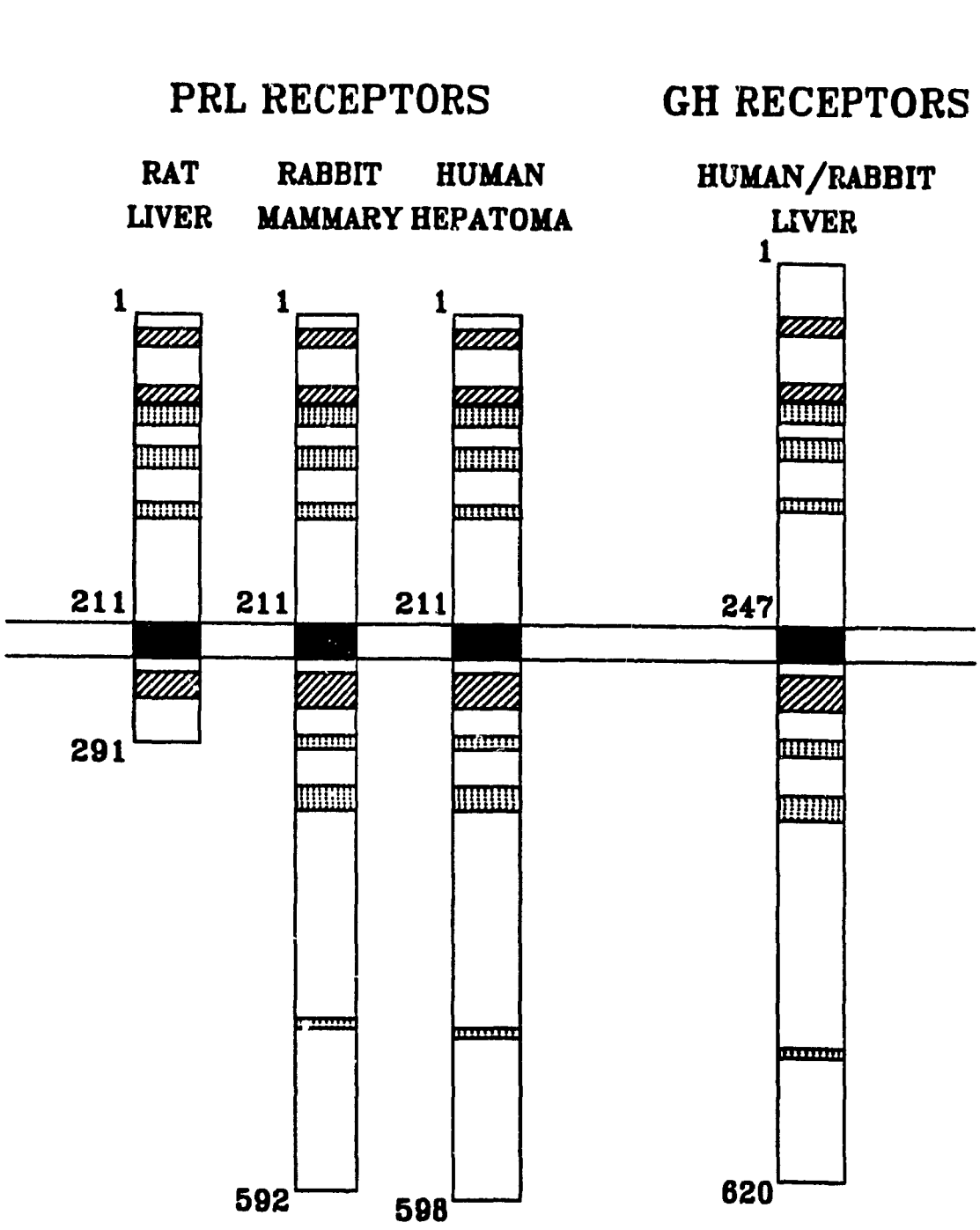

FIG. 5. Schematic representation of the PRL receptor structures from three different species (rat, rabbit and human) compared with the human/rabbit GH receptors.

Numbers indicate the position of the first amino acid, the first amino acid of the transmembrane region (black), and the last amino acid of the mature receptor. Regions of high similarity with the GH receptors (>68%) are cross-hatched and those of moderate similarity (38-60%) are stippled.

receptor contains three additional sequences similar to the GH receptor, as is the case for the rabbit receptor (19). In the absence of a consensus sequence for a tyrosine kinase domain or any potential ATP binding site, the presence of these highly conserved sequences in the cytoplasmic domain of all the PRL and GH receptors strengthens the hypothesis that they may be potentially involved in the process of signal transduction of both hormones. Transfection of full-length PRL receptor cDNAs will permit a systematic evaluation of structure-function relationships; studies involving mutagenesis and hybrid constructs in functional cellular systems may help clarify the mechanism of action of these hormones.

Since there has been no reliable means of accurately measuring PRL receptor levels in small quantities of human tissues, due to the relatively low level of binding, compared with PRL receptor levels in experimental animals, the human PRL receptor cDNA constitutes an important new tool. It will be possible to measure hPRL receptor mRNA levels in different breast cancer cell lines and biopsies, and under various physiological conditions. Moreover, the deduced amino acid sequence of the receptor will permit the production of synthetic peptides and antibodies to the extracellular and cytoplasmic domains, which will be critical for the identification of prolactin receptors by immunocytochemistry. Therefore, the availability of a human PRL receptor cDNA should permit a better understanding of prolactin's role in the development and growth of human breast cancer, in the regulation of human reproductive processes, and potentially, as an immunomodulatory hormone.

MATERIALS AND METHODS

Screening of the Human Libraries

A size-selected hepatoma cDNA library prepared in λgt10 was obtained from J. C. Edman in W. J. Rutter's laboratory at the University of California, San Francisco. An oligo(dT) primed T-47D cDNA library was prepared as described previously (18) except that the cDNAs were size-selected on Bio-Gel P-60 and ligated to λgt10 arms. Low stringency hybridization with F3 cDNA probe, labelled by random priming (27), was carried out for 16 h at 42 C in 20% formamide, 5 x Denhardt's, 5 x SSPE (28), 0.1% SDS, 0.1 mg/ml salmon sperm DNA, and 6% dextran sulphate; washing was at 42 C in 1 x SSC, 0.1% SDS 1mM EDTA. High stringency hybridization with a H1 RNA probe was performed as described previously (18). Suitable restriction fragments of the cDNA clones were subcloned into M13 derivatives or in Bluescript vector (Stratagene) and both strands were sequenced by the dideoxy chain termination method (29) using modified T7 DNA polymerase (Sequenase, US Biochemicals).

Northern Blot Analysis

Human chorion laeve and T-47D polyadenylated mRNAs (25 μg each) were isolated using the guanidium thiocyanate/CsCl procedure (30), treated for 60 min at 50 C in 1M glyoxal, 50% DMSO, 10 mM phosphate buffer, pH6.5 and run through a 1.5% agarose gel in 10 mM phosphate buffer. RNA was transferred to a nylon membrane (Genescreen, NEN/Dupont), baked and hybridized to a H1 RNA probe as previously described (18). Hybridization and washing temperatures were 65 C and 75 C, respectively. The filter was then autoradiographed overnight at -70 C with an intensifying screen.

Stable Expression in Eukaryotic Cells

A 2.5 kb cDNA containing the entire PRL receptor coding sequence was constructed by joining H1 and H2 cDNAs at their Nco I site, and assembled at the Eco RI site of the mammalian expression vector pECE (31). Stable CHO cell lines were established by cotransfection of 10 μg of this construct with 2 μg of pSV2 neo using a pulse generator constructed in the laboratory. A volume of 0.5 ml of cell suspension (~10^6 cell/ml) in 275 mM sucrose, 1 mM phosphate buffer, pH 7.4 and 1mM $MgSO_4$ received an electric shock of 120 V, 10 pulses/sec, 100 μsec/pulse for 5 minutes. Cells were transferred to a tube containing the two plasmids for 20 mins at 4° C, suspended in Ham's F12 medium with 10% fetal bovine serum and distributed in five P100 petri dishes. Selection was made with 400 μg/ml of active G418, the eukaryotic neomycin analog. After isolation of the transfectant clones, cells were harvested, washed and lysed in Eppendorf tubes by repeated freeze-thawing cycles. Membranes were prepared by centrifugation (10,000 g) for 2 min and the pellet was resuspended in 25 mM tris-HCl, pH 7.5, 25 mM $MgCl_2$, 1 mM PMSF, 10 μM leupeptin, 1 μg/ml aprotinin. Three hundred fifty μg of suspension and 30,000 cpm of ^{125}I-hGH were used per tube for binding studies that were performed as previously described (32). Control CHO cells specifically bound 1.4% of added radioactivity compared to 8.2% in the transfected CHO cells. Human GH used for radiolabeling and competition curves was the recombinant hormone (GO42A) kindly provided by Genentech (San Francisco, CA). Human PRL was generously supplied by Dr. H. G. Friesen, University of Manitoba, Winnipeg, Manitoba. Rat PRL (NIADDK rPRL-I-5; 30 IU/mg), rat GH (NIADDK rGH-I-5; 2.0 IU/mg) were kindly supplied by the National Hormone

and Pituitary Program. Bovine insulin (24.5 IU/mg) was obtained from Sigma (St. Louis, MO) and human placental lactogen (hPL) from ICN Pharmaceuticals, Inc. (Cleveland, Ohio).

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REFERENCES

1. Nicoll CS, Bern HA 1972 On the actions of prolactin among the vertebrates: is there a common denominator? In: Wolstenholme GEW and Knight J (eds) Lactogenic Hormones. Churchill Livingston, Edinburgh, pp 299-317

2. Meites J (1988) Biological functions of prolactin in mammals. In: Hoshino K (ed) Prolactin Gene Family and its Receptors. Elsevier Science Publishers, Amsterdam, pp 123-130

3. De Ziegler D, Gurpide E 1982 Production of prolactin by cultures of cells from human decidua. J Clin Endocrinol Metab 55:511-515

4. Heffner LJ, Iddenden DA, Little CR 1986 Electrophoretic analyses of secreted human endometrial proteins: identification and characterization of luteal phase prolactin. J Clin Endocrinol Metab 62:1288-1295

5. Hiestand PC, Mekler P, Nordmann R, Grieder A, Permmongkol C 1986 Prolactin as a modulator of lymphocyte responsiveness provides a possible mechanism of action for cyclosporin. Proc Natl Acad Sci USA 83:2599-2603

6. Di Mattia GE, Gellersen B, Bohnet HG, Friesen HG 1988 A human β-lymphoblastoid cell line produces prolactin. Endocrinology 122:2508-2517

7. Hughes JP, Elsholtz HP, Friesen HG 1985 Growth hormone and prolactin receptors. In: Posner BI (ed) Polypeptide Hormone Receptors. Dekker, New York, pp 157-199.

8. Tyson JE, Mowat GS, McCoshen JA 1984 Simulation of a probable biologic action of decidual prolactin on fetal membranes. Am J Obstet Gynecol 148:296-300

9. McNatty P, Sowers RS, McNeilly AS 1974 A possible role for prolactin in control of steroid secretion by the human Graafian follicle. Nature 250:653-655

10. Russell DH 1988 Prolactin and immunomodulation. In: Hoshino K (ed) Prolactin Gene Family and its Receptors. Elsevier Science Publishers, Amsterdam, pp 155-165

11. Welsch CW, Nagasawa H 1977 Prolactin and murine mammary tumorigenesis: a review. Cancer Res 37:951-963

12. Subramanian MG, Gala RR 1986 Do prolactin levels measured by RIA reflect biologically active prolactin? J Clin Immunoassay 9:42-52

13. Manni A, Wright C, Davis G, Glenn J, Joehl R and Feil P, 1986 Promotion by prolactin of the growth of human breast neoplasms cultured in vitro in the soft agar clonogenic assay. Cancer Res 46:1669-1672

14. Biswas R, Vonderhaar BK 1987 Role of serum in the prolactin responsiveness of MCF-7 human breast cancer cells in long-term tissue culture. Cancer Res 47:3509-3514

15. Murphy LJ, Murphy LC, Vrhovsek E, Sutherland RL, Lazarus L 1984 Correlation of lactogenic receptor concentration in human breast cancer with estrogen receptor concentration. Cancer Res 44:1963-1968

16. Bonneterre J, Peyrat JP, Beuscart R, Lefebvre J, Demaille A 1987 Prognostic significance of prolactin receptors in human breast cancer. Cancer Res 47:4724-4728

17. Di Carlo R, Muccioli G, Di Carlo LF, Faggiuolo R, Berruit A, Maschietto M, Dogliotti L 1988 Prognostic value of prolactin receptors in human breast tumors. In: Hoshino K (ed) Prolactin Gene Family and its Receptors. Elsevier Science Publishers, Amsterdam, pp 481-484

18. Boutin JM, Jolicoeur C, Okamura H, Gagnon J, Edery M, Shirota M, Banville D, Dusanter-Fourt I, Djiane J, Kelly PA 1988 Cloning and expression of the rat prolactin receptor, a member of the growth hormone/prolactin receptor gene family Cell 53:69-77

19. Edery M, Jolicoeur C, Levi-Meyrueis C, Dusanter-Fourt I, Pétridou B, Boutin JM, Lesueur L, Kelly PA, Djiane J 1989 Identification and sequence analysis of a second form of prolactin receptor by molecular cloning of complementary DNA from rabbit mammary gland. Proc Natl Acad Sci (USA) 86:2112-2116

20. Kozak M 1986 Point mutations define a sequence flanking the AUG initiator codon that modulates translation by eukaryotic ribosomes Cell 44:283-292

21. Kozak M 1986 Bifunctional messenger RNAs in eukaryotes. Cell 47:481-483

22. Peyrat JP, Djiane J, Kelly P, Vanderwalle B, Bonneterre J, Demaille A 1984 Characterization of prolactin receptors in human breast cancer. Breast Cancer Res and Treatment 4:275-281

23. Murphy LJ, Vrhovsek E, Sutherland RL, Lazarus L 1984 Growth hormone binding to cultured human breast cancer cells. J Clin Endocrinol Metab 58:149-156

24. Herington AC, Graham J, Healy DL 1980 The presence of lactogen receptors in human chorion laeve. J Clin Endocrinol Metab 51: 1466-1468

25. Mori C, Inoue K, Arii S, Tobe T, Hoshino K, 1988 Prolactin receptor detected immunohistochemically in the liver of hepatoma patients. In: Hoshino K (ed) Prolactin Gene Family and its Receptors. Elsevier Science Publishers, Amsterdam, pp 345-348

26. Leung DW, Spencer SA, Cachianes G, Hammonds RG, Collins C, Henzel WJ, Barnard R, Waters MJ, Wood WI 1987 Growth hormone receptor and serum binding protein: purification, cloning and expression. Nature 330:537-543

27. Feinberg AP, Vogelstein B A 1983 A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal Biochem 132:6-13

28. Maniatis T, Fritsch EF, Sambrook J 1982 Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

29. Sanger F, Nicklen S, Coulson AR 1977 DNA sequencing with chain terminating inhibitors. Proc Natl Acad Sci USA. 74:5463-5467

30. Chirgwin JM, Przybyla AE, Macdonald RJ, Rutter WJ 1979 Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. Biochemistry 18:5294-5299

31. Ellis L, Clauser E, Morgan DO, Edery M, Roth RA, Rutter WJ 1986 Replacement of insulin receptor tyrosine residues 1162 and 1163 compromises insulin-stimulated kinase activity and uptake of 2-deoxy- glucose. Cell 45:721-732

32. Posner BI, Kelly PA, Shiu RPC, Friesen HG, 1974 Studies of insulin, growth hormone and prolactin binding: tissue distribution, species variation and characterization. Endocrinology 96:521-531

PART III

GENERAL DISCUSSION

CHAPTER 8

GENERAL DISCUSSION

8.1 Cloning of the PRL receptor

Cloning a very low abundance protein like the PRL receptor represented a difficult task, especially in 1984, when we undertook this study. Indeed, at that time, only a few receptors had been cloned (see Introduction), most of the work achieved following the purification of the receptor protein which provided amino acid sequence information. In the absence of homogenously purified PRL receptor, and with the availability of anti-PRL receptor poly- and monoclonal antibodies, two of the techniques presented in Chapter I appeared potentially applicable to cloning of the PRL receptor: expression systems with immunoscreening and gene transfer. The direct expression systems in Xenopus oocytes and in COS cells were developed only in 1987. On the other hand, the techniques of differential hybridization and cDNA subtraction required positive and negative tissues or cells that contained, in general, the same mRNAs, except for the PRL receptor mRNA. These requirements are not fulfilled by any cell system known to express the PRL receptor. Therefore, I decided to use an expression system with immunoscreening and keep the gene transfer technique as an alternative method, in case of failure of the first approach. The decision to begin with immunoscreening of an expression system was based on the fact that the libraries that were used were prepared in λgt11. These libraries could also be screened with oligonucleotide probes, if and when they became available. This was indeed the case and the first positive clones for the PRL receptors were identified in a polysome-enriched rat liver cDNA library that was originally prepared in the expression vector λgt11 and screened with poly- and monoclonal antibodies.

Although immunoscreening of an expression library is a very attractive approach, since antibodies to partially purified receptor preparations are relatively easily available, the success rate of this approach is not high. In fact, there are certainly many more unpublished, unsuccessful studies, than the few successful reports that have been published. One notable failure of immunoscreening of a λgt11 library with poly- and monoclonal antibodies is the insulin receptor, reported by Ebina et al., (1985). Most of the successful cloning of receptors and other proteins with this technique involved screening with polyclonal antibodies. Only one membrane receptor, the α1 subunit of the DHP-sensitive calcium channel (Ellis et al., 1988), has been successfully cloned by screening an expression library with a monoclonal antibody. On the other hand, two steroid hormone receptors, the progesterone and the vitamin D receptors (Conneely et al., 1986; Jeltsch et al., 1986; Loosfelt et al., 1986; McDonnell et al., 1987) have been successfully cloned using this approach. In these studies, more than 10^6 clones had to be screened in order to find only one or a few positive clones. Moreover, most groups had a panel of mAbs, from which they chose the most appropriate, usually by testing their ability to detect receptor by immunoblot analysis. It is thought that only mAbs capable of identifying a signal on an immunoblot will be effective for screening. For the PRL receptor, 2 mAbs (E21 and E29) against the rat liver PRL-R were available at the time of immunoscreening, and only E21 was able to identify the PRL receptor on an immunoblot (Kaloh et al., 1987). The high specificity of mAbs for certain epitopes of a receptor make them attractive probes for screening, but at the same time limit their

usefulness, since it is only when these epitopes are expressed in the right configuration that they can be recognized. As shown in Chapter 4, I was unable to detect the immature form of the PRL receptor synthesized in reticulocyte lysates (Fig 1 and 7) using the mAb E21. Even the mature form synthesized in frog oocytes (Fig. 3) was not detected by immunoprecipitation with the mAb E21, although it was able to immunoprecipitate solubilized or partially purified receptor (Katoh et al., 1987). These results suggested that the immunoprecipitation studies were not sensitive enough to detect the PRL-receptor or that the change in glycosylation of the receptor found both in vitro and in frog oocytes was sufficient to somehow change the epitope recognized by E21. Such a change is certainly expected in the fusion proteins expressed in the λgt11 system and can explain the failure to identify specific positive recombinants with the mAbs.

Another problem involves the λgt11 itself, since only one-sixth of the inserted cDNAs will be expressed in the right sense and the correct coding frame. This reduces the chances of finding a positive clone, especially when the receptor is present in very low abundance, as is true for the PRL receptor. In the case of the vitamin D receptor, for which the cell concentration is similar to the PRL receptor, McDonnell et al., (1987) screened 10^7 independent clones with mAbs, and only found a single positive clone. Such a low concentration of receptors requires extremely good libraries or the enrichment of the receptor mRNAs prior to preparation of a library. The immunopurification of polysomes that I used resulted in a 300 to 500-fold enrichment, a result which is quite good, especially when compared to the 10-fold enrichment obtained by

other techniques, such as mRNA size fractionation (Maniatis et al., 1982). Moreover, the 32 kDa band precipitated by the antiserum no 212 from the in vitro translation products of this mRNA is highly compatible with the M_r of 33,368 predicted from the cDNA structure for the nonglycosylated rat liver PRL receptor. This form was not detected by polyclonal Abs when non-enriched mRNAs were used. The number of positive recombinants found when the enriched cDNA library was screened with antisera confirmed the extent of enrichment. However, an important proportion of these positive recombinants probably represented proteins present in the partially purified preparation of the PRL receptor and were unrelated to the receptor. For this reason, screening a non-enriched, size-selected library with these antisera gave many false positives. In fact, successful cloning of other membrane receptors by using an expression vector (see Table I) were usually obtained with monospecific polyclonal antibodies that were prepared by receptor affinity chromatography. We were unable to produce this quality of polyclonal antibody due to the low degree of purity ($\sim$1%) of our partially purified PRL receptor preparation at that time. Nevertheless, in the absence of any alternative cloning procedure, it is quite probable that a thorough examination of some of the 10,000 positive recombinants identified in the enriched library detected by the polyclonal antiserum would have resulted in one or more PRL receptor cDNA clone.

The turning point in the experimental approach of the cloning of the PRL receptor was certainly the purification to homogeneity of the rat liver PRL receptor. This purification was made possible by the use of immunoaffinity chromatography with the specific monoclonal antibody E21

(Okamura et al., 1989a). This step offers several advantages compared to hormone affinity chromatography previously described: the maintenance of Triton X-100, which is a more efficient detergent for solubilizing rat liver membranes; the decrease in degradation of the receptor, due principally to the fact that the affinity chromatography was performed at 4 C instead of at 20 C; and the larger yield of receptor. These advantages resulted in a 1000-fold purification over microsomal receptors, with a recovery of 52%. In spite of this improved purification step, the purified receptor was still only 4-6.5% pure. Thus, a preparative SDS gel electrophoresis step was necessary to obtain a homogeneous preparation.

Once the amino acid sequence of some peptide fragments was obtained, peptide sequences were chosen and oligonucleotide probes were synthesized that represented the best predicted sequence (PRLR-2) and two mixtures of oligonucleotides, that encoded most of the possible sequences (PRLR-1, PRLR-2A). As discussed in Chapter 1, both types of synthetic oligonucleotides have been successfully used to clone membrane receptors. Table III compares the sequences of the synthetic oligonucleotides with that of the F3 cDNA. Based on these comparisons, one mismatch of the four choices was seen in the 24-mixmer PRLR-1, 10 mismatches for 14 choices in the 45-mer, best predicted sequence PRLR-2 and 0 mismatches for two choices in the 23-mixmer PRLR-2A. The two positive recombinants E_1 and E_2 were obtained by screening the enriched library with probe PRLR-2A. Seventy-one per cent of the choices of the 45-mer best predicted sequence were incorrect. This explains the lack of positives obtained with this probe and shows the limitation of prediction of codon usage by

TABLE III: COMPARISON BETWEEN THE SYNTHETIC OLIGONUCLEOTIDES AND F3 cDNA SEQUENCES[1]

PRLR-1 (peak 13)[2]	CCA	GTA	GC**T**/C	A/**G**TC	**A**/T GG	CTT	A/**G**CA								
			*												
F3 cDNA	CCA	GTA	TCC	ATG	TGG	CTT	GCA								
	210						203								
PRLR-2 (peak 21-1)[2]	CTT	GAA	CTG	GGT	CTG	GTG	GCC	GGT	GAA	GTG	GAT	CTC	CCA	CTC	CTC
	*	*		*	*	*	*	*	*	*					*
F3 cDNA	TTT	AAA	CTG	TGT	TTG	ATG	ACC	TGT	AAA	ATG	GAT	CTC	CCA	CTC	TTC
	187														173
PRLR-2A (peak 21-1)[2]	GT	A/**G**AA	A/G TG	**A**/G AT	T/C TC	CCA	T/C TC	T/C TC							
F3 cDNA	GT	AAA	ATG	GAT	CTC	CCA	CTC	TTC							
	180						173								

[1] Bold letters represent choices made at this base position. The numbers under the F3 cDNA sequence indicate the amino acid position deduced from the cDNA sequence (Boutin et al., 1988).

[2] The peak number represents the tryptic fragment, separated by reverse-phase HPLC, that was used to deduce the sequence of the synthetic oligonucleotides (Okamura et al., 1989a).

the published tables.

8.2 Structure of the PRL receptor

The cloning and expression of the rat liver PRL receptor presented in chapter 5 confirmed previous biochemical studies which suggested that the PRL binding subunit had a mol. wt. of 35,000-45,000 (Chapter 3) but failed to explain the higher M_r forms of PRL-R seen under certain conditions. Moreover, Northern blot analysis with mRNAs from different rat tissues in Fig. 5 of Chapter 5, showed that the PRL-R mRNA signal presented a marked size heterogeneity varying with the tissue examined. In some tissues the 2 kb band seen in liver on Northern blot analysis (Chapter 5), was not the major band, suggesting the existence of different sizes of untranslated regions or of different coding regions of the receptor. Using the F3 rat liver cDNA as a probe, cDNA libraries from other tissues and species were screened, in order to find other forms of PRL receptor. In Chapter 7, the cloning of the human PRL receptor was presented. Although this receptor showed a high sequence identity with the extracellular, transmembrane and part of the intracellular domains of the rat liver PRL-R, it contained a much longer cytoplasmic domain, that was homologous to the rabbit and human GH receptors. Our collaborators in France identified a similar long form of PRL-R in rabbit mammary gland using the rat PRL-R cDNA probe (Edery et al., 1989). Northern blot analysis of different rabbit tissues with this long cDNA form used as a probe revealed three bands similar to those reported in Fig. 2, Chapter 7 for the human and, in contrast to the rat, mRNAs in the rabbit and human do not vary in different tissues tested (unpublished data). The human and the rabbit PRL receptors have a strong

sequence identity (75%), even in their long cytoplasmic domains, and they also show a strong identity with the rat PRL receptor that abruptly ends at residue 261 of the cytoplasmic domain of the mature rat PRL-R.

Davis and Linzer (1989), using an oligonucleotide probe designed from a region of the F3 cDNA clone, isolated 3 different PRL-R cDNA clones from a mouse liver cDNA library. All these clones were nearly identical to the rat liver PRL cDNA except that two of them (PR-1 and PR-2) encoded proteins with divergent carboxyl-terminal sequences after residue 261, of the rat PRL-R, that is at the same site of divergence between the sequences of the short rat liver form and of the long human and rabbit forms. Thus PR-1 encodes a mature protein of 284 aa and PR-2, a mature protein of 273 aa. Moreover, they found a third cDNA form (PR-3) which had a similar sequence to the rat clone after residue 261 but contained two single nucleotide changes in its 5'-region compared to the two other mouse forms: one base substitution in the second codon of the signal sequence and one base deletion at codon 78 that brings a translational termination codon into frame after 19 additional residues. The authors suggested that this truncated from of only 78 aa may be secreted but did not test binding to PRL.

Recently, we screened size-selected libraries from rat ovary, estrogen-treated liver and kidney, using a RNA probe derived from the cDNA F3, in order to detect a long form of PRL receptor in the rat, since there is biochemical evidence of larger forms of the PRL receptor (Bonifacino and Dufau, 1984; Haldosén and Gustafsson, 1987). Such screening revealed the existence of a large form of the PRL receptor (PRL-R2) in the rat, which is a major form in ovary and is present to a much lower extent in liver (Shirota et al., unpublished). The predicted

size of the mature form is 591 amino acids. The cDNAs isolated are identical to F3, except that they diverge after residue 261 to give a larger cytoplasmic domain. This long form of the PRL-R shows strong overall sequence identity with the corresponding residues of the rabbit (65%) and human (64%) PRL-Rs, and maintains the same four regions of localized identity with the rabbit and human GH receptors, described previously (Chapter 7, Fig. 5). Moreover, mRNAs specific to the short and long forms of the PRL-R are present in both the ovary and liver of the rat, as indicated by Southern blot analysis of cDNA amplified by DNA polymerase chain reaction (PCR) of RNA (Shirota et al., unpublished).

The common site of divergence of many sequences at residue 262 of the rat PRL-R suggests that these forms arise by alternative RNA splicing. This has been confirmed by the identification of the structural arrangement of the PRL-R gene (Banville et al., unpublished). Indeed, the PRL-R gene includes at least 11 exons spread over >60 kb. The short (PRL-R1) form contains the first nine exons plus exon 11, exon 10 being spliced out. The long (PRL-R2) form is formed by the first 10 exons (Fig. 1). It will be interesting to see if other exons corresponding to the two different carboxyl-terminal sequences of the mouse PR-1 and PR-2 can be found in the rat genome. It is probable that other exons will be found to explain the complex pattern of mRNAs for certain tissues (e.g., ovary) of the rat (Shirota et al., unpublished). Moreover, preliminary results of primer extension experiments indicate that several different start sites of PRL-R mRNAs can be detected in rat liver (Banville et al., unpublished). These results suggest a pattern of receptor heterogeneity that was suspected but not well appreciated by previous biochemical studies.

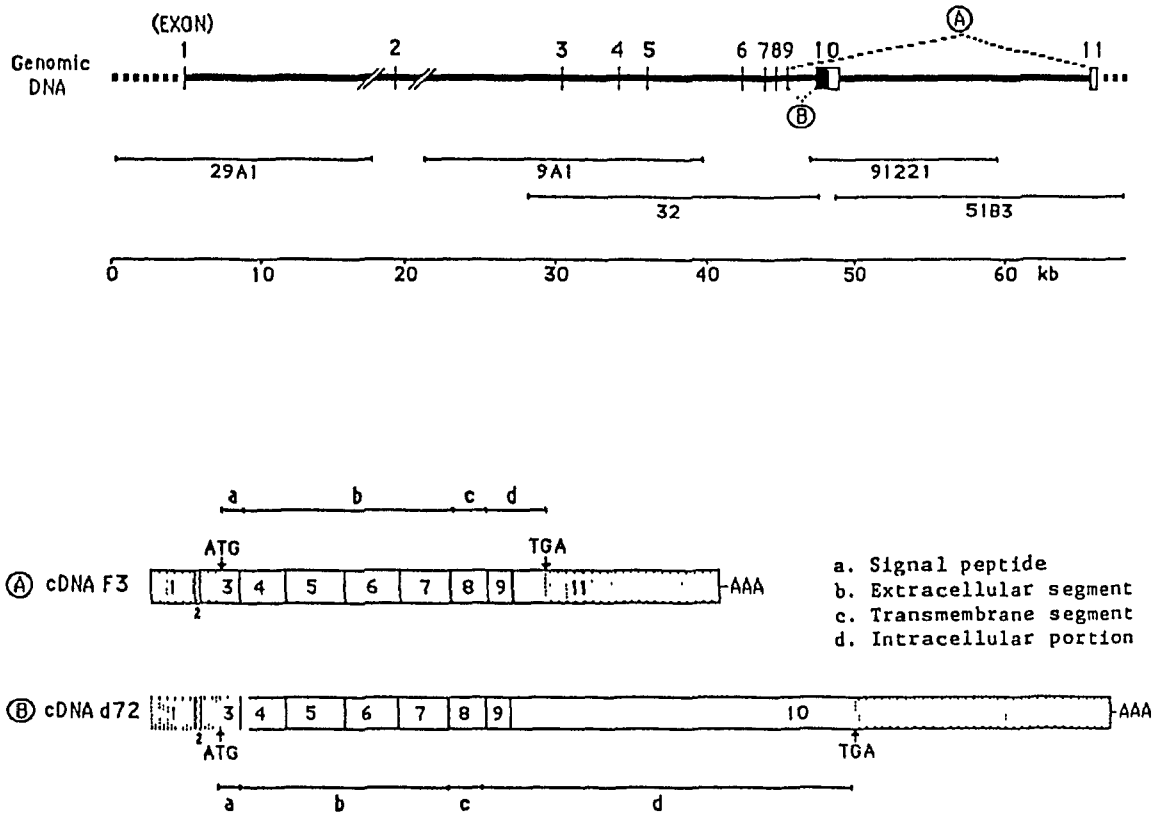

FIG. 1 Genomic organization of the rat prolactin receptor.

The upper panel shows a map of the rat PRL-R gene. The position of the exons are indicated by vertical bars and boxes. The filled part of the boxes indicates the translated sequences and the empty part, the untranslated sequences. The name as well as the size of each genomic clone is noted. The circled A and B indicate the alternative splicing processes which result in the short and long forms of the PRL-R, respectively. In the lower panel are shown the cDNAs corresponding to the alternative splicing products which result in the short (A) and long (B) forms of the receptor. The stippled areas indicate the untranslated regions and the numbered boxes correspond to the exons. The four different domains of the proteins are indicated by the letters a,b,c,d (Banville et al., unpublished).

The long form of the rabbit mammary gland PRL-R was not expected from cross-linking experiments (Haeuptle et al., 1983; Katoh et al., 1985), or immunoprecipitation (Dusanter-Fourt et al., 1987). These studies identified a major M_r band of 32,000-40,000. Only the development of more sensitive techniques using mAbs recently allowed the detection of a larger band of 77,000 in this tissue (Sakai et al., 1988; Okamura et al., 1989b). This larger form probably represents the glycosylated form of the rabbit mammary gland PRL-R, which has a theoretical M_r of 75,000-80,000 according to the cDNA sequence (Edery et al., 1989). Difficulty to detect this large form by biochemical techniques can be explained by degradation of the receptor during these procedures. In the human, a similar form is expected, based on the cDNA sequence, although there is no reliable biochemical data available for comparison.

The 84,000 band that has been detected in liver and ovary of the rat (Bonifacino and Dufau, 1984; Haldosén and Gustafsson, 1987; Okamura et al., 1989b) is probably the result of dimer formation of the small 42,000 form. First a homogeneous preparation of [^{125}I]-labeled purified PRL-R, obtained by electroelution from a gel slice corresponding to a mol. wt of 38,000-43,000, was stored at -20 C for 3 weeks and then analyzed on SDS-PAGE followed by autoradiography. A larger M_r form (84,000) could be generated from the smaller (42,000) preparation (Okamura et al., 1989a). Second, immunoblot analysis with specific anti-rat liver PRL-R mAbs of extracts of CHO cells transfected with F3 cDNA (see Chapter 5) revealed the presence of both 42,000 and 84,000 forms, although the F3 cDNA encodes only the short form (unpublished results). Finally, the baculovirus expression system (Smith et al., 1983) was used

to overexpress the short form of the rat liver PRL-R by using the F3 cDNA. When the synthetic products were analyzed by immunoblot analysis, two forms of 38,000 and 76,000 were detected, corresponding to a differently glycosylated form of RPL-R1 and a probable homodimer of the 38,000 subunit (Okamura et al., unpublished data). Thus these results strongly suggest that the larger form represents a homodimer of 42,000 M_r form but at present the nature of the link (disulfide or other non-covalent bonds) has not been established. If the 84,000 M_r band detected in rat ovary does not correspond to the long form of the PRL-R, it is probable that the 51,000 band, recently discovered (Okamura et al., 1989b), corresponds to a degradation product of long PRL-R2 form.

Although the PRL-Rs do not contain a cysteine-rich region like the RTKs and other membrane receptors (see Chapter 2), they do contain highly conserved regions between the first two pairs of cysteines, suggesting an important functional role of these cysteines in the formation of inter or intramolecular disulfide bonds. These bonds are probably crucial for the conformation of the PRL-Rs and preliminary mutagenesis studies suggest that they are necessary for the binding of PRL to the PRL receptor.

Previous biochemical studies have suggested the presence of carbohydrate moieties in PRL receptors. Concanavalin A can affect the binding of PRL to its receptors and solubilized PRL receptors can be retained by concanavalin A-Sepharose column and eluted with specific sugars (Costlow and Gallagher, 1979; Bhattacharya and Vonderhaar, 1982; Sasaki et al., 1982). However, studies of Necessary et al (1984) on purified rabbit mammary gland PRL-R and of Vonderhaar et al., (1985) on purified mouse liver PRL receptors, did not confirm retention on concanavalin A-sepharose column, suggesting that the core binding unit of

the receptor does not contain con A-specific carbohydrate residues. Cloning of the rat PRL receptor clearly showed the presence of N-linked carbohydrates. Indeed, two of the three potential N-linked glycosylation chains, as assessed by cDNA structure, were confirmed to be glycosylated by protein sequencing of the corresponding peptide fragments (see Chapter 5 and Okamura 1989a). In fact all membrane receptors that have been cloned (Chapter 2) are glycoproteins and their unglycosylated precursor forms are always smaller than their mature forms.

8.3 Regulation and functions of PRL receptors

In Chapter 6, we showed that steady-st te mRNA and binding levels of PRL receptors are both regulated by development and estrogens, but that binding does not exactly parallel mRNA levels. From these results, it was concluded that PRL-R gene expression in rat liver is regulated at both the pretranslational and translational levels. However, the mechanism involved in this dual regulation is still completely unknown. One such a mechanism involved in the regulation of transferrin-receptor has been recently described by Klausner and coworkers. They discovered a family of regulatory RNA sequences, iron-responsive elements (IREs), located in the 5'-untranslated region of ferritin mRNA and the 3'-untranslated region of transferrin receptor mRNA, that can mediate iron-dependent control of ferritin translation and transferrin receptor mRNA stability (Hentze et al., 1987; Casey et al., 1988). The IREs are conserved stemloop structures that bind a cytosolic protein (IRE-BP) and free-sulfhydryl groups are involved in this RNA-protein interaction (Hentze et al., 1989). Although there is actually no evidence for the

existence of similar sequences in the untranslated regions of the PRL-Rs mRNAs, the possibility that other types of mRNA control sequences are involved remain attractive, especially with the discovery of multiple forms of PRL-R mRNAs which could contain different responsive elements and be expressed accordingly in various tissues. On the other hand, short open-reading frames are present in the 5'-untranslated regions of the human, rabbit and to a lesser extent, mouse PRL-Rs but not of the rat PRL-R. It has been suggested that these sequences may be involved in translational control (Mueller and Hinnebusch, 1986). In the case of the human PRL-R we tried to improve expression of the receptor in transfected CHO cells by deleting this 5'-upstream short open-reading frame of the cDNA sequence but no change was observed. Therefore, from the data available, no possible function of these sequences in the control of the PRL-R gene expression can yet be assigned.

As discussed in section 3.4, sex steroid hormones are able to induce hepatic PRL-R via increased pituitary secretion of PRL and GH. Moreover, PRL can cause both up and down regulation of its own receptor, depending on its concentration and the species studied. The availability of the PRL-R cDNAs will permit the study of this autoregulation at both the mRNA and protein levels. Furthermore, using probes specific to the different forms of the PRL-R, it will be possible to find if differential tissue or species expression is due to the regulation of specific forms of the receptor. These probes can be either a cDNA or a cRNA covering one specific C-terminus region, after residue 261 of the rat PRL, and used on Northern blot analyses, S1 (or RNase A) mapping and <u>in situ</u> hybridization. Alternatively, antibodies directed against these same

specific regions of the PRL-R can be used in immunoblot studies and for immunocytochemistry.

It is also possible that more than one gene encodes the multiple forms of PRL-R. In the rat, the present data suggest only one PRL-R gene, since the pattern of hybridization to genomic DNA digested with various restriction enzymes (Banville, unpublished) and the evidence for alternative RNA splicing are compatible with the formation of various mRNAs from a single gene. However, in the mouse, Davis and Linzer (1989) suggest that the existence of at least two PRL-R genes as being necessary to account for receptor heterogeneity in the liver. Expression of different PRL-R genes, varying according to the tissue or species studied would be a possible explanation for some paradoxical hormonal effects on PRL-R. More work on genomic organization of the PRL-R is required to resolve this issue and to isolate the 5'-flanking region of the PRL-R gene(s). With the availability of this region of the gene, it will be possible to link this sequence to reporter genes and identify the presence of responsive elements to different DNA binding factors by mutagenesis and transfection techniques, in order to better understand the transcriptional regulation of the PRL-R.

An important result of the cloning of the PRL-R has been the discovery of regions of striking similarity with the human and rabbit GH receptors, suggesting that the two receptors originated from a common ancestor. It is expected that the placental lactogen (PL) receptor belongs to this new receptor family. Indeed the PL-R has been recently purified from fetal and maternal sheep liver and shown to be a protein of M_r 44,000 binding oPL with a potency 30-50 times greater than that of oGH

and 500-1000 times greater than that of oPRL (Freemark and Comer, 1989). Interestingly, while there is little or no specific binding of GH or PRL to fetal tissues, as demonstrated in rat liver (Chap. 6; Kelly et al., 1974· Dhanireddy and Ulane, 1984), specific PL binding sites are detected in the liver of the fetal lamb (Freemark et al., 1986), fetal mouse (Harigaya et al., 1988) and human fetus (Hill et al., 1988). As seen in Chapter 6, the absence of PRL binding sites in rat fetal liver corresponds to undetectable mRNA levels, suggesting that the PRL-R gene is not yet transcriptionally activated at this early stage. The isolation of the PL cDNA would allow the clarification of this striking ontogenic difference between the two distinct but related receptors. It is probable that different transcriptional regulation occurs in the 5'-flanking region of both genes.

The structure of the PRL and GH receptors, as revealed by their cDNA sequences, suggests they form a new family of receptors that are part of the single membrane-spanning class of receptors. In fact, they do not have any domain related to structures known to be involved in one of the classical transduction systems. An ATP binding site, like that found in the cytoplasmic domain of the RTKs, is absent and there is no potential auto-phosphorylation site in the cytoplasmic domains of either the PRL or GH receptors. There is a growing list of single membrane-spanning receptors, for which the cDNA sequence failed to reveal their mechanism of transduction, as has been discussed in Chapter 2. This is particularly true for the "Miscellaneous" family into which PRL and GH receptors can be placed. In this class of receptors, there is evidence

that most (IL-2-R, NGF-R and IFN-γ-R) are associated with a second effector subunit that may or may not bind the ligand, and interacts with a cytoplasmic transduction system (see Chap. 2). There is actually no evidence for the existence of such a second subunit linked to either the PRL or GH receptors. As discussed previously, it is possible that the very large mol wt forms of PRL-R found under non-dissociating conditions, if not an artifact, may represent oligomers including other subunits. Contrary to IL-2-R and NGF-R, another subunit is not required for high affinity binding, since transfection of the cloned rat and human PRL-R in frog oocytes or eukaryotic cells allows the expression of receptors with a K_a similar to that reported for the membrane-bound or solubilized receptors.

Comparison of the cytoplasmic domain of the PRL-Rs, especially the long forms, with that of the GH-Rs revealed regions of strong localized sequence identity. These sequences may potentially interact with some unknown transduction system to transduce the message for both hormones. The fact that multiple forms of PRL-Rs were found with various carboxyl-terminal regions may indicate that varied actions of PRL involve multiple receptors that are linked to distinct signal transduction pathways, as suggested by Davis and Linzer (1989). On the other hand, the pattern of the short and long forms of the PRL-R is similar to the tandem ANP-C and GC/ANP-R (see Table I and Chap. 2). The ANP-C is a truncated form of the GC/ANP-R with a similar extracellular and transmembrane regions but with a short cytoplasmic domain lacking the guanylate cyclase domain of the latter. For this reason the ANP-C is a transporter, like the LDL and transferrin receptors, unable to transduce the ANF signal which requires

the GC domain of the GC/ANP-R. It is therefore possible that the short form of the PRL-R is uniquely a transporter, incapable of transmitting a signal inside the cell, while the long form contains the cytoplasmic sequences required to transduce the PRL message.

In order to determine the post-receptor events involved in PRL actions, the development of highly sensitive systems to measure the stimulatory effects of PRL is essential. Thus far, lactogenic and mitogenic effects in the mammary gland have been used as a model system. This tissue, however, consists of a mixed population of PRL sensitive and insensitive cells, making it difficult to detect minor intracellular changes in response to PRL. The establishment and use of cell lines which are highly sensitive to PRL is a necessary step. The rat Nb2 lymphoma cell line has been extensively used to study the mechanism of action of PRL, but lactogenic hormones affect mainly mitogenesis in these cells without the metabolic effects found in mammary gland. Moreover, there is evidence that Nb2 cells may express a different form of PRL receptor (Okamura et al., 1989b), suggesting that studies with this cell type may reflect only a particular action of PRL.

Usually, cell lines isolated from the mammary gland lose their ability to produce milk proteins when maintained in culture. Recently, some mammary epithelial cell lines, COMMA-1D (Danielson et al., 1984), HC11 (Ball et al., 1988) and HBL-100 (Gaffney, 1982), were shown to respond to PRL by the production of casein. These cell lines have been used to study the effect of PRL and other hormones on the transcription of casein gene promoter constructs (Ball et al., 1988; Doppler et al., 1989). However, these cells have a very low number of endogenous PRL

receptors and the magnitude of response of the casein gene is low. With the availability of PRL-R cDNAs, it will be possible to transfect these cells with a short or long cDNA form to amplify the casein response to PRL and test the ability of each form to transduce a PRL signal. Further studies with mutagenesis of the conserved cytoplasmic regions coupled with such transfections should identify the functional importance of these sequences. Finally, construction of PRL/GH receptor chimeras and subsequent transfection into functional systems, will help clarify the role of the different receptor domains involved in both PRL and GH actions.

CONTRIBUTION TO ORIGINAL KNOWLEDGE

The following novel findings and observation have been demonstrated in this thesis:

1) Evaluation of the poly and monoclonal antibodies by testing their ability to immunoprecipitate the PRL-R synthesized from mRNA *in vitro* and *in vivo* gave a good appreciation of their value for screening λgt11 expression libraries, for the isolation of the PRL-R cDNA clone (Chapter 4).

2) The primary structure of the rat liver and human prolactin receptors have been deduced from their respective cDNA clones (Chapters 5 and 7).

3) Regions of strong localized sequence identity between the PRL and GH receptors and the absence of sequence identity with any other reported protein suggest that the GH and PRL receptors form a new receptor gene family of the single membrane-spanning type (Chapters 5 and 7).

4) There are at least two forms of PRL-R. The long one (human PRL-R) shares the same extracellular, transmembrane and part of the cytoplasmic domains with the short one (rat liver PRL-R) but contains a much longer cytoplasmic domain, which is similar in size to that of the GH-R, (Chapters 5 and 7).

5) Both the short and long forms of the PRL-R direct the expression of protein binding PRL with high affinity in eukaryotic cells, suggesting that the PRL-R contains a single binding subunit (Chapters 5 and 7).

6) The PRL-R gene expression in rat liver is regulated by estrogen and during development at both the pre-translational and translational levels (Chapter 6).

7) In the absence of any classical concensus sequence in the cytoplasmic domains of the PRL and GH receptors, the finding of highly conserved sequences suggests that they may be involved in the transfer of the hormonal message (Chapters 5 and 7).

BIBLIOGRAPHY

Agius L, Robinson AM, Girard JR, Williamson DH 1979 Biochem J 180:689-692

Aguet M, Dembic Z, Merlin G 1988 Cell 55:273-280

Alhadi T, Vonderhaar BK, 1982 Endocrinology 110:254-259

Allen DG, Eisner DA, Wray SC 1985 Nature 316:674-675

Amit T, Barkey RJ, Gavish M, Youdim MBH 1985 Mol Cell Endocrinol 39:21-29

Anderson TR, Mayer GL, Nicoll CS 1981 J Cyclic Nucleotide Res 7:225-233

Aragona C, Bohnet HG, Friesen HG 1975 Endocrinology 99:1017-1022

Auger KR, Serunian LA, Soltoff SP, Libby P, Cantley LC 1989 Cell 57:167-175

Auld VJ, Goldin AL, Krafte DS, Marshall J, Dunn JM, Catterall WA, Lester HA, Davidson N, Dunn RJ 1988 Neuron 1:449-461

Ball RK, Friis RR, Schönenberger CA, Dopppler W, Groner B 1988 EMBO J 7:2089-2095

Ballivet M, Patrick J, Lee J, Heinemann S 1982 Proc Natl Acad Sci USA 79:4466-4470

Barkey RJ, Calvo JC, Dufau ML, 1988 Biochem Biophy Res Commun 156:776-782

Barnard EA 1988 Nature 335:301-302

Baumann A, Krah-Jentgens I, Müller R, Müller-Holt Kemp F, Seidel R, Kecskemethy N, Casal J, Ferrus A, Pongs O 1987 EMBO J 6:3419-3429

Baxter RC, Zaltsman Z, Turtle JR 1984 Endocrinology 114:1893-1901

Baxter RC, Zaltsman Z 1984 Endocrinology 115:2009-2014

Bellussi G, Muccioli G, Ghè C, Di Carlo R 1987 Life Sciences 41:951-959

Benovic JL, Strasser RH, Caron MG, Lefkowitz RJ 1986 Proc Natl Acad Sci USA 83:2797-2801

Berczi I, Nagy E In: Hormones and immunity, Berczi I, Kovacks K (eds), MTP Press Limited, Lancaster 1986 pp 145-171

Bergeron JJM, Posner BI, Josefsberg Z, Sikstrom R, 1978 J Biol Chem 253:4058-4066

Bern HA, Nicoll CS 1968 Rec Progr Horm Res 24:681-713

Bernton EW, Meltzer MS, Holaday JW 1988 Science 239:401-404

Berridge MJ 1984 Biochem J 220:345-360

Bertics PJ, Weber W, Cochet C, Gill GN 1985 J Cell Biochem 29:195-208

Bertics PJ, Gill GN 1985 J Biol Chem 260:14642-14647

Bevilacque MP, Stengelin S, Gimbrone MA JR, Seed B 1989 Science 243:1160-1165

Beyer EC, Paul DL, Goodenough DA 1987 J Cell Biology 105:2621-2629

Bhattacharya A, Vonderhaar BK 1979 Proc Natl Acad Sci USA 76:4489-4492

Birnbaum MJ, Haspel HC, Rosen OM 1986 Proc Natl Acad Sci USA 83:5784-5788

Birnbaum MJ 1989 Cell 57:305-315

Biswas R, Vonderhaar BK 1987 Cancer Res 47:3509-3514

Blair LAC, Levitan ES, Marshall J, Dionne VE, Barnard EA 1988 Science 242:577-579

Blumberg PM 1988 Cancer Res 48:1-8

Bohnet HG, Shiu RPC, Grinwich LD, Friesen HG 1978 Endocrinology 102:1657-1661

Bolander Jr. FF 1985 J Endocrinol 104:29-34

Bonifacino JS, Dufau ML 1984 J Biol Chem 259:4542-4549

Bonner TI, Buckley NJ, Young AC, Bronn MR 1987 Science 237:527-532

Bonner TI, Young AC, Braun MR, Buckley NJ 1988 Neuron I 403-410

Bonneterre J, Peyrat JP, Beuscart R, Lefebvre J, Demaille A 1987 Cancer Res 47:4724-4728

Boulter J, Evans K, Goldman D, Martin G, Treco D, Heinemann S, Patrick J 1988 Nature 319:368-374

Boutin JM, Jolicoeur C, Okamura H, Gagnon J, Edery M, Shirota M, Banville D, Dusanter-Fourt I, Djiane J, Kelly PA 1988 Cell 53:69-77

Bouvier M, Hausdorff WP, De Blasi A, O'Dowd BF, Kobilka BK, Caron MG, Lefkowitz RJ 1988 Nature 333:370-373

Brandl CJ, Green NM, Korczak B, MacLennan DH 1986 Cell 44, 597-607

Buckley AR, Crowe PD, Russel DH, 1988 Proc Natl Acad Science USA 85:8649-8653

Buckley AR, Montgomery DW, Kibler R, Putnam CW, Zukoski CF, Gout PW, Beer CT, Russell DH 1986 Immunopharmacology 12:37-51

Bunzow JR, Van Tol HHM, Grandy DK, Albert P, Salon J, MacDonald C, Machida CA, Neve KA, Civelli O 1988 Nature 336:783-787

Cameron CM, Rillema JA 1983 Endocrinology 113:1596-1600

Casey JL, Hentze MW, Koeller DM, Coughman SW, Rouault TA, Klausner RD, Harford JB 1988 Science 240:924-928

Catterall WA 1988 Science 242:50-61

Chang C, Kokontis J, Liao S 1988 Science 240:324-326

Changeux JP, Devillers-Thiery A, Chermouille P 1984 Science 225:1335-1345

Chao MV, Bothwell MA, Ross AH, Koprowski H, Lanahan AA, Buck CR, Sehgal A 1986 Science 232:518-521

Charron MJ, Brosius III FC, Alper SL, Lodish HF 1989 Proc Natl Acad Sci USA 86:2535-2539

Chen HW, Hamer DH, Heininger H, Meier H 1972a Biochem Biophys Acta 287:90-97

Chen HW, Meier H, Heiniger HJ, Huebner RJ 1972b J Natl Cancer Inst 49:1145-1154

Chen WS, Lazar CS, Poenie M, Tsien RY, Gill GN, Rosenfeld MG, 1987 Nature 328:820-823

Chien Y-H, Becker DM, Lindsten T, Okamura M, Cohen DI, Davis MM 1984 Nature 312:31-35

Chinkers M, Garbers DL, Chang MS, Lowe DG, Chin H, Goeddel DV, Schulz S 1989 Nature 338:78-83

Chou CK, Dull TJ, Russell DS, Gherzi R, Lebwohl D, Ullrich A, Rosen OM 1987 J Biol Chem 262:1842-1847

Conneely OM, Sullivan WP, Toft DO, Birnbaumer M, Cook RG, Maxwell BL, Zarucki-Schulz T, Greene GL, Schrader WT, O'Malley BW 1986 Science 233:767-770

Cosman D, Cerretti DP, Larsen A, Park L, March C, Dower S, Gillis S, Urdal D 1984 Nature 312:768-771

Costlow ME, Gallagher PC 1979 Biochem Biophys Acta 587:192-201

Cotecchia S, Schwinn DA, Randall RR, Lefkowitz RJ, Caron MG, Kobilka BK 1988 Proc Natl Acad Sci USA 85:7159-7163

Coughin SR, Escobedo JA, Williams LT 1989 Science 243:1191-1194

Coussens L, Yang-Feng TL, Liao Y-C, Chen E, Gray A, McGrath J, Seeburg PH, Libermann TA, Schlessinger J, Francke U, Levinson A, Ullrich A 1985 Science 230: 1132-1139

Czech MP, Klarlund JK, Yagaloff KA, Bradford AP, Lewis RE 1988 J Biol Chem 263:11017-11020

Dahms NM, Lobel P, Breitmeyer J, Chirgwin JM, Kornfeld S 1987 Cell 50:181-192

Danielson KG, Oborn CJ, Durban EM, Butel JS, Medina D 1984 Proc Natl Acad Sci USA 81:3756-3760

Dave JR, Knazek RA 1980 Proc Natl Acad Sci USA 77:6597-6600

Davis MM, Bjorkman PJ 1988 Nature 334:395-402

Davis JA, Linzer DIH 1989 Mol Endo 3:674-680

Davis RJ, Czech MP 1985 Proc Natl Acad sci (USA) 82:1974-1978

Davis CG, Goldstein JL, Südhof TC, Anderson RGW, Russell DW, Brown MS 1987 Nature 326:760-765

Deneris ES, Connolly J, Boulter J, Wada E, Wada K, Swanson LW, Patrick J, Heinemann S, 1988 Neuron I 45-54

Deneris ES, Boulter J, Swanson LW, Patrick J, Heinemann S 1989 J Biol Chem 264:6268-6272

Dhanireddy R, Ulane RE 1984 Life Sci 35:733-740

DiMattia CE, Gellersen B, Bohnet HG, Friesen HG 1988 Endocrinology 122:2508-2517

Dixon RAF, Kobilka BK, Strader DJ, Benovic JL, Dohlman HG, Frielle T, Bolanowski MA, Bennett CD, Rands E, Diehl RE, Mumford RA, Slater EE, Sigal IS, Caron MG, Lefkowitz RJ, Strader CD 1986 Nature 321:75-79

Dixon RAF, Sigal IS, Rands E, Register RB, Candelore MR, Blake AD, Strader CD 1987 Nature 326:73-77

Djiane J and Durand P 1977 Nature 266:641-643

Djiane J, Delouis C, Kelly PA 1979a Proc Soc Exp Biol Med 162:342-348

Djiane J, Clauser H, Kelly PA 1979b Biochem Biophys Res Commun 90:1371-1380

Djiane J, Houdebine L-M, Kelly PA, 1981 Proc Natl Acad Science USA 78:7445-7448

Djiane J, Houdebine L-M, Kelly PA 1982 Endocrinology 110:791-795

Doglio A, Dani C, Grimaldi P, Ailhaud G, 1989 Proc Natl Acad Science USA 86:1148-1159

Dohlman HG, Caron MG, Lefkowitz RJ 1987 Biochemistry 26:2657-2664

Doppler W, Groner B, Ball RK 1989 Proc Natl Acad Sci (USA) 86:104-108

Downward J, Yarden Y, Mayes E, Scrace G, Trotty N, Stockwell P, Ullrich A, Schlessinger J, Waterfield MD 1984 Nature 307:521-527

Downward J, Waterfield MD, Parker PJ 1985 J Biol Chem 260:14538-14546

Dusanter-Fourt I, Djiane J, Kelly PA, Houdebine LM, Teyssot B 1984 Endocrinology 114:1021-1027

Dusanter-Fourt I, Kelly PA, Djiane J 1987 Biochimie 69:639-646

Ebina Y, Ellis L, Jarnagin K, Edery M, Graf L, Clauser E, Ou J-h, Masiarz F, Kan YW, Goldfine ID, Roth RA, Rutter WJ 1985 Cell 40:747-758

Ebina Y, Araki E, Taira M, Shimada F Mori M, Craik CS, Siddle K, Pierce SB, Roth RA, Rutter WJ 1987 Proc Natl Acad Sci USA 84:704-708

Edery M, Djiane J, Houdebine L-M, Kelly PA 1983 Cancer Res 43:3170-3174

Edery M, Jolicoeur C, Levi-Meyrueis C, Dusanter-Fourt I, Pétridou B, Boutin JM, Lesueur L, Kelly PA, Djiane 1989 J Proc Natl Acad Sci USA 86:2112-2116

Edwards III, CK, Ghiasuddin SM, Schepper JM, Yunger LM, Kelley KW 1988 Science 239:769-771

Ellis SB, Williams ME, Ways NR, Brenner R, Sharp AH, Leung AT, Campbell KP, McKenna E, Koch WJ, Hui A, Schwartz A, Harpold MM 1988 Science 241:1661-1664

Esser V, Limbird LE, Brown MS, Goldstein JL, Russell DW 1988 J Biol Chem 26:13282-13290

Etindi RN and Rillema JA, 1987 Biochem Biophys Acta 927:345-349

Etindi RN, Rillema JA 1988 Biochem Biophys Acta 968:385-391

Evans RM 1988 Science 240:889-895

Farese RV 1988 Am J Med 85 (Suppl 5A):36-43

Fargin A, Raymond JR, Lohse MJ, Kobilka BK, Caron MG, Lefkowitz RJ 1988 Nature 335:358-360

Ferland LH, Djiane J, Houdebine L-M, Kelly PA 1984a Mol and Cell Endocrinol 35:25-31

Ferland LH, Djiane J, Houdebine L-M Kelly PA 1984b Endocrinology 115:1842-1849

Fisher ER, Fisher B 1963 Cancer Res 23:1532-1540

Francis MJO, Hill DJ 1975 Nature 255 167-168

Fraser CM, Chung F-Z, Wang C-D, Venter JC 1988 Proc Natl Acad Sci (USA) 85:5478-5482

Frawley LS, Schwabe C, Miller III HA, Betts JG, Hoeffler JP, Simpson MT In: Prolactin gene family and its receptors, Hoshino K (ed) Elsevier Science Publishers, Amsterdam, pp 49-59

Frazier RP, Costlow ME 1982 Exp Cell Res 138: 39-45

Freemark M, Comer M, Handwerger S 1986 Am J Physiol 251:328-333

Freemark M, Comer M 1989 J Clin Invest 83:883-889

Frielle T, Daniel KW, Caron MG, Lefkowitz RJ 1988 Proc Natl Acad Sci USA 85:9494-9498

Frielle T, Collins S, Daniel KW, Caron MG, Lefkowitz RJ, Kobilka BK 1987 Proc Natl Acad Sci USA 84:7920-7924

Fukuda K, Higashida H, Kubo T, Maeda A, Akiba I, Bujo H, Mishina M, Numa S 1988 Nature 335:355-358

Fukumoto H, Seino S, Imura H, Seino Y, Eddy RL, Fukushima Y, Byers MG, Shows TB, Bell GI 1988 Proc Natl Acad Sci (USA) 85:5434-5438

Fuller F, Porter JG, Arfsten AE, Miller J, Schilling JW, Scarborough RM, Lewicki JA, Schenk DB 1988 J Biol Chem 263:9395-9401

Gaffney EV 1982 Cell Tissue Res 227:563-568

Gertler A, Walker A, Friesen HG 1985 Endocrinology 116: 1636-1644

Gertler A, Friesen HG 1986 Mol Cell Endocrinol 48:221-228

Geuze HJ, Slot JW, Strous GJ AM, Lodish HF, Schwartz AL 1983 Cell 32:277-287

Gilman AG 1987 Ann Rev Biochem 56:615-649

Giraudet J, Dennis M, Heidmann T, Haumont P-Y, Lederer F, Changeux JP 1987 Biochemistry 26:2410-2418

Gocayne J, Robinson DA, Fitzgerald MG, Chung FZ, Kerlavage AR, Lentes KU, Lai J, Wang C-D, Fraser CM, Venter JC 1987 Proc Natl Acad Sci USA 84:8296-8300

Goldman D, Deneris E, Luyten W, Kochar A, Patrick J, Heinemann J 1987 Cell 48:965-973

Gout PW, Beer CT, Noble RL 1980 Cancer Res 40:2433-2436

Greene WC, Robb RJ, Svetlik PB, Rusk CM, Depper JM, Leonard WJ 1985 J Exp Med 162:363-368

Grenningloh, Rienitz A, Schmitt B, Methfessel C, Zensen M, Beyreuther K, Gundelfinger Ed, Betz H 1987 Nature 328:215-220

Gronwald RGK, Grant FJ, Haldeman BA, Hart C.E, O'Hara PJ, Hagen FS, Ross R, Bowen-Pope DF, Murray MJ 1988 Proc Natl Acad Sci USA 1988 85:3435-3439

Gullick WJ, Downward J, Waterfield MD 1985 EMBO J 4:2869-2877

Guy HR, Hucho F 1987 TINS 10:318-321

Guyette WA, Matusik RJ, Rosen JM 1979 17:1013-1023

Haeuptle MT, Aubert ML, Djiane J, Kraehenbuhl JP 1983 J Biol Chem 258:305-314

Haldosén L-A, Gustafsson J-A 1987 J Biol Chem 262:7404-7411

Harigaya T, Smith WC, Talamantes F 1988 Endocrinology 122:1366-1372

Hatakeyama M, Minamoto S, Uchiyama T, Hardy RR, Yamada G, Taniguchi T 1985 Nature 318:467-470

Hediger MA, Coady MJ, Ikeda TS, Wright EM 1987 Nature 330:379-381

Hedrick SM, Cohen DI, Nielsen EA, Davis MD 1984 Nature 308:149-153

Helfman DM, Feramisco JR, Fiddes JC, Thomas GP, Hughes SH 1983 Proc Natl Acad Sci USA 80:31-35

Hempstead B, Schleifer LS, Chao MV 1989 Science 243:373-375

Hentze MW, Coughman SW, Rouault TA, Barrio-Canal JG, Doncis LA, Harford JB, Klausner RD 1987 Science 238:1570-1573

Hentze MW, Rouault TA, Harford JB, Klausner RD 1989 Science 244:357-359

Herz J, Hamann U, Rogne S, Myklebost O, Gausepohl H, Stanley KK 1988 EMBO J 7:4119-4127

Hiestand PC, Mekler P, Nordmann R, Grieder A, Permmongkol C 1986 Proc Natl Acad Sci USA 83:2599-2603

Hill DJ, Freemark M, Strain AJ, Handwerger S, Milner RDG 1988 J Clin Endocrinol Metab 66:1283-1290

Hollenberg SM, Weinberger C, Ong ES, Cerelli G, Oro A, Lebo R, Thompson EB, Rosenfeld MG, Evans RM 1985 Nature 318:635-641

Honegger AM, Szapary D, Schmidt A, Lyall R, Van Obberghen E, Dull TJ, Ullrich A, Schlessinger J 1987 Mol Cell Biol 7:4568-4571

Honegger AM, Kris RM, Ullrich A, Schlessinger J 1989 Proc Natl Acad Sci USA 86:925-929

Houdebine L-M, Djiane J, 1980 Mol Cell Endocrinol 17:1-15

Houdebine L-M, Djiane J, Dusanter-Fourt I, Martel P, Kelly PA, Devinoy E, Servely J-L 1985 J Dairy Sci 68:489-500

Hucho F, Oberthur W, Lottspeich F 1986 FEBS Lett 205:137-142

Huganir RL, Greengard P 1983 Proc Natl Acad Sci (USA) 80:1130-1134

Huganir RL, Mibs K, Greengard P 1984 Proc Natl Acad Sci (USA) 81:6968-6972

Huganir RL, Delcour AH, Greengard P, Hess GP 1986 Nature 321:774-776

Hughes JP, Elsholtz HP, Friesen HG 1985 In: Polypeptide Hormone Receptors, Posner BI (ed) Dekker, New York, pp 157-199

Hunter T, Ling N, Cooper JA 1984 Nature 311:480-483

Hunter T, Cooper JA 1985 Ann Rev Biochem 54:877-930

Huynh V, Young RA, Davis RW 1985 In: DNA cloning: A practical approach. Glover DM (ed) IRL, Oxford, U.K., vol I, pp 49-78

Hynes RO 1987 Cell 48:549-554

Iacopetta BJ, Rothenberger S, Kühn LC 1988 Cell 54:485-489

Imoto K, Busch C, Sakmann B, Mishina M, Konno T, Nakai J, Bujo H, Mori Y, Fukudo K, Numa S 1988 Nature 335:645-648

Jackson TR, Blain LAC, Marshall J, Goedert M, Hanley MR 1988 Nature 335:437-440

James DE, Strube M, Mueckler M 1989 Nature 338:83-87

Jan LY, Jan YN 1989 Cell 56:13-25

Jeltsch JM, Krozowski Z, Quirin-Stricker C, Gronemeyer H, Simpson RJ, Garnier JM, Krust A, Jacob F, Chambon P 1986 Proc Natl Acad Sci USA 83:5424-5428

Johnson D, Lanahan A, Buck CR, Sehgal A, Morgan C, Mercer E, Bothwell M, Chao M 1986 Cell 47:545-554

Johnston GI, Cook RG, McEver RP 1989 Cell 56:1033-1044

Josefsberg Z, Posner BI, Patel B, Bergeron JJM, 1979 J Biol Chem 254:209-214

Julius D, MacDermott AB, Axel R, Jessell TM, 1988 Science 241:558-564

Jung V, Rashidbaigi A, Jones C, Tischfeld JA, Sows TB, Pestka S 1987 Proc Natl Acad Sci USA 84:4151-4155

Kahn CR, White MF 1988 J Clin Invest 82:1151-1156

Kamb A, Iverson LE, Tanouye MA 1987 Cell 50:405-413

Katoh M, Djiane J, Leblanc G, Kelly PA 1984 Mol Cell Endocrinol 34:191-200

Katoh M, Djiane J, Kelly PA 1985 Endocrinology 116:2612-2620

Katoh M, Raguet S, Zachwieja J, Djiane J, Kelly PA 1987 Endocrinology 120:739-749

Kavathas P, Herzenberg LA 1983 Proc Natl Acad Sci USA 80:524-528

Kavathas P, Sukhatme VP, Herzenberg LA, Parnes JR 1984 Proc Natl Acad Sci USA 81:7688-7692

Kavathas P 1985 In: Hybridoma technology in the Biosciences and Medicine, Springer TA (ed) Plenum, New York, Chap 23, pp 383-395

Kayamo T, Noda M, Flockerzi V, Takahashi H, Numa S 1988 FEBS Lett 228:187-194

Kayano T, Fukumoto H, Eddy RL, Fan Y-S, Byers MG, Shows TB, Bell GI 1988 J Biol Chem 263:15245-15248

Kelly PA, Posner BI, Tsushima T 1974 Endocrinology 95:532-539

Kelly PA, Posner BI, Friesen HG, 1975 Endocrinology 97:1408-1415

Kelly PA, Djiane J, Deléan A 1980 Prog Reprod Biol 6:124-136

Kelly PA, Djiane J, Leblanc G 1983 Proc Soc Exp Biol Med 172:219-224

Kelly PA, Katoh M, Djiane J, Ferland LH 1986 In: The Receptors Vol 111, Conn M. (ed) Academic Press Inc., Orlando, Florida, pp 355-391

Kelly PA, Gould D, Okamura H, Djiane J 1988 In: Prolactin and Lesions in Breast Uterus and Prostate, Nagasawa H (ed), CRC Press, Boca Raton, Florida, pp 61-94

Kelly PA, Okamura H, Zachwieja J, Raguet S, Gould D, Boutin JM, Jolicoeur C, Mélançon R, Djiane J 1988b Progress in Cancer Research and Therapy, vol 35, Hormones and Cancer 3, Bresciani LF, King RJB, Lippman ME, Raymond JP (eds), Raven Press Ltd, New York, pp 75-80

Khan MN, Posner BI, Verma AK, Khan RJ, Bergeron JJM 1981 Proc Natl Acad Sci USA 78:4980-4984

Kiess W, Blickenstaff GD, Sklar MM, Thomas CL, Nissley SP, Sahagian GG 1988 J Biol Chem 263: 9339-9344

Kiess W, Thomas LCL, Greenstein LA, Lee L, Sklar MM, Rechler MM, Sahagian GG, Nissley SP 1989 J Biol Chem 264:4710-4714

Kledzik GS, Marshall S, Campbell GA, Gelato M, Meites J 1976 Endocrinology 98: 373-379

Klein HH, Freidenberg GR, Kladde M, Olefsky JM 1986 J Biol Chem 261:4691-4697

Kobilka BK, Dixon RA, Frielle T, Dohlman HG, Bolanowski MA, Sigal IS, Yang-Feng TL, Francke U, Caron MG, Lefkowitz RJ 1987a Proc Natl Acad Sci USA 84:46-50

Kobilka BK, Matsui H, Kobilka TS, Yang-Feng TL, Francke U, Caron MG, Lefkowitz RJ, Regan JW 1987b Nature 238:650-656

Kobilka BK, Frielle T, Collins S, Yang-Feng T, Kobilka TS, Francke U, Lefkowitz RJ, Caron MG 1987c Nature 329:75-79

Kobilka BK, MacGregor C, Daniels K, Kobilka TS, Caron MG, Lefkowitz RJ 1987d J Biol Chem 262:7321-7327

Kobilka BK, Kobilka TS, Daniel K, Regan JW, Caron MG, Lefkowitz RJ 1988 Science 240:1310-1315

Kopito RR and Lodish HF 1985 Nature 316:234-238

Koppelman MCS, Dufau ML 1982 Endocrinology 111:1350-1357

Kubo T, Fukuda K, Mikami A, Maeda A, Takahashi H, Mishima M, Haga T, Haga K, Ichiyama A, Kangawa K, Kojima M, Matsuo H, Hirose T, Numa S 1986a Nature 323:411-416

Kubo T, Maeda A, Sugimoto K, Akiba I, Mikami A Takahashi H, Haga T, Haga K, Ichiyama A, Kangawa K, Matsuo H, Hirose T, Numa S 1986b FEBS Lett 209:367-372

Kühn H, Dreyer WJ 1972 FEBS Lett 20:1-6

Kühn LC, Mcclelland A, Ruddle FH 1984 Cell 37:95-103

Kumar NM, Gilula NB 1986 J Cell Biol 103:767-776

Langosch D, Thomas L, Betz H 1988 Proc Natl Acad Sci USA 85:7394-7398

Larsen JL, Dufau ML, 1988 Endocrinology 123:438-444

Lax I, Bellot F, Howk R, Ullrich A, Givol D, Schlessinger J 1989 EMBO J 8:421-427

Lefkowitz RJ, Caron MG 1988 J Biol Chem 263:4993-4996

Leonard RJ, Labarca CG, Charnet P, Davidson N, Lester HA 1988 Science 242:1578-1581

Leonard WJ, Depper JM, Crabtree GR, Rudikoff S, Pumphrey J, Robb RJ, Krönke M, Svetlik PB, Peffer NJ, Waldmann TA, Greene WC 1984 Nature 311:626-631

Leonard WJ, Depper JM, Kanehisa M, Krönke M, Peffer NJ, Svetlik PB, Sullivan M, Greene WC 1985 Science 230:633-639

Leung CKH, Shiu RPC 1981 Cancer Res 41:546-551

Leung DW, Spencer SA, Cachianes G, Hammonds RG, Collins C, Henzel WJ, Barnard R, Waters MJ, Wood WI 1987 Nature 330:537-543

Levitan ES 1988 TINS 11:41-43

Levitan ES, Schofield PR, Burt DR, Rhee LM, Wisden W, Köhler M, Fujita N, Rodriguez HF, Stephenson A, Darlison MG, Barnard EA, Seeburg PH 1988 Nature 335:76-79

Lin CR, Chen WS, Kruiger W, Stolarsky LS, Weber W, Evans RM, Verma IM, Gill GN and Rosenfeld MG 1984 Science 224:843-848

Liscia DS, Vonderdhaar BK 1982 Proc Natl Acad Sci USA 79:5930-5934

Liscia DS, Alhali T, Vonderhaar BK 1982 J Biol Chem 257:9401-9405

Littman DR, Thomas Y, Maddon PJ, Chess L, Axel R 1985 Cell 40:237-246

Lobel P, Dahms NM, Breitmeyer J, Chirgwin JM, Kornfeld S 1987 Proc Natl Acad Sci USA 84:2233-2237

Lobel P, Dahms NM, Kornfeld S 1988 J Biol Chem 263:2563-2570

Loewenstein WR 1987 Cell 48:725-726

Loosefelt H, Atger M, Misrahi M, Guiochon-Montel A, Meriel C, Logeat F, Benarous R, Milgrom E 1986 Proc Natl Acad Sci (USA) 83:9045-9049

Low MG, Saltiel AR 1988 Science 239:268-275

Lowenthal JW, Green WC 1987 J Exp Med 166:1156-1161

Lubahn DB, Joseph DR, Sullivan PM, Williard HF, French FS, Wilson EM 1988 Science 240:327-330

MacDonald RG, Pfeffer SR, Coussens L, Tepper MA, Brocklebank CM, Mole JE, Anderson JK, Chen E, Czech MP, Ullrich A 1988 Science 239:1134-1137

MacDonald RG, Tepper MA, Clairmont KB, Perregaux SB, Czech MP 1989 J Biol Chem 264:3256-3261

MacLennan DH, Brandl CJ, Korczak B, Green NM 1985 Nature 316:696-700

Madon PJ, Littman DR, Godfrey M, Maddon DE, Chess L, Axel R 1985 Cell 42:93-104

Mamalaki C, Stephenson FA, Barnard EA 1987 EMBO J 6:561-565

Maniatis T, Fritsch EF, Sambrook J 1982 Molecular Cloning: A laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York

Manni A, Chambers MJ, Pearson OH 1978 Endocrinology 103:2168-2171

Manni A, Wright C, Davis G, Glenn J, Joehl R, Feil P 1986 Cancer Res 46:1669-1672

Marshall S, Kledzik GS, Gelato M, Campbell GA, Meites J 1976 Steroids 27: 187-195

Marshall S, Bruni JF, Meites J 1978 Proc Soc Exp Biol Med 159:256-259

Masu Y, Nakayama K, Tamaki H, Harada Y, Kuno M, Nakanishi S 1987 Nature 329:836-838

Matsui T, Heidaran M, Miki T, Popescu N, LaRochelle W, Kraus M, Pierce J, Aaronson S 1989 Science 243:800-804

McClain DA, Macgawa H, Lee J, Dull TJ, Ullrich A, Olefsky JM 1987 J Biol Chem 262:14663-14671

McDonnell DP, Mangelsdorf DJ, Pike JW, Haussler MR, O'Malley BW 1987 Science 235:1214-1217

McLelland A, Kühn LC, Ruddle FH 1984 Cell 39:267-274

Meites J 1988 In: Prolactin gene family and its receptors, Hoshino K (ed)

Elsevier Science Publishers, Amsterdam, pp 123-130

Mick CCW, Nicholl CS 1985 Endocrinology 116:2049-2053

Miller C 1989 Neuron 2:1195-1205

Mishina M, Tobimatsu T, Imoto K, Tanaka K-I, Fujita Y, Fukuda K, Kurasaki M, Takahashi H, Morimoto Y, Hirose T, Inayama S, Takahashi T, Kuno M, Numa S 1985 Nature 313:364-369

Mitani M, Dufau ML 1986 J Biol Chem 261:1309-1315

Mittra I, 1980 Biochem Biophys Res Commun 95:1760-1767

Moltz H, Leidahl LC 1977 Science 196:81-83

Montgomery DW, Zukoski CF, Shah GN, Buckley AR, Pacholczyk T, Russell DH 1987 Biochem Biophys Res Commun 145:692-698

Morgan DO, Edman JC, Standring DN, Fried VA, Smith MC, Roth RA, Rutter WJ 1987 Nature 329:301-307

Mueckler M, Caruso C, Baldwin SA, Panico M, Blench I, Morris HR, Allard WJ, Lienhard GE, Lodish HF, 1985 Science 229:941-945

Mueller PP, Hinnebusch AG 1986 Cell 45:201-207

Murphy LC, Lee-Wing M, Goldenberg GJ, Shiu RPC 1987 Cancer Res 47:160-4164

Murphy LJ, Tachibana K, Friesen HG 1988a Endocrinology 122:2027-2033

Murphy PR, DiMattia GE, Friesen HG 1988b Endocrinology 122:2476-2485

Nathans J, Hogness DS 1983 Cell 34:807-814

Nathans J, Hogness DS 1984 Proc Natl Acad Sci USA 81:4851-4855

Nathans J, Thomas D, Hogness DS 1986 Science 232:193-202

Necessary PC, Humphrey PA, Mahajan PB Ebner KE, 1984 J Biol Chem 259:6942-6946

Necessary PC, Humphrey PA, Mahajan PB, Ebner KE 1984 J Biol Chem 259:6942-6946

Nicoll CS 1980 Fed Proc 39:2563-2566

Nicoll CS, Delidow BC, English DE, Herbert NJ, Russell SM 1988 In: Prolactin gene family and its receptors, Hoshino K (ed) Elsevier Science Publishers, Amsterdam, pp 349-357

Nikaido T, Shimizu A, Ishida N, Sabe H, Teshigawara K, Maeda M, Wehiyama T, Yodoi J, Honjo T 1984 Nature 311:631-635

Nishi M, Ishida Y, Honjo T 1988 Nature 331:267-269

Noda M, Takahashi H, Tanabe T, Toyosato M, Furutani Y, Hirose T, Asai M, Inayama S, Miyata T, Numa S 1982 Nature 299:793-797

Noda M, Takahashi H, Tanabe T, Toyosato M, Kikyotani S, Hirose T, Asai Takashima H, Inayama S, Miyata T, Numa S 1983a Nature 301:251-255

Noda M, Takahashi H, Tanabe T, Toyosato M, Kikyotani S, Furutani Y, Hirose T, Takashima H, Inayama S, Miyata T, Numa S 1983b Nature 302:528-532

Noda M, Ikeda T, Kayano T, Suzuki H, Takeshima H, Kurasaki M, Takahashi H, Numa S 1986a 320:188-192

Noda M, Ikeda T, Suzuki H, Takeshima H, Takahashi T, Kuno M, Numa S 1986b Nature 322:826-828

Noda M, Shimizu S, Tanabe T, Takai T, Kayano T, Ikeda T, Takahashi H, Nakayama H, Kanaoka Y, Minamino N, Kangawa K, Matsuo H, Reftery MA, Hirose T, Inayamaq S, Hayashida H, Miyata T, Numa S 1989 312: 121-127

Nolin JM, Witorsch RJ, 1976 Endocrinology 99:949-958

Norstedt G, Mode A, Eneroth P, Gustafsson JA 1981 Endocrinology 108: 1855-1861

Norstedt G 1982 Endocrinology 110:2107-2112

Norstedt G, Andersson G, Gustafsson JA 1984 Endocrinology 115:672-680

O'Dowd BF, Hnatowich M, Regan JW, Leader WM, Caron MG, Lefkowitz RJ 1988 J Biol Chem 263:15985-15992

Ofenstein JP, Rillema JA 1987 Proc Soc Exp Biol Med 185:147-152

Oka T, Perry JW 1976 J Biol Chem 251:1738-1744

Okamura H, Raguet S, Bell A, Gagnon J, Kelly PA 1989a J Biol Chem 264:5904-5911

Okamura H, Zachwieja J, Raguet S, Kelly PA 1989b Endocrinology 124:2499-2508

Oshima A, Nolan CM, Kyle JW, Grubb JH, Sly WS 1988 J Biol Chem 263:2553-2562

Papazian DM, Schwarz TL, Tempel BL, Jan YN, Jan LY 1987 Science 237:749-753

Paul DL 1986 J Cell Biol 103:123-134

Peralta EG, Winslow JW, Peterson GL, Smith DH, Ashkenazi A, Ramachandran J, Schimerlik MI, Capon D 1987a Science 236:600-605

Peralta EG, Ashkenazi A, Winslow JW, Smith DH, Ramachandran J, Capon DJ 1987b EMBO J 6:3923-3929

Peralta EG, Ashkenazi A, Winslow JW, Ramachandran J, Capon DJ 1988 Nature 334:434-437

Pohlmann R, Nagel G, Schmidt B, Stein M, Lorkowski G, Krentler C, Cully J, Meyer HE, Grzeschik KH, Mersmann G, Hasilik A, von Figura K, 1987 Proc Natl Acad Sci USA 84:5575-5579

Posner BI, Kelly PA, Friesen HG 1974 Proc Natl Acad of Sci USA 71: 2407-2410

Posner BI, Kelly PA, Friesen HG, 1975 Science 188:57-59

Posner BI, Josefsberg Z, Bergeron JJM, 1979 J Biol Chem 254:12494-12499

Pritchett DB, Bach AWJ, Wozny M, Taleb O, Toso RD, Shih JC, Seeburg PH 1988a EMBO J 1988 7:4135-4140

Pritchett DB, Sontheimer H, Gorman GM, Kettenmann H, Seeburg PH, Schofield PR 1988b Science 242:1306-1308

Pritchett DB, Sontheimer H, Shivers BD, Ymer S, Kettenmann H, Schofield PR, Seeburg PH 1989 Nature 338:582-585

Radeke MJ, Misko TP, Hsu C, Herzenberg LA, Shooter EM 1987 Nature 325:593-597

Regan JW, Kobilka TS, Yang-Feng TL, Caron MG, Lefkowitz RJ, Kobilka BK 1988 Proc Natl Acad Sci USA 85:6301-6305

Richards JF 1975 Biochem Biophys Res Commun 63:292-299

Richards JF, Beer CT, Bourgeault C, Chan K, Gout PW 1982 Mol Cell Endocrinol 26:41-49

Rillema JA 1975 Horm Metab Res 7:45-49

Rillema JA 1980a Fed Proc 39:2593-2598

Rillema JA 1980b Endocrinology 106:1360-1364

Rillema JA, Wing L-YC, Foley KA 1983 Endocrinology 113:2024-2028

Rillema JA 1984 Horm Metab Res 16:532-534

Rogers SA, Hammerman MR 1989 J Biol Chem 264:4273-4276

Rosa AAM, Ferland LH, Djiane J, Houdebine LM, Kelly PA 1985 Endocrinology 116:1288-1294

Rosen OM, Herrera R, Olowe Y, Petruzzelli LM, Cobb MH 1983 Proc Natl Acad Sci USA 80:3237-3240

Roth RA, Stover C, Hari J, Morgan DO, Smith MC, Sara V, Fried VA 1987 Biochem Biophys Res Commun 149:600-606

Roth RA 1988 Science 239:1269-1271

Rothenberger S, Iacopetta BJ, Kühn LC 1987 Cell 49:423-431

Ruddle FH 1985 In: Hybridoma technology in the Biosciences and Medicine, Springer TA (ed) Plenum, New York, Chap 22, pp 375-381

Ruoslahti E, Pierschbacher MD 1986 Cell 44:517-518

Russell DW, Yamamoto T, Schneider WJ, Slaughter CJ, Brown MS, Goldstein JL 1983 Proc Natl Acad Sci USA 80:7501-7505

Russell DW, Schneider WJ, Yamamoto T, Luskey KL, Brown MS, Goldstein JL 1984 Cell 37:577-585

Russell DH, Martisian L, Kibler R, Larson DF, Poulos B, Magun BE 1984 Biochem Biophys Res Comm 121:899-906

Russell DH, Kibler R, Matrisian L, Larson DF, Poulos B, Magun BE 1985 J Immunol 134:3027-3031

Russell DH, Buckley AR, Montgomery DW, Larson NA, Gout PW, Beer CT, Putnam CW, Zukoski CF, Kibler R 1987 J Immun 138:276-284

Russell DH 1988 In: Prolactin gene family and its receptors, Hoshino K (ed) pp 155-165

Sakai S, Ike F, Murakami H, Kohmoto K 1988 In: Prolactin gene family and its receptors, Hoshino K (ed) pp 115-118

Sardet C, Franchi A, Pouysségur J 1989 Cell 56:271-280

Sasaki N, Tanaka Y, Imai Y, Tsushima T, Matsuzaki F 1982 Biochem J 203:653-662

Schlessinger, J 1988a Biochemistry 27:3119-3123

Schlessinger J 1988b TIBS 13:443-447

Schmieden V, Grenningloh G, Schofield PR, Betz H 1989 EMBO J 8:695-700

Schneider C, Kurkinen M, and Greaves M 1983 EMBO J 2:2259-2263

Schneider C, Owen MJ, Banville D, Williams JG 1984 Nature 311:675-678

Schofield PR, Darlison MG, Fujita N, Burt DR Stephenson FA, Rodriguez H, Rhee LM, Ramachandran J, Reale V, Glencorse TA, Seeburg PH, Barnard EA 1987 328:221-227

Seed B 1987 Nature 329:840-842

Seed B, Aruffo A 1987 Proc Natl Acad Sci USA 84:3365-3369

Shafie SM, Grantham FH 1981 J Natl Cancer Inst 67:51-56

Sharon M, Klausner RD, Cullen BR, Chizzonite R, Leonard WJ 1986 Science 234:859-863

Sherr CJ, Rittenmier CW, Sacca R, Roussel MF, Look AT, Stanley ER 1985 Cell 41:665-676

Shia MA, Lodish HF 1989 Proc Natl Acad Sci (USA) 86:1158-1162

Shirota M, Banville D, Jolicoeur C, Boutin JM, Edery M, Djiane J, Kelly PA 1989 (Submitted for publication)

Shiu RPC, Friesen HG 1974 J Biol Chem 249:7902-7911

Shiu RPC, Friesen HG 1976a Biochem J 157:619-626

Shiu RPC, Friesen HG 1976b Science 192:259-261

Shiu RPC, Friesen HG 1980 Ann Rev Physiol 42:83-96

Shiu RPC, Elsholtz HP, Tanaka T, Friesen HG, Gout PW, Beer CT, Nobel RL 1983 Endocrinology 113:159-165

Shiu RPC, Iwasiow BM 1985 J Biol Chem 260:11307-11313

Shiu RPC, Murphy LC, Tsuyuki D, Myal Y, Lee-Wing M, Iwasiow B 1987 Recent Prog Horm Res 43:277-303

Shull GE,Schwartz A, Lingrel JB 1985 Nature 316:691-695

Shull GE, Lingrel JB 1986 J Biol Chem 261:16788-16791

Shull GE, Greeb J, Lingrel JB 1986a Biochemistry 25:8125-8132

Shull GE, Lane LK, Lingrel JB 1986b Nature 321:429-431

Sibley DR, Benovic JL, Caron MG, Lefkowitz RJ 1987 Cell 48:913-922

Siegal JP, Sharon M, Smith PL, Leonard WJ 1987 Science 238:75-78

Sims JE, March CJ, Cosman D, Widmer MB, MacDonald HR, McMahan CJ, Grudin CE, Wignall JM, Jackson JL, Call SM, Friend D, Alpert AR, Gillis S, Urdal DL, Dower SK 1988 Science 241:585-589

Smith GE, Summers MD, Frasee MJ 1983 Mol Cell Biol 3:2156-2165

Spiess M, Schwartz AL, Lodish HF 1985a J Biol Chem 260:1979-1982

Spiess M, Lodish HF 1985b Proc Natl Acad Sci (USA) 82:6465-6469

Stamenkovic I, Amiot M, Pesando JM, Seed B 1989 Cell 56:1057-1062

Standaert ML, Pollet RJ 1988 FASEB J 2:2453-2461

Stein R, Pinkas-Kramarski R, Sokolovsky M 1988 EMBO J 7:3031-3035

Steinbach JH, Hume C 1989 TINS 12:3-6

Stoolman LM 1989 Cell 56:907-910

Strader CD, Sigal IS, Register RV, Candelore MR, Rands E, Dixon RAF 1987a Proc Natl Acad Sci (USA) 84:4384-4388

Strader CD, Dixon RAF, Cheung AH, Candelore MR, Blake AD, Sigal IS 1987b J Biol Chem 262:16439-16443

Strader CD, Sigal IS, Candelore MR, Rands E, Hill WS, Dixon RAF 1988 J Biol Chem 263: 10267-10271

Südhof TC, Goldstein JL, Brown MS, Russell DW 1985 Science 228:815-822

Suzuki H, Beckh S, Kubo H, Yahag N, Ishida H, Kayano T, Noda M, Numa S 1988 FEBS Lett 228:195-200

Swenson KI, Jordan JR, Beyer EC, Paul DL 1989 Cell 57:145-155

Tanabe T, Takeshima H, Mikami A, Flockerzi V, Takahashi H, Kanagawa K, Kojima M, Matsuo H, Hirose T, Numa S 1987 Nature 328:313-318

Tanabe T, Beam KG, Powell JA, Numa S 1988 Nature 336:134-139

Tanaka LT, Shiu RPC, Gout PW, Beer CT, Noble RL, Friesen HG 1980 J Clin Endo Metab 51:1058-1063

Tanouye MA, Ferrus A, Fujita SC 1981 Proc Natl Acad Sci USA 78:6548-6552

Tempel BL, Papazian DM, Schwarz TL, Jan YN, Jan LY 1987 Science 237:770-775

Tempel BL, Jan YN, Jan LY, 1988 Nature 332:837-839

Teyssot B, Houdebine LM 1980 Eur J Biochem 110:263-270

Thorens B, Sarkar HK, Kaback HR, Lodish HF 1988 Cell 55:281-290

Tong PY, Tollefson SE, Kornfeld S 1988 J Biol Chem 263:2585-2588

Too CKL, Cragoe Jr. EJ, Friesen HG 1987 Endocrinology 121:1512-1520

Tsudo M, Kozak RW, Goldman CK, Waldman TA 1986 Proc Natl Acad Sci USA 83:9694-9698

Tycko B, Maxfield FR 1982 Cell 28: 643-651

Ullrich KJ 1979 Ann Rev Physiol 41:181-195

Ullrich A, Coussens L, Hayflick JS, Dull TJ, Gray A, Tam AW, Lee J, Yarden Y, Libermann TA, Schlessinger J, Downward J, Mayes ELV, Whittle N, Waterfield MD, Seeburg PH 1984 Nature 309:418-425

Ullrich A, Bell JR, Chen EY, Herrera R, Petruzzelli LM, Dull TJ, Gray A, Coussens L, Liao Y-C, Tsubokawa M, Mason A, Seeburg PH, Grunfeld C, Rosen OM and Ramachandran J 1985 Nature 313:756-761

Ullrich A, Gray A, Tam AW, Yang-Feng T, Tsubokawa M, Collins C, Henzel W, Le Bon T, Kathuria S, Chen E, Jacobs S, Francke U, Ramachandran J and Fujita-Yamaguchi Y 1986 EMBO J 5:2503-2512

van Driel IR, Goldstein JL, Südhof TC, Brown MS 1987 J Biol Chem 262:17443-17449

van Der Gugten AA, Waters MJ, Murthy GS, Friesen HG 1980 Endocrinology 106: 402-411

Vandlen RL, Wu WC-S, Eisenach JC, Raftery MA 1979 Biochemistry 18:1845-1854

Vesely DL 1984 Biochem Biophys Res Comm 123:1084-1090

Vonderhaar BK, Bhattacharya A, Alhadi T, Liscia DS, Andrew EM, Young JK, Ginsburg E, Bhattacharjee M, Horn TN 1985 J Dairy Sci 68:466-488

Wada K, Ballivet M, Boulter J, Connolly J, Wada E, Deneris ES, Swanson LW, Heinemann S, Patrick J, Heinemann J 1988 Science 240:330-334

Waters SB, Rillema JA, 1989 Mol Cell Endocrinol 63:159-166

Welsch CW, Nagasawa H 1977 Cancer Res 37:951-963

Welsch CW, Swim EL, McManus MJ 1981 Cancer Lett 14:309-313

White and Littman DR 1989 Cell 56:725-728

Wilde CJ, Hasan HR, White DA, Mayer RJ 1981 Biochem Biophys Res Commun 103:934-942

Williams AF, Barclay AN 1988 Ann Rev Immunol 6:381-405

Willingham MC, Pastan I 1980 Cell 21:67-77

Xu Y-H, Ishii S, Clark AJL, Sullivan M, Wilson RK, Ma DP, Roe BA, Merlino GT and Pastan I 1984 Nature 309:806-810

Yamamoto T, Davis CG, Brown MS, Schneider WJ, Casey ML, Goldstein JL, Russell DW 1984 Cell 39:27-38

Yamasaki K, Taga T, Hirata Y, Yawata H, Kawanishi Y, Seed B, Taniguchi T, Hirano T, Kishimoto T, 1988 Science 241:825-828

Yarden Y, Escobedo JA, Kuang WJ, Yang-Feng TL, Daniel TO, Tremble PM, Chen EY, Ando ME, Harkins RN, Francke U, Fried VA, Ullrich A, Williams CT 1986 Nature 323:226-232

Yarden Y, Rodriguez H, Wong SK-F, Brandt DR, May DC, Burnier J, Harkins RN, Chen EY, Ramachandran J, Ullrich A and Ross EM 1986b Proc Natl Acad Sci USA 83:6795-6799

Yarden Y, Kuang WJ, Yang-Feng T, Coussens L, Munemitsu S, Dull TJ, Chen E, Schlessinger J, Francke U, Ullrich A 1987 EMBO J 6:3341-3351

Yarden Y, Ullrich A 1988a Ann Rev Biochem 57:443-478

Yarden Y, Ullrich A 1988b Biochemistry 27:3113-3119

Young D, Waitches G, Birchmeier C, Fasano G, Wigler M 1986 Cell 45:711-719

Yu K-T, Czech MP 1986 J Biol Chem 261:4715-4722