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CONSTRUCTION OF A TARGETING VECTOR FOR THE ANALYSIS OF THE NEUROENDOCRINE FUNCTION OF CHROMOGRANIN A

by

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Abstract

Parathyroid hormone and Chromogranin A (CgA), are the two major secreted products of the parathyroid gland. CgA is the major member of the granin family of acidic glycoproteins which are expressed in all endocrine and neural cells. Granins are thought to play a role in secretory granule formation and targeting of peptide hormones and neurotransmitters to granules of the secretory pathway.

The CgA gene is a single copy gene, that has been characterized in human, bovine, mouse and rat where it was found to be composed of eight exons with conserved exon-intron boundaries. It has been found that several of the peptides encoded within the CgA molecule inhibit hormone and neurotransmitter release in either an autocrine or paracrine fashion. The biosynthesis of CgA is regulated by many different factors, including steroid hormones and a number of intracellular messenger systems, such as intracellular calcium, Protein Kinase A (PKA) and Protein Kinase C (PKC).

Although a number of studies have been conducted on CgA, the precise role that it plays in neuroendocrine function remains unclear. Therefore, to gain insight into the important functions of CgA, this project involved isolation of the CgA gene through screening of a mouse strain genomic DNA library. Restriction fragments of the phage DNA were subcloned into a plasmid vector, sequenced and were confirmed to contain seven of the eight exons of the mouse CgA gene including, eight kilobases of the 5' flanking region. Through the sequence information obtained, a targeting vector was constructed containing approximately nine kilobases of genomic DNA. This vector in turn, will be used for the functional disruption of the CgA gene in mice through the technique of homologous recombination.

Résumé

Les cellules parathyroïdienne synthétisent, stockent et sécrètent principalement l'hormone parathyroïdienne et la chromogranine A. Les chromogranines sont une famille de glycoprotéines acides localisées exclusivement dans les granules de sécrétion des cellules endocrines et des neurones. La fonction biologique des chromogranines n'est pas établie de façon définitive, mais plusieurs arguments étayent l'hypothèse selon laquelle elles seraient impliquées dans la formation des granules de sécrétion et le ciblage des hormones peptidiques et des neurotransmetteurs vers la voie de sécrétion régulée.

Le gène de la chromogranine A est à copie unique et est composé de huit exons dont les frontières intron-exon sont conservées chez l'Homme, le boeuf, la souris et le rat. Plusieurs peptides issus de la chromogranine A inhibent la sécrétion d'hormones et de neurotransmetteurs de façon autocrine et paracrine, établissant ainsi un rôle hormonal.

La régulation de la biosynthèse de la chromogranine A est contrôlée par différents facteurs dont les hormones stéroïdiennes, et plusieurs composantes du système de messagerie intracellulaire tels le calcium intracellulaire et les protéines kinases A (PKA) et C (PKC).

Mon projet consistait à construire un vecteur de ciblage du gène de la chromogranine A qui sera utilisé pour effectuer la disruption de ce gène chez la souris par la technique de recombinaison homologue afin d'élucider le rôle précis de la chromogranine A dans le système neuroendocrinien. Nous avons isolé le gène de la chromogranine A par cribblage d'une banque d'ADN génomique murine et les fragments de restriction obtenus de l'ADN de phages isolés ont été sous-clonés dans un vecteur plasmidique. Le séquençage de ces fragments a confirmé la présence de sept des huit exons du gène et

huit kilobases de la région flanquant le gène en 5'. Le vecteur de ciblage contient neuf kilobases du gène de la chromogranine A et est prêt à être transfecté.

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Dedication

I dedicate this thesis to my parents, for their support, love and encouragement throughout my studies.

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Abbreviations

$1,25\ (OH)_2D_3$	Vitamin D
bp	Base pairs
Ca^{2+}	calcium ion
cAMP	Cyclic adenosine-3',5'-monophosphate
cDNA	Complementary DNA
CgA	Chromogranin A
CgB	Chromogranin B
CgC	Chromogranin C
cpm	Counts per minute
CRE	cAMP response element
CREB	cAMP response element binding protein
DNA	Deoxyribonucleic acid
ES cells	Embryonic stem cells
FBS	Fetal bovine serum
FSH	Follicle stimulating hormone
g	Gram
GRE	Glucocorticoid response element
kb	Kilobase pairs
kDa	Kilodalton
LH	Luteinizing hormone
M	Molar

ml	Millilitre
mM	Millimolar
mRNA	Messenger RNA
PBS	Phosphate buffered saline
PC1	Prohormone Convertase 1
PC2	Prohormone Convertase 2
PCR	Polymerase chain reaction
PKA	Protein Kinase A
PKC	Protein Kinase C
POMC	Pro-opiomelanocortin
PTH	Parathyroid hormone
PTHrP	Parathyroid hormone related protein
SgI	Secretogranin I (CgB)
SgII	Secretogranin II (CgC)
SgIII	Secretogranin III (1B1075)
SgIV	Secretogranin IV (HISL-19)
SgV	Secretogranin V (protein 7B2)
µl	Microlitre

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Chapter I

Chromogranin A

Introduction

Granins

The granins are a group of acidic proteins that bind calcium and aggregate in its presence. They are found in the trans golgi network (TGN), the secretory granule of the cell and may be subject to proteolytic processing. Presently, there are seven members to the granin family: chromogranin A (CgA; 1,2), chromogranin B (CgB; 3), chromogranin C (CgC/SgII; 4), the 1B1075 gene product (SgIII; 5), HISL-19 antigen (SgIV; 6), the protein 7B2 (SgV; 7,8) and a new novel chromogranin like precursor, NESP55 (SgVI?; 9) (Fig. 1).

The granins are exclusively expressed in endocrine and neuroendocrine cells. They have a widespread distribution which not only reflects their important function in endocrine cells but in recent times, they have become useful diagnostic and prognostic tools for neuroendocrine tumours (73-84).

The granins share a number of biochemical properties. They have acidic isoelectric points and are hydrophilic which can be attributed to their high number of acidic amino acids (glutamic and aspartic acid residues). Three of the granins have a disulphide loop, CgA, CgB and the 7B2 protein. In addition, three of the granins have shown the ability to bind calcium, CgA, CgB and CgC (42). Lastly, the granins possess a number of paired basic amino acids, which are thought to be sites for proteolytic processing (Table 1).

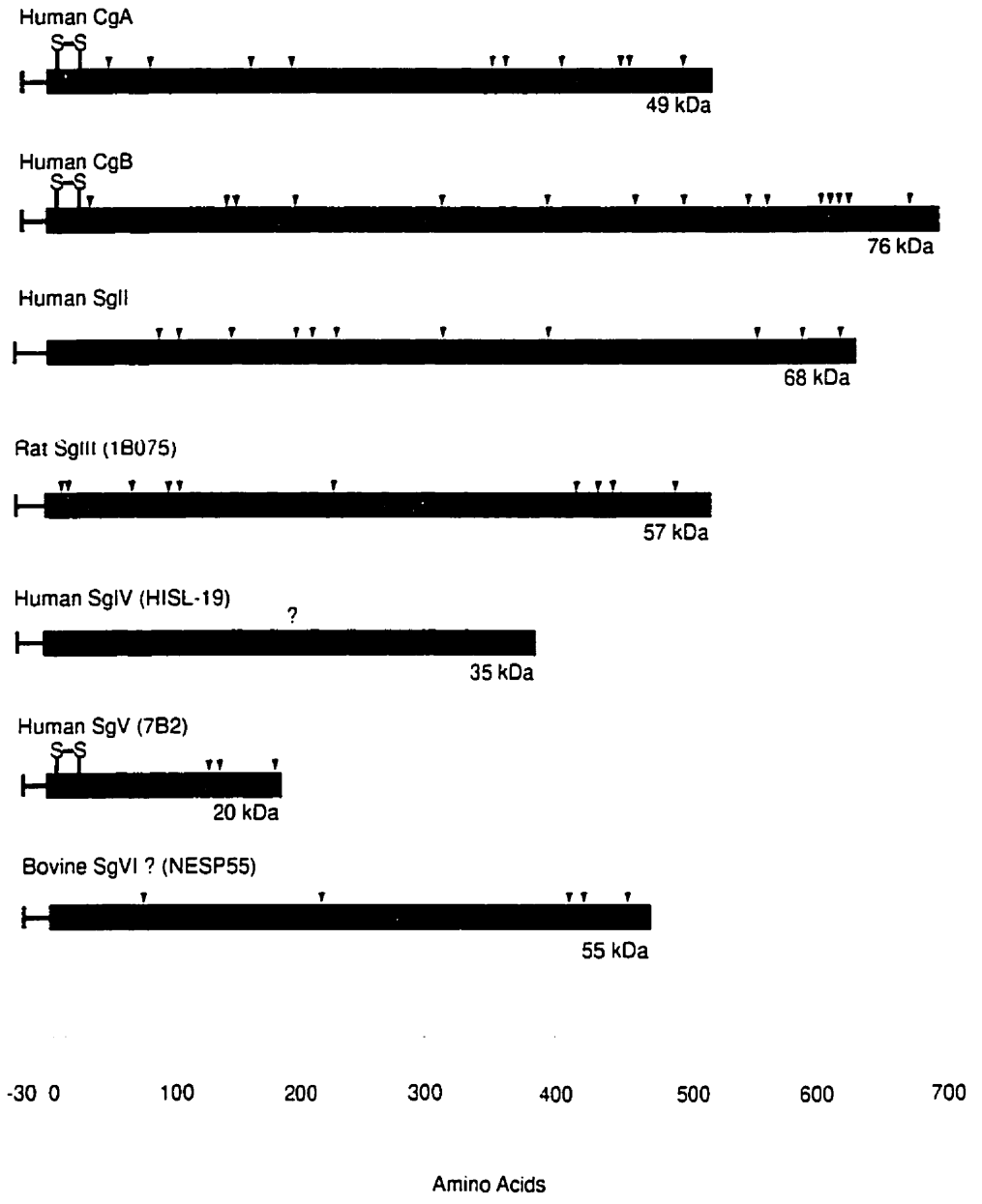


Figure 1. The granin family of endocrine secretory proteins. There are at least seven members of this family of secretory proteins. The human proteins are shown (except for SgIII where only the rat cDNA has been cloned). They possess multiple pairs of basic amino acid residues (indicated by arrows). In CgA, CgB and 7B2, a disulphide loop is present in the amino-terminal region. ? denotes that SgIV cDNA has not yet been cloned nor has the protein been sequenced. It is not known whether SgIV contains pairs of basic amino acid residues. See text for a description of other features of the CgA molecule. (taken from Mouland, A., 1993)

Table 1. General Properties of the Granin Family of Proteins[1]

Granin[2]	Mass (kDa)	mRNA (kB)	Chromosome Assignment	pI	% Glu/Asp	Dibasic Sites	Heat Stability	Calcium Binding	Disulphide Loop
CgA	49	2.1	14q32.2-32.3	4.9	25	10	Yes	Yes	Yes
CgB	76	2.5	20pter-p12	5.1	24	15	Yes	Yes	Yes
SgII	68	2.5	ND[3]	5.0	20	9	Yes	Yes	No
SgIII (1B1075)	57	2.2	9*	5.1	19	10	ND	ND	No
SgIV (HISL-19)	ND	ND	ND	5.6	ND	ND	ND	ND	ND
SgV (7B2)	21	1.4	15q11-q15	5.2	16	3	Yes	ND	No
SgVI? (NESP55)	55	0.7	ND	4.8	21	5	Yes	ND	ND

1 Adapted from Huttner et al., (1991) and Fisher-Colbrie et al., (1997)

2 Data for the human granins are shown, except for SgII where that for the rat (and mouse*) are shown as well as for SgVI where that for the bovine is shown.

3 not determined (ND)

Function

The granins are thought to have multiple functions. Intracellularly, granins are thought to participate in the regulated secretory pathway, by having a role in processing proteins which are synthesized along with constitutively secreted proteins, but whose secretion is regulated by signals from the extracellular environment (42,60). By virtue of their ability to aggregate in the low pH, high calcium environment of the trans-Golgi network, granins have been proposed to have a role in targeting peptide hormones and neurotransmitters to granules of the regulated pathway. Extracellularly, granins serve a different range of functions. Encoded within the granin sequence are biologically active peptides which are released by proprotein convertases within the secretory granule (59). These peptides are thought to function in an autocrine or paracrine fashion and serve to regulate cellular secretion of other peptides (59), see Table 2. However, other granin functions have been found to exist. Recently, Secretogranin V, better known as 7B2, has been determined to function as a neuroendocrine chaperone for the prohormone convertase PC2. It has been found to be a bifunctional molecule that is essential in the transport and maturation of proPC2 in the regulated secretory pathway. More specifically, it prevents the premature autocatalytic conversion of the proenzyme until the appropriate site for activation in the secretory pathway has been reached.

Table 2. Granin derived peptides and their biological effects. The species from which the sequences of CgA, CgB, and SgII are derived are abbreviated as follows: r, rat; b, bovine; p, porcine; h, human. The cleavage sites are indicated using the single letter amino acid code: NH_2, amino terminus: COOH, carboxy-terminus. (59)

Granin	Peptide	Sequence	Cleavage	Origin	Effect	Target Tissue	Reference
CgA	β-granin	1-128(r)	NH_2-KR	Islet β-cell	Unknown	Unknown	30
	β-granin like peptide	1-113(b)	NH_2-KR	Parathyroid	↓ PTH/CgA secretion	Parathyroid	33
	Vasostatin	1-76(b)	NH_2-KK	Adrenal Medulla	↓ vascular tone	Arteries, veins	35,36
	Chromostatin[a]	124-143(b)	K-K	Adrenal Medulla	↓ catecholamine secretion	Adrenal medulla	34,44
	Pancreastatin	240-288(p)	R-KR	Islet β-cell	↓ isulin secretion ↓ PTH secretion	Islet β-cell Parathyroid	26,32
	Parastatin	347-419(p)	K-K	Parathyroid*	↓ PTH secretion	Parathyroid	37
CgB	GAWK	420-493(h)	KR-KR	Pituitary	Unknown	Unknown	29
	BAM-1745	547-560(b)	KR-RKK	Adrenal Medulla	Unknown	Unknown	45,46
	CCB	597-653(h)	RKK-COOH	Pituitary	Unknown	Unknown	29
SgII	Secretoneurin	154-186(b)	KR-KR	Brain	↑ Dopamine release	Caudateputamen	38,39
	Frog secretoneurin related peptide[b]	157-189(f)	KR-KR	Brain	Unknown	Unknown	47
	AMENM	582-586(b)	KR-COOH	Adrenal Medulla	Unknown	Unknown	48

[a] Note that a recent correction (44) indicates that the inhibition of catecholamine secretion is not due to chromostatin but maybe to another CgA-derived peptide. The asterisk indicates the fact that parastatin was produced by exogenous protease. [b] The sequence position of the frog secretoneurin-related peptide (47) was according to the mouse SgII sequence (16). Only direct effects of the granin-derived peptides on target tissues are listed. PTH, parathyroid hormone. (59)

Chromogranin A

Chromogranin A, CgA, is a major member of the granin family of proteins present in all endocrine and neuroendocrine cells. It is an acidic glycoprotein that is found in the core of secretory vesicles, that is co-released through exocytosis with a variety of neurotransmitters as well as amine or peptide hormones. More than 25 years have elapsed since its discovery in adrenal chromaffin granules (10).

To date, CgA is the best-studied member of the granin family. Although its precise role in the endocrine/neuroendocrine system is not known for certain, emerging evidence has shown that it plays several biological roles, both within the secretory granules and after release from neuroendocrine cells. In addition, the gene's widespread yet restricted (endocrine/neuroendocrine) pattern of expression provides additional evidence that CgA may function as a modulator of cell secretory activity.

So far, many studies have looked at the regulation of biosynthesis and secretion of CgA in an attempt to determine its exact role. However, most of the information collected so far seems to reaffirm previous studies that show CgA functions in an intracellular as well as extracellular capacity, in addition to providing more data on the protein's biochemical properties. It is for this reason that this project was undertaken. Hopefully, through the targeted ablation of the CgA gene, its functions within the neuroendocrine system will become clearer.

Biochemistry

Pre-chromogranin A is synthesized with an 18 amino acid hydrophobic signal sequence present at the NH_2-terminus, that targets it to the endoplasmic reticulum. CgA is encoded by a single copy gene. It has a predicted molecular mass of 49 kDa and varies in amino acid length, from 430 in the porcine to 445 in the murine species (11-18). CgA has an isoelectric point that ranges between 4.5-5.0, which is attributed to the high proportion of glutamic and aspartic acid residues (25%) in addition to quite a few proline residues (10%).

CgA possesses several paired basic amino acid residues (the precise number depending on the species), which are presumed to be sites for proteolytic processing that when cleaved, result in several biologically active peptides that include: pancreastatin, β-granin, vasostatin parastatin, and chromostatin. A number of conserved oligo-glutamic acid clusters are present within the CgA molecule which represent candidates for calcium binding domains as well as determining the secondary and tertiary structure of the protein. In addition, due to two cysteine residues located at the NH_2-terminus, there is an intramolecular disulphide loop present, which has been found to be conserved among species.

Within the murine species there exists a polymorphic poly-glutamine region of 11-19 consecutive glutamine residues, that are encoded by a polymorphic $[CAG]_n$ repeat towards the N-terminus.

In addition, most species contain an RGD sequence [Arg-Gly-Asp] located at the N-terminus of the CgA molecule. The only exception is the rat, where the RGD sequence is located at the COOH-terminus. Although no evidence exists to date on

whether the RGD sequence is functional within the CgA gene, RGD sequences are known to be involved in the attachement of anchorage dependent cells to the extracellular matrix or in cell to cell adhesion.

Although two exceptions exist (the cleaved signal peptide as well as the disulphide loop which are hydrophobic), the hydropathy plot of CgA is quite hydrophilic and is attributed to the high content of acidic amino acid residues.

In addition to having shown that CgA undergoes pH and calcium dependent conformational changes (55,92), further studies have revealed that CgA exists in a primarily tetrameric state at the intravesicular pH of 5.5 and in a primarily dimeric state at the near physiological pH of 7.5 (103). This suggests a dimeric existence of CgA in the endoplasmic reticulum and cis-Golgi cisternae, and a tetrameric existence in the TGN and secretory vesicles. These different dimerization states were found to be due to the acidic pH-induced conformational changes that exposed additional sites of interaction (55,56,92). In addition, the conserved C-terminal region was found to be responsible for the dimerization and tetramerization of CgA. Furthermore, studies have shown that CgA interacts with the vesicle membrane at the intravesicular pH of 5.5 and is released from it at a pH of 7.5, and that the membrane binding ability of CgA is thought to be due to the pH induced anchor of the conserved near N-terminal region of CgA (102).

CgA has been found to interact with several integral membrane proteins of secretory vesicles, including a 260 kDa protein reactive to inositol 1,4,5-triphosphate receptor antibody (91). This pH-dependent binding of integral membrane proteins, including the IP_3 receptor, by CgA has profound physiological importance in elucidating the calcium mobilization mechanism of the IP_3 sensitive store of calcium, as well as in

understanding the membrane protein sorting process that occurs during secretory vesicle biogenesis.

In addition, recent studies have identified the secretory vesicle membrane binding region of CgA. It is located also within the conserved NH_2-terminal region of CgA, residues 18-37 (85).

Lastly, CgA migrates aberrantly on an SDS polyacrylamide gel with an apparent Mr of 75-80 kDa. This is also due to the high content of acidic residues in addition to post-translational modifications: glycosylation, phosphorylation, sulfation and carboxymethylation (19,20,21), the occur within the protein.

Post-Translational Modifications

Phosphorylation

CgA has been found to be phosphorylated in vitro on serine and tyrosine residues but very little on threonine residues (21). The importance of phosphorylation still remains unclear. Recent studies on bovine adrenal medullary CgA have shown that it contains four phosphorylation sites on serine residues; Ser81(N-terminal region), Ser307, Ser372 and Ser376 (C-terminal region). An additional site was present on a tyrosine residue Tyr173 (19).

Glycosylation

Parathyroid CgA has been found to be glycosylated, containing 18% carbohydrate (93). However later studies showed that the percentage of carbohydrate was similar to that of the adrenal medulla CgA, having approximately 5.0% (94). Both samples showed

the majority of glycosylation sites to be O-linked. Studies involved to determine whether N-linked glycosylation takes place on CgA, have indicated that no such modifications occur within the endoplasmic reticulum (68), even though human and rat CgAs possess consensus sites for N-glycosylation (10). Recent studies have identified two O-linked glycosylation sites in bovine adrenal medullary CgA, one on Ser186 and one on Thr231 (19).

Carboxymethylation

Carboxymethylation is thought to be a requirement for a protein which is destined for endoproteolytic attack (95). Since CgA is processed to a number of biologically active peptides, such post-translational modification may be necessary for this process. For example, evidence of cotranslational carboxymethylation of CgA in adrenal medulla chromaffin cells has been identified.

Sulphation

CgA in bovine chromaffin cells has been shown to be sulphated. This sulphate moiety is mainly bound to carbohydrates and not to tyrosine residues (68). Such post-translational modification is thought to have a role in rendering a precursor protein amenable to proteolytic attack in addition to enhancing the biological activity of certain peptides. For example, a positive relationship between proteolytic processing and sulphation of the gastrin precursor is suggested to exist (96). In addition, studies have shown that sulphated cholecystokinin and leu-enkephalin are more potent in eliciting their biological effects than the nonsulphated forms (97).

Disulphide Bond Formation

CgA contains two amino-terminal cysteine residues (Cys17-Cys38) that participate in the formation of a disulphide loop (3). This structure is conserved in CgA in all species and is also present in two other granins, 7B2 and CgB. Recently, CgB studies have shown that reduction of the disulphide bond causes mis-sorted of CgB to the constitutive pathway, but is not required for CgB aggregation in the trans-Golgi network (98). In addition, other studies involving a deletion mutant of hCgB lacking the highly conserved disulphide-bonded loop at the level of the TGN as well as the mature secretory granule, reaffirm that the disulphide loop is essential for sorting to immature secretory granules (101). Since CgB and CgA are more closely related to each other than to any other granin, in addition to the disulphide bridge of CgA being located in an identical region as in the CgB protein, it would be reasonable to assume that the disulphide bridge of CgA has the same function.

Structure of the CgA gene

Chromogranin A is encoded within eight separate exons in the mouse, rat, bovine and human genome (12,16,22) (Fig. 2). The 5' untranslated region of the CgA mRNA and most of the signal peptide is encoded within exon one. Within exons 2 through 5, the putative biologically active peptides β-granin and vasostatin are encoded. The two cysteine residues of the conserved disulphide loop of CgA are contained within exon three. Exon five contains the poly(Q) domain which is specific to murine CgA. Exon six contains the peptide designated as chromostatin and is the exon containing the most

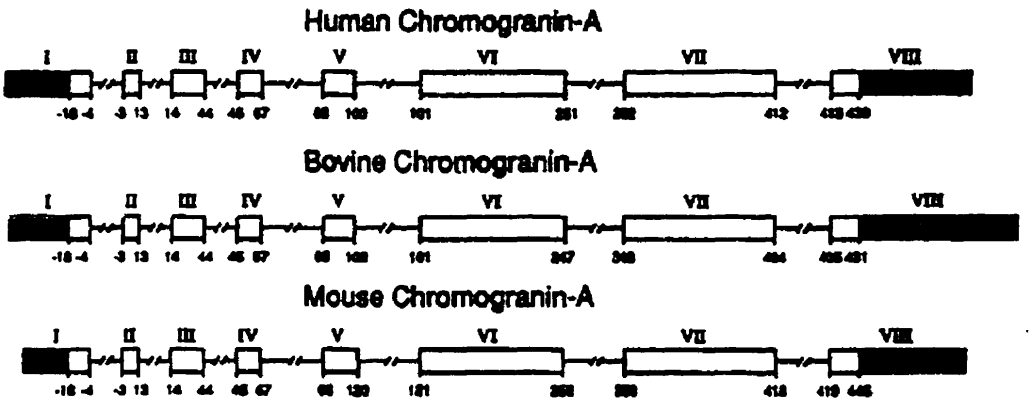


Figure 2. Comparison of the exon/intron organization of the CgA genes. The relative sizes of the 8 exons (roman numerals) of the human, bovine and mouse CgA genes. Filled boxes denote noncoding regions; open boxes indicate coding regions. The numbers at the intron/exon borders denote the amino acids of the preproteins with the first amino acid of the mature proteins indicated by +1. (33)

variable peptide sequence across species. Exon seven contains the sequence encoding pancreastatin and the 3' end of exon seven as well as the 5' end of exon eight encode the sequences of the paracrine factor, parastatin. Exon eight of the gene, contains the C-terminus of the protein, including the last dibasic amino acid pair, and the 3' untranslated region of the CgA mRNA (Fig. 3).

Regulation of CgA Gene Transcription.

A: Steroid Hormones

CgA is expressed in a wide variety of neuroendocrine cell types, each of which co-express a unique and cell specific polypeptide hormone or neurotransmitter (10). For this co-expression to exist, the chromogranin A gene is able to respond to a wide variety of developmental and hormonal signals that drive polypeptide expression in each of the neuroendocrine cell types in which it is expressed. For example, due to the very high expression of CgA in the pituitary, adrenal medulla and parathyroid, a number of studies have looked at the regulation of CgA biosynthesis by estrogen, glucocorticoids, calcium and vitamin D. In these studies, it was found that estrogen suppresses CgA mRNA and protein abundance in the pituitary (25,26,27) but is upregulated by glucocorticoids in the adrenal and pituitary (22,29,30,87).

Recently, a glucocorticoid response element (GRE) was identified within the rCgA promoter and studies involving site directed mutagenesis as well as promoter constructs have shown that glucocorticoids directly activate CgA gene expression (86).

Studies involving the parathyroid gland have shown that CgA biosynthesis is enhanced by $1,25(OH)_2D_3$ (30,31), yet PTH biosynthesis is inhibited by $1,25(OH)_2D_3$ (31). Under these type of conditions, CgA is released at a higher rate, yet total amount

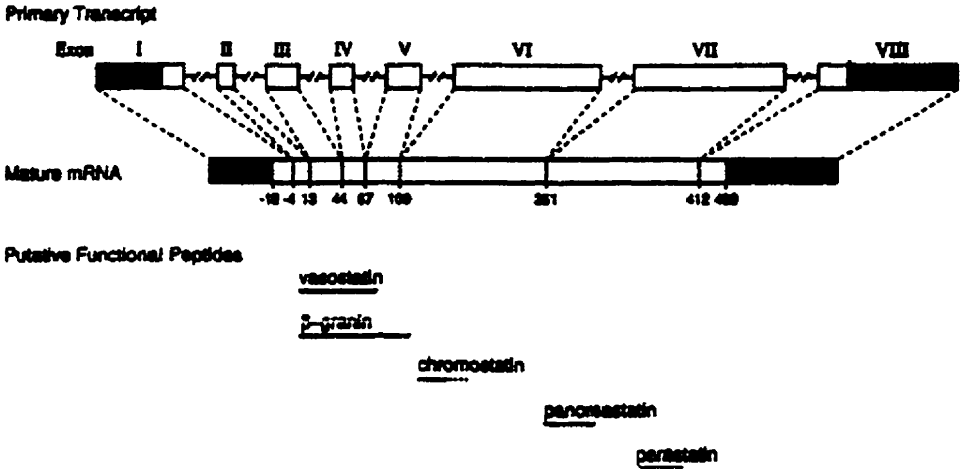


Figure 3. Relationship between the functional peptide coding regions. The exon/intron organization of the CgA gene is related to the known CgA-derived peptides which have been reported to be biologically active. (33)

present in the cell is little affected (30). In addition, in vivo studies of CgA mRNA expression in the parathyroid have shown that increased $1,25(OH)_2D_3$ concentrations stimulate CgA mRNA expression, while a decrease in concentration showed reduction in CgA mRNA expression (88). Overall, $1,25(OH)_2D_3$ has been shown to have a post translational effect on CgA synthesis by decreasing peptide chain elongation and hence CgA translatability.

In the majority of these cases, steroid hormones have shown to have a stimulatory effect on CgA synthesis and an inhibitory effect on the resident hormone. For example, glucocorticoids and $1,25\ (OH)_2D_3$ decrease POMC and PTH synthesis in the pituitary corticotroph and parathyroid chief cell respectively. The only exception is estrogen. It seems to have an inhibitory effect on CgA synthesis and a stimulatory effect on the biosynthesis of the resident hormone, such as FSH and LH. This indicates that the neuroendocrine cell has some sort of autonomous secretory control mechanism through the differential regulation of CgA and the resident hormone. Secretory cell stimulation would result in an increase in CgA biosynthesis with the release of the resident hormone. At the same time, this rise in CgA or CgA peptides would act to suppress further release of the resident hormone.

B: Intracellular Messengers

A number of intracellular messengers have been implicated in the regulation of CgA biosynthesis. One example is calcium. Many studies to date have shown calcium as being an important signal in CgA biosynthesis. Cholinergic agonists such as nicotine

and histamine stimulate calcium influx into the cell by activating a voltage dependent calcium channel. In conjunction with this calcium influx, an increase in CgA biosynthesis is exhibited (32-35).

On the other hand, when intracellular stores of calcium are released through stimulation with factors such as bradykinin, the levels of CgA within the cell rise. It seems that calcium release from intracellular stores contribute to the induction of CgA biosynthesis. Recently, studies have shown that activation of CgA transcription via secretory stimulation in chromaffin cells is highly dependent on the calcium route into the cytosol. Only when calcium entered into the cytosol through the voltage gated ion channels did an increase in CgA transcription occur (36).

Other factors which have been shown to have involvement in CgA biosynthesis are the PKC and PKA signalling pathways.

A calcium dependent membrane bound form of PKC, is activated by diacylglycerol and has been shown to be involved in CgA biosynthesis regulation. For example, the use of phorbol esters (which mimic effects of diacylglycerol), have been shown to stimulate CgA synthesis in chromaffin cells and some neuroendocrine cell lines (32,37). Recently however, it has been shown that cholinergic stimulation of CgA transcription is dependent on the activation of PKC. Studies have shown that when PKC was inhibited, no transcriptional activation was observed, yet when PKC was activated, it mimicked the nicotinic effects (36).

PKA on the other hand, works in a different fashion. It is a cAMP dependent protein kinase that phosphorylates protein substrates that in turn act directly on the gene level to alter protein synthesis. One substrate of PKA is CREB, the cAMP response

element binding protein, which is a member of the family of activating transcription factors. When activated by phosphorylation, CREB will bind to the cAMP response element found in the promoters of a number of genes which then leads to the upregulation of gene transcription. Such a CRE has been identified within the promoter of CgA yet studies that involved either cAMP directly or forskolin which acts directly on AC, adenylate cyclase, produced little or no increase in CgA levels (33,38,39).

On the other hand, the study of PKC and nicotine stimulated activation of CgA biosynthesis found that CREB was needed and essential to this stimulation, by acting as the protein that relays the transcriptional signal to the CRE present in cis. When CREB was mutated, no transcriptional activation was achieved even with stimulation using nicotine, phorbol esters or membrane depolarization (36). In addition, one study has shown that the CRE is indispensable in the tissue specific expression of the chromogranin A gene. When deletion or point mutations were done in the CRE, promoter activity was abolished in neuroendocrine cells (89).

Furthermore, recent gene transfer experiments using an hCgA gene promoter / Cat reporter gene construct has shown that the CRE plays a key role in both basal and cAMP stimulated expression of the CgA gene in neuroendocrine cells. For example, upon deletion or mutation of the CRE, a loss of neuroendocrine cell specific transcriptional activity was observed. In addition, the importance of the CRE in basal transcription was demonstrated when mutation in 5' extended constructs (-2300 to +32 and -700 to +32) of the CRE, resulted in a 50-75% decrease in basal activity in neuroendocrine cells (99).

The conflicting data with respect to the CRE and its effects on CgA expression may be due to different experimental methods involved in the above studies. For

example, the use of different cell types could account for the unobserved increases in CgA levels when forskolin was used (33,38,39).

Lastly, prior studies involving hCgA have identified a DRE (distal regulatory element) of 27bp which has been found to act as an enhancer in CgA gene transcription (40). It was found to be located between -576 and -550 bp and shown to interact with the CRE to enhance gene transcription in neuroendocrine cells. To date, this DRE has been characterized and has been shown to be dependent on a unique neuroendocrine specific DRE binding factor (DBF) that specifically and directionally binds the DRE to assemble and activate a functional transcriptional complex (40,41). These studies have only begun to unravel the complexities behind the biosynthesis of CgA. In addition, they have implicated the CRE as an important and integral part to this regulation.

Chromogranin A Functions

Extracellular

The granins are a family of acidic, heat stable proteins present in secretory granules of a wide variety of endocrine cells and neurons (42). The classical three members of this protein family are (in order of discovery), chromogranin A (CgA), secretogranin II (SgII) and chromogranin B (CgB/secretogranin I). To date, research has focused on many aspects of the granins. Some areas include, their structure and biochemical properties, expression patterns in endocrine cells and neurons, their use as serum markers in health and disease related topics, their sorting to secretory granules (intracellular localization) in addition to their possible roles as precursors to biologically active peptides.

Proteolytic processing of CgA results in the production of a number of peptides that are thought to function as modulators of endocrine cell secretory activity. For example, Pancreastatin, a carboxyl-terminally amidated 49 amino acid residue peptide inhibits glucose stimulated insulin release from the perfused rat pancreas. Processing can occur both intracellulary and extracellularly and a number of papers have shown that processing is important for the expression of biological activity (23,43).

An obvious prerequisite for proteolytic processing is the presence of appropriate recognition site(s) for the processing enzyme. The elucidation of the primary structure of CgA has shown that it contains multiple potential sites for processing by proteases that recognize distinct single or pairs of basic amino acids. Some of the granin derived peptides studied to date have been generated by cleavage at such sites.

Evidence in support of proteolytic processing of CgA into smaller biologically active peptides started with the isolation from porcine pancreas of a 49 amino acid, carboxyl-terminally amidated peptide, pancreastatin, that inhibited glucose-stimulated insulin release from rat pancreatic islets (44,45). In addition to the full length peptide, synthetic replicates of the C-terminal region of pancreaststin also decreased insulin release. Structural characterization of the cDNA encoding porcine CgA indicated that the biologically active peptide corresponds to residues 240 to 280 of the mature protein (11). This suggested that the parent CgA molecule gives rise to pancreastatin through proteolytic processing.

Other cell types have been shown to produce pancreastatin activity. Although little processing of CgA to peptides occurs within the parathyroid cell, it was demonstrated that pancreastatin had an inhibitory effect on PTH secretion from porcine

parathyroid cells in culture (43), and that pancreastatin evokes a pertussis toxin reversible decrease in intracellular cyclic AMP levels in the histamine stimulated, isolated rabbit parietal cell (46).

In addition to pancreastatin, a number of other sequenced peptides containing the N-terminal region of CgA have been isolated and have shown some biological activity. For example, β-granin, a 20kDa N-terminal CgA fragment (amino acids 1-113) was isolated from a rat insulinoma (48). Although its function in the pancreas is unknown, β-granin has been shown to inhibit parathyroid cell secretion in vitro (49). Within the β-granin sequence is vasostatin (amino acids 1-76). It has been found to have inhibitory effects on tension development in isolated blood vessels (50).

Another biologically active peptide termed parastatin, generated by endoprotease Lys-C digestion of CgA (porcine CgA 347-419) (51), has been found to inhibit PTH secretion at low extracellular calcium. Lastly, chromostatin, another CgA fragment generated by endoproteinase Lys-C digestion in vitro, has been shown to inhibit catecholamine secretion from bovine adrenal medullary cells (52) and exhibits saturable binding to a putative receptor (53). However, it should be mentioned that some controversy exists with respect to the last study since many of the observed effects were as a result of a contaminant formed during the chemical synthesis of the chromostatin peptide (24).

In addition, studies have shown peptides with antibacterial activity are present as water soluble compounds of bovine chromaffin granules and are released during secretion. CgA specifically, has been found to generate a large natural fragment corresponding to residues 79-431, and this has been shown to inhibit the growth of Gram

+ve and Gram -ve bacteria. The identification of such antibacterial peptides suggests a potential role in host defense against microbial infection (100).

In conclusion, the above studies indicate that CgA may in fact be a precursor to a number of biologically active peptides that modulate hormone secretion from endocrine cells. In addition, a recent study has implicated both CgA and its fragments as having a role in the regulation of cell adhesion (90).

Intracellular

A number of experimental studies have shown that CgA functions to modulate cell secretory activity through co-localization and co-secretion with the resident hormone in secretory granules.

CgA is a high capacity, low affinity calcium binding protein (54,55,56). Since calcium binding is influenced by pH (54,91), magnesium and ionic strength (54), binding may change during the maturation and acidification of secretory granules. In addition, hydrogen ion and calcium concentration have been shown to induce conformational changes in the protein (55,56,92), and that calcium enhances CgA association with the granule membrane (57). All of these factors provide a mechanism by which CgA can act to sort hormones into the regulated secretory pathway and package hormones into secretory granules.

Lastly, due to the multiple pairs of basic amino acid sites, CgA could have a role in regulating prohormone processing by acting as a competitive substrate for serine protease-like activity in cells (58) (Fig.4).

Figure 4

Putative Functions for Chromogranin A

EXTRACELLULAR

- Precursor to biologically active peptides

- Regulation of cell adhesion

INTRACELLULAR

- Role as an intracellular calcium-binding protein

- Role in the packaging of peptide hormones and neuropeptides, granule condensation and exocytosis

- Intracellular regulator of prohormone processing

Proteolytic processing

A number of bioactive peptides are known today to be generated by proteolytic processing of CgA at the multiple pairs of basic amino acid residues within the protein. A number of the granin-derived peptides studied so far have been generated by cleavage at such sites (59). Studies on proteolytic processing first started when it was revealed that multiple forms of chromogranins existed in various endocrine tissues (60). These studies then led to the discovery that the lower molecular weight chromogranins were related to CgA, that they were contained within the CgA sequence and that the pattern of processing could be predicted based on the placement of the pairs of basic amino acids within the CgA molecule (18,61,62). Eventually, the granins were established as being propeptides by the discovery that CgA had the potential to be the precursor to pancreastatin (11).

The extent of CgA processing has been shown to be highly tissue dependent and occurs to varying degrees in many endocrine tissues. In the parathyroid and adrenal medulla, very little processing of CgA occurs, however, in the intestine, stomach, peripheral nerves and pancreas there is extensive proteolytic processing (33,48,63-66).

Information to date indicates that CgA processing seems to occur in qualitatively different patterns, yielding tissue specific products rather then complete proteolysis in all endocrine tissues (65). This has brought into question the importance of the expression of the CgA processing enzymes and how critical they are to the distribution of CgA derived peptides in various endocrine tissues. For example, in the pancreas, the level of PC2 expression is far higher than in the adrenal medulla (67). This may explain why there are more processed forms of CgA in the former tissue. In brief, PC2 is a member of

the eukaryotic subtilisin-like family of serine endoproteases that function in the biosynthetic processing of peptides into smaller bioactive peptides. Studies have shown that PC2 cleavage occurs at pairs of basic amino acids and that this conversion occurs within the secretory granule where the high calcium ion concentration and acidic pH is necessary for the proper functioning of this convertase (67,71).

Another factor which has been shown to affect proteolytic processing is the presence or absence of post-translational modification. Before the granins are processed, they are phosphorylated on serine residues as they pass through the trans-golgi cisternae. This phosphorylation has been implicated in having an influence on the efficiency of processing at the basic residues within the granins (68-70). The same can be said for other post translational processing events including O-glycosylation and sulfation; events that occur during granin transport along the secretory pathway (59,68-70). These events it seems may in part be responsible for the differences in proteolytic processing seen in the parathyroid and adrenal gland (65).

From a different viewpoint, CgA has also been implicated as being a possible competitive inhibitor of PC2 and other processing enzymes, which therefore affect the processing rates of other co-stored hormones (58,65). It is well documented that hormonal treatment using vitamin D or glucocorticoids up-regulate CgA levels, and through this up-regulation, CgA may act as a competitive substrate therby modulating the rate of post-translational processing and therefore the availability of tissue unique hormones (65). In other words, the granins may compete with co-packaged peptide hormone or neuropeptide precursors as substrates for the PC enzymes and therefore directly act as inhibitors of peptide precursor processing.

One must be aware that the mere presence of an appropriate recognition site for processing does not render this a functional cleavage site unless it is accessible to the relevant protease. Factors such as folding of the granins can affect protease accessibility (59). Although very little information is available on this subject, it is likely to be extremely complex.

To date, studies have shown CgA to be a substrate for PC1 as well as furin in vivo, and that processing of this type influences its trafficking into the regulated secretory pathway. In addition, these same studies have linked PC1 activity to be specific to CgA, which confirms the fact that certain processing enzymes may be transcriptionally regulated in a particular cell type (71). Reserpine, which induces proenkephalin processing and induces the activity of the peptidyl-glycine amidating enzyme to produce bioactive enkephalins, has been found to induce CgA processing. For example, it has been recently shown that bovine chromaffin cell cultures treated with reserpine decreased catecholamine levels, which in turn led to an increase in proteolytic processing of all secretory peptides (72). This reaffirms the fact that granins, can act as inhibitors of peptide proteolysis.

CgA as a neuroendocrine tumour marker

Due to the widespread distribution of CgA, more so than any other granin, CgA in recent times has begun to play a role as a diagnostic and prognostic tool for neuroendocrine tumours.

Over 15 years have passed since the first radioimmunoassay for purified hCgA from pheochromocytoma vesicles was developed (73). This was the very first study to find that patients with pheochromocytomas presented themselves with elevated

circulating levels of CgA relative to those of normal patients. Immediately, this led to studies of a wide variety of neoplastic disorders with the assumption that CgA could be a useful marker in diagnosing endocrine neoplasms. This was in fact the case. Many studies found increased levels of circulating CgA in neuroendocrine neoplasms and today CgA is regarded as the best general neuroendocrine serum marker available (83). When compared to other neuroendocrine markers such as neuron-specific enolase (NSE) and the alpha-subunit of glycoprotein hormones (alpha-SU), CgA is found to have the highest specificity out of all three for the detection of neuroendocrine tumours (83). For example, patients that presented themselves with pheocromocytomas have shown elevated CgA plasma levels in addition to patients with pancreatic and carcinoid tumours (33,74,83). In addition, patients with parathyroid adenomas and primary parathyroid hyperplasia also have shown to have elevated CgA levels (74).

Circulating CgA has been correlated to both tumour burden and disease stage. For example, in small cell lung carcinoma, CgA levels were correlated with the extent of the disease. In cases where the disease had progressed significantly, CgA levels were elevated, whereas in the earlier stages of the same disease, CgA levels were found to be low (77,78). Plasma levels of CgA have also been found to be directly proportional to tumour mass in cases of neuroblastomas. These studies have shown that although other factors were found to be elevated in tumour bearing animals, only plasma CgA correlated with the volume of the tumour (79,83).

Circulating CgA has also been found useful in diagnosing nonfunctioning endocrine tumours. In instances where no hormonal product could be detected, elevated blood CgA levels were found. In addition, some cases showed that the measurement of

CgA gene expression was helpful in diagnosing nonfunctioning tumours as well as endocrine tumours that arose in nonendocrine tissues (80,81).

It is important to mention that in some instances, patients have presented themselves with a particular disease that exhibited elevated CgA levels, that had no correlation with the primary tumour. Rather, it was found that the elevated CgA levels originated from another source, stimulated by factors that were produced by the tumour (75,76).

To date, a number of new techniques have been developed to better detect chromogranins. For example, the use of immunohistochemistry and serology techniques have been developed as new in vitro diagnostic tools for endocrine tumours (82). Recently, an in vivo method for targeting CgA has been developed for the detection of pituitary adenomas by immunoscintigraphy. This new technique, which is based on the use of an anti-CgA monoclonal antibody and on the avidin biotin three step method (Cg-3S-ISG), detected endocrine tumours in vivo, with higher diagnostic accuracy (93%) than conventional techniques (66%), such as ultrasonography, magnetic resonance imaging and planar radiography (82).

Although the specificity of CgA cannot compete with that of specific hormonal secretion products of many neuroendocrine tumours, many studies to date show that measurement of CgA seems to have useful clinical application in subjects with neuroendocrine tumours that either have no marker available or have markers which are inconvenient for routine clinical use.

Chapter 2

Gene Targeting

Introduction

DNA is an extraordinary molecule. It is the blueprint and archive that is handed down virtually unchanged from generation to generation, that encodes to a large degree the information that specifies our physical form. Within the sequence of DNA, is the hereditary information that determines the structure of proteins in all eukaryotic and prokaryotic organisms. All living creatures refer to their DNA for instructions in a constant and dynamic give and take. It is DNA which carries the instructions by which cells grow, divide and differentiate in addition to providing a basis for the evolutionary process both within and between related species. This information is continually accessed, read out and acted upon.

DNA, is the information carrying molecule that comprises the GENES, or units of inheritance, which are arranged in linear arrays along the chromosomes of the cell. Genes are the blueprints for all RNA and protein molecules in the cell.

A gene can be subdivided into two basic regions, the coding region and the noncoding region. The coding region contains the genetic code that is read by the translational machinery in the cytoplasm and is defined by the translation initiation codon (usually AUG) and one or other of the three translation termination codons (UAA, UAG or UGA). The noncoding region represents those DNA sequences which are required for the expression of the genetic information but which are not translated into a polypeptide sequence. One exception to this latter generalization is the intron sequences which, are noncoding sequences found embedded in the coding sequences of the vast majority of eukaryotic but not prokaryotic genes. These sequences appear to have no role in gene

function or expression *per se* but rather are discarded during the processing of the primary RNA transcript.

Much of today's knowledge about the physiological function of genes is derived through the study of existing mutant organisms. Traditionally, identification of mutated genes has been based on the analysis of a resultant phenotype characterized by a variety of biochemical and or morphological criteria. A number of different approaches have been developed to aid in the determination of a link between the descriptive analysis of a mutant phenotype and it's primary molecular defect. Some examples include the study of temperature sensitive mutants in yeast and positional cloning in mice and humans (110,119). Usually however, a gene is cloned following the purification of an encoded protein with no prior knowledge of its physiological function. Its function on the other hand can be deduced from the biochemical properties of the protein product, its expression pattern,...etc. For example, in some cases a gene defect may disturb a metabolic pathway, leading to the accumulation of a metabolite in the affected organism. Therefore, through the study of the pathway and the purification of the protein encoded by this particular gene, one could begin to define the molecular basis of the gene defect. This, for example, can be done through the cDNA cloning of the defective allele or by the analysis and comparison of the normal and mutated genomic loci.

Although all these methods are effective, the ideal way to determine the function of an encoded protein is through the use of genetics and is based on the availability of organisms with an inactive gene product. In other words, through the study of the mutant phenotype (via the functional elimination of a gene), rather than it's overexpression, can

one begin to characterize the protein's role in normal physiology. Unfortunately, the availability of higher organisms such as mammals having spontaneous mutations at a particular locus is not always possible. This is due in part to the generation time of mammals as well as the limitations imposed by rates of random integration. Therefore, other methods have been devised to help infer the function of particular gene products in an intact animal: the most common of these methods being tissue ablation. For example, excision of the parathyroid glands would lead to hypocalcemia accompanied by low levels of circulating PTH levels (109). This technique however, is only suitable for proteins whose synthesis is limited to a defined tissue. One runs into problems when that particular protein is expressed in both a spatial and temporal fashion such as PTHrP. Hence a different approach was needed. This is where transgenic technology has come into play. To date, it is the leading technique for accessing and changing the information contained in DNA, since it is no longer contingent on the availability of spontaneous mutations. Rather, transgenic technology provides the researcher with the capabilities of gene introduction through DNA microinjection. With this, one can attain a model specimen exhibiting either a gain of function or loss of function of a particular gene. Today, a number of transgenic experiments carried out in various laboratories have resulted in the production of mutant mouse strains through the introduction of selective mutations in the murine mouse genome using homologous recombination (104,110,116,130).

Recombination, in general, allows for reassortment at the subchromosome level. Through the study of recombination-defective mutants, it has been found that recombination has an important role in the repair of damaged DNA. Homologous

recombination, on the other hand, is defined as genetic recombination that occurs between DNA with long stretches of homology (eg., between homologous chromosomes during meiosis) and which is mediated by enzymes that show no sequence specificity (104). There are a number of models detailing the events leading to recombination such as the Double Strand Break Repair model, the Meselson-Radding model,...etc, yet these will not be discussed here. What is important to remember is that without homologous recombination, the ability to disrupt genes in the murine embryonic stem cells as well as transmission of the disrupted gene through the germline of mice would never have been possible. Gene targeting as a whole, has opened a vast and new dimension in mammalian genetics and has proven extremely useful in the generation of animal models for human diseases, on which therapeutic approaches are then tested. (123,132).

Gene Targeting-overview

Gene targeting allows for the modification of genetic information in a living organism. In other words, it allows for the direct isolation of mutant organisms. Gene targeting is distinguished from other techniques which manipulate genomes by the following three reasons. First, it is a process which is specific, such that a predetermined sequence can be inserted or substituted at the target locus. Secondly, it is a process that is directed, affecting only the locus of choice, and finally, for practical significance, this process is efficient. Therefore, through the use of gene targeting, the selective removal of a single gene from the mouse genome can be achieved and hence the function of that particular gene can be ascertained.

In general, gene targeting has become a feasible approach for the study of gene function for the following two reasons:

- - the development of methods for the screening and selection of rare homologous events in mammalian cells through the introduction of selectable markers in the transfected piece of DNA.
- - the successful isolation of pluripotent murine embryonic stem cells that can be grown and manipulated in tissue culture.

The underlying approach of gene targeting by homologous recombination is relatively simple. A targeting vector carrying a selectable marker (usually the neomycin resistance gene) flanked by sequences homologous to the genomic target gene is constructed through digestion of the target gene with restriction enzymes followed by ligation of the fragments into the targeting vector. Restriction enzymes are specialized proteins that bind specifically to and cleave double-stranded DNA at specific sites within or adjacent to a particular sequence known as a recognition sequence. The vector is then introduced by transfection into an embryonic stem (ES) cell line. The homologous flanking sequences enable targeted insertion into the genome and the gene mutated by the selectable marker replaces the original wildtype sequence. Subsequently, those ES cells having undergone the correct recombination event are isolated and injected into blastocysts (3.5 day embryos, 32 cell stage) and contributes to all tissues in the developing embryo including the germ line. Usually, a color tag is used to identify the chimeric animals. Subsequently, through germ line transmission, mice which are homozygous for the mutation are generated and the phenotypic consequences of the alteration are then studied and analyzed (Fig.5).

Most of today's studies involve the generation of null mutations within the mouse genome to determine the roles of various genes. This is achieved through a targeted disruption, whereby recombination occurs between homologous sequences present within the targeting vector and the genome of murine ES cells. Within this targeting vector are two selectable markers, the bacterial neomycin gene (neo^r) and the herpes simplex virus thymidine kinase (hsv-tk) gene at one end. These two genes are used for what is known as positive-negative selection whereby one screens a number of cells to determine which of the constructs integrated into the genome randomly, thereby enriching for homologous recombinants. Once a successful targeted ES cell is located, the researcher knows that a "knock out " of that particular gene has been achieved since the targeted gene segments have been replaced by the neomycin selectable marker. In addition, the researcher is able to differentiate between those cells having undergone homologous recombination and those that underwent random integration through the hsv-tk gene, which is placed outside the region of homology. Homologous recombination would eject this gene whereas random integration would incorporate the gene. Then, as stated above, positive ES cells are then injected into a blastocyst and placed into a pseudo-pregnant mouse. The progeny are then screened to determine which have incorporated the disruption and then brother and sister are crossed to produce a homozygous null mutation. Then begins the analysis for phenotypic changes to determine the function of the gene.

In summary, due to the technological advances in gene targeting by homologous recombination in mammalian systems, one is able to create mutations in any gene desired. As such, one produces a number of mutant mouse strains and mutant cell lines.

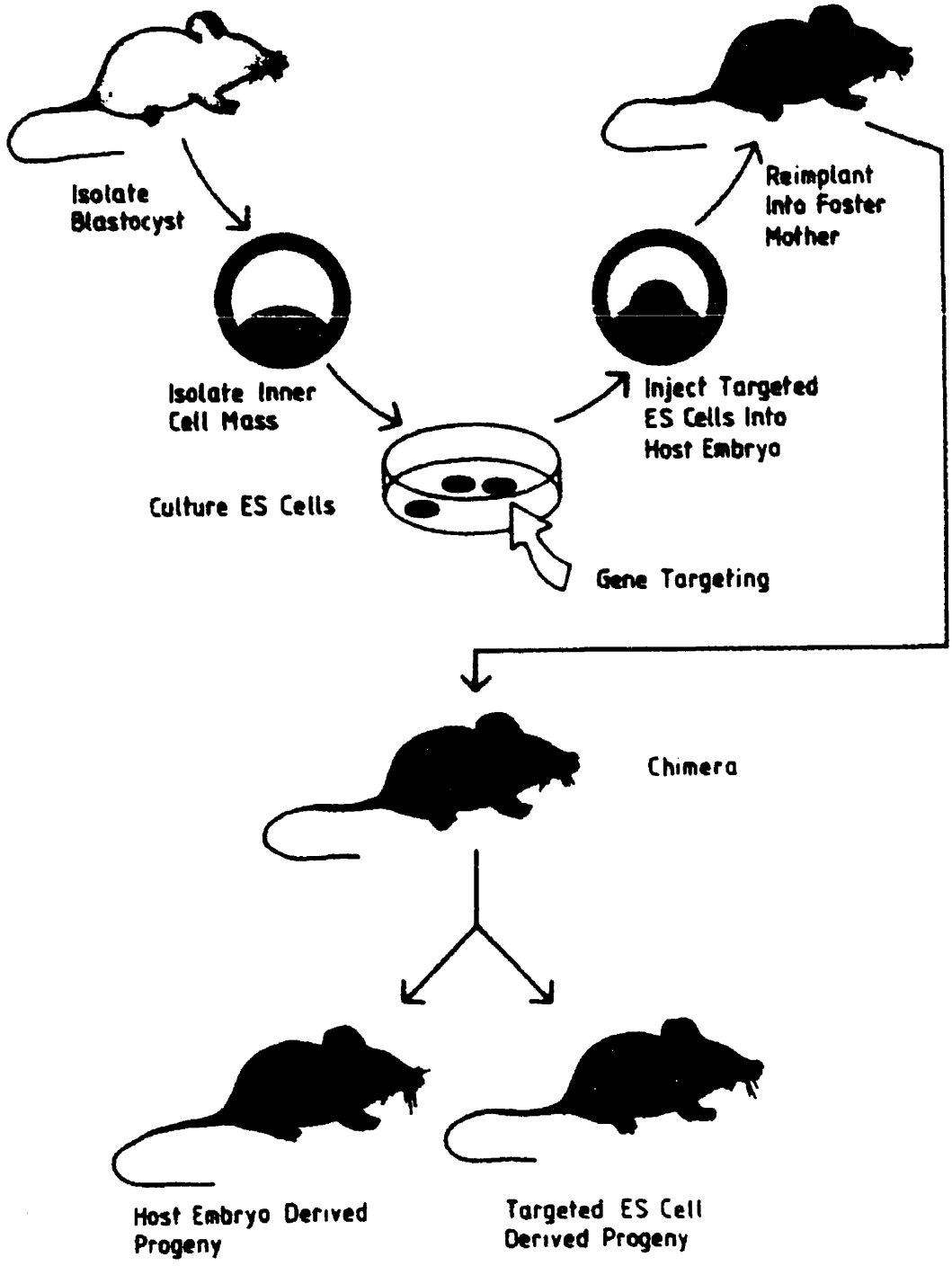


Figure 5. Generation of mutant mice by gene targeting. ES cells are isolated from the inner cell mass of a mouse blastocyst. These pluripotent cells are cultured in the presence of differentiation inhibiting factors while a targeting vector is introduced into the genome. Following selection, appropriatley targeted clones are introduced by microinjection into blastocysts, tranferred into the uterus of a pseudopregnant female, and development is allowed to progress to birth. Chimeric animals will transmit the mutation through the germline and eventually breed mice heterozygous and homozygous for the modified gene. (110)

However, since most mammalian cells are diploid, containing two copies of each gene or allele, both of them are needed to be inactivated to produce a discernible phenotypic change in a mutant.

Rather than reviewing the strategic approaches and the detailed protocols involved in gene targeting experiments, this chapter will focus more or less on the technology behind this technique. Topics such as embryonic stem cells and targeting vectors will be emphasized as well as a brief discussion on the relatively new targeting technique involving the Cre-loxP recombination system of bacteriophage P1.

Embryonic Stem Cells

ES cell lines are derived from pluripotent, uncommitted cells of the inner cell mass of preimplantation blastocysts. These cell lines are cultivated in the presence of differentiation inhibiting factors. In general, the most common genetic background of ES cell lines is 129Sv, a mouse strain which tends to develop spontaneous teratocarcinomas and is widely used for studies of early embryonic development (116). Overall, ES cells are preferred for gene targeting experiments for the three following reasons:

1. They can be screened in culture for rare homologous recombination events.
2. Homologous recombination with some vectors occurs in these cells at a high frequency.
3. The cells can be returned from in vitro culture to a host embryo where they become incorporated into the developing mouse.

The most important aspect, however, is the fact that mouse embryonic stem cells can give rise to cells of all tissues, including germ cells. But before more is said about

mouse ES cells, a little understanding of the basic developmental events that occur during the first nine days of mouse embryogenesis is needed.

The function of embryonic development is to produce from a single egg a three dimensional organism with a body plan that is predetermined by the genetic code. In short, the process of embryogenesis involves a progressive loss of the developmental potential of cells. In other words, when an egg is fertilized, it has the capacity to give rise to all cell types within the adult mouse and is said to be totipotent. But as the embryo cells divides and matures, this ability is slowly lost and the resultant cells retain only a restricted capacity to develop into certain tissues. Such a cell has been defined as one that has differentiated. Therefore it can be said that development involves many differentiation events that occur in series and in parallel.

Embryonic development begins with fertilization of the egg and the subsequent joining of the two haploid nuclei to form a diploid zygote. At this stage the egg is surrounded by the zona pellucida, a layer of mucopolysaccharides. Development first occurs within the first four days in the uterus and oviduct prior to implantation in the uterine wall, yet there is no growth of the embryo since no external source of nutrition is available. When implantation does occur, rapid growth is observed due to the direct contact with the mother's blood supply.

The first two days of development are characterized by the formation of an eight cell embryo known as a morula. Here, all the cells are totipotent and thus have an equivalent developmental potential. During the next 24 hours, more rounds of cell division are followed to produce an embryo with two layers of cells. At this stage, the outside of the embryo has differentiated to the trophectoderm while the interior remains

undifferentiated totipotent cells, which are known as the inner cell mass cells (ICM). Eventually, a fluid filled cavity or blastocoel is formed in the interior and the ICM ends up as a clump of cells attached to one end of the outer layer of the trophectoderm. Here, the 32 cell embryo is referred as a blastocyst. However, it is the ICM cells, that when transferred into culture, give rise to the embryonic stem cell lines.

From a practical point of view, ES cells differ from most somatic cells in two important ways. The first is that they are much smaller. This is an advantage, since from a tissue culture dish tenfold more cells can be recovered. The second difference, a disadvantage, is the ES cells have very specific growth requirements, such as a feeder layer or LIF, Leukemia Inhibitory Factor, and a rigid protocol of passaging the cells frequently. Careful culturing of the cells is crucial for the targeted cells to maintain their ability to contribute to the germline in ES cell chimeras. A chimera is an animal that is made up of cells from two different embryos. Such animals are made by mixing two morula stage embryos or by injecting ICM cells from one embryo into another host blastocyst. The embryos are then transferred into the uterus of a pseudopregnant mouse where they implant and continue embryonic development. The resulting chimeras are composed of a mixture of cells from both embryos.

To determine whether the germ cells are derived from either or both embryos, each embryo is marked with a genetic trait that is recognized later on in the chimera and the offspring. In these gene targeting experiments, the most common genetic marker is coat color. For example, if the ICM cells from a white mouse homozygous for a recessive mutation at the albino locus (c/c) are injected into the blastocyst of a black mouse homozygous for the wildtype albino gene (C/C), then any resulting chimeras are

identified by the patches of white and black fur. Then, to test whether the germlines of the chimeras are made up from cells from either or both genotypes, the chimeras are bred with a white mouse (c/c). Since black coat color is dominant, any white offspring must be homozygous (c/c) and have been derived from the injected ICM cells, whereas any black mice are heterozygous (C/c), having been derived from the host blastocyst. Both Figs. 6 and 7 depict the use of coat color as a genetic marker.

As stated earlier, ES cells must be grown in very strict conditions in order to maintain their ability to give rise to the germ cells. One of the most important requirements is to inhibit differentiation and promote proliferation as well as retention of a diploid number of chromosomes. To date, there are a number of different factors that retain the pluripotency of ES cells. The first factor is defined as a requirement for the proliferation of ES cells and it involves the plating of a layer of fibroblast cells under the ES cells. Before plating, these fibroblast cells are treated either through gamma-irradiation or with drugs such as mitomycin, in order to inhibit further cell division (mitotically inactive) but not metabolism. The importance of fibroblast cells resides in their production and secretion of a number of proteins, some of which are essential for ES cell proliferation, hence the nickname FEEDER cells. When these cells are not present, the ES cells tend to divide only a few times while differentiating into cell types typical of an early embryo (such as a primitive endoderm), where further division is inhibited. Recently, a protein factor called the Differentiation Inhibiting Factor (DIF) or Leukemia Inhibiting Factor (LIF) has been discovered (127,132), which allows for ES cell proliferation in the absence of feeder cells. Although growing ES cells in LIF is much

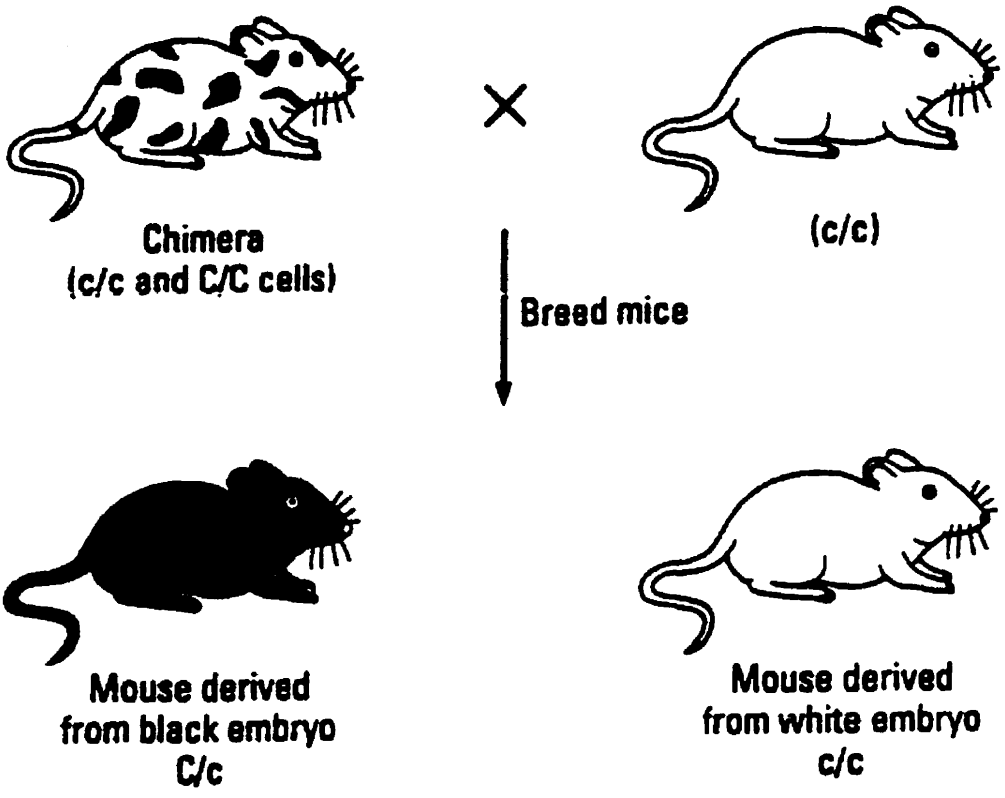


Figure 6. Breeding scheme for determining the genotypes of the cells that have populated the germline. The chimera is black and white, indicating that the genotypes of the two embryos used to form the chimera were homozygous wild type for albino (C/C), giving a black coat color phenotype, and homozygous mutant for albino (c/c), giving a white coat color. The wild type allele is dominant, and thus the offspring of a chimera bred with a white (c/c) mouse can either be black and heterozygous for albino (C/c) or white and homozygous mutant for albino (c/c). The C/c mice are derived from the C/C morula and the c/c mice from the c/c morula. (113)

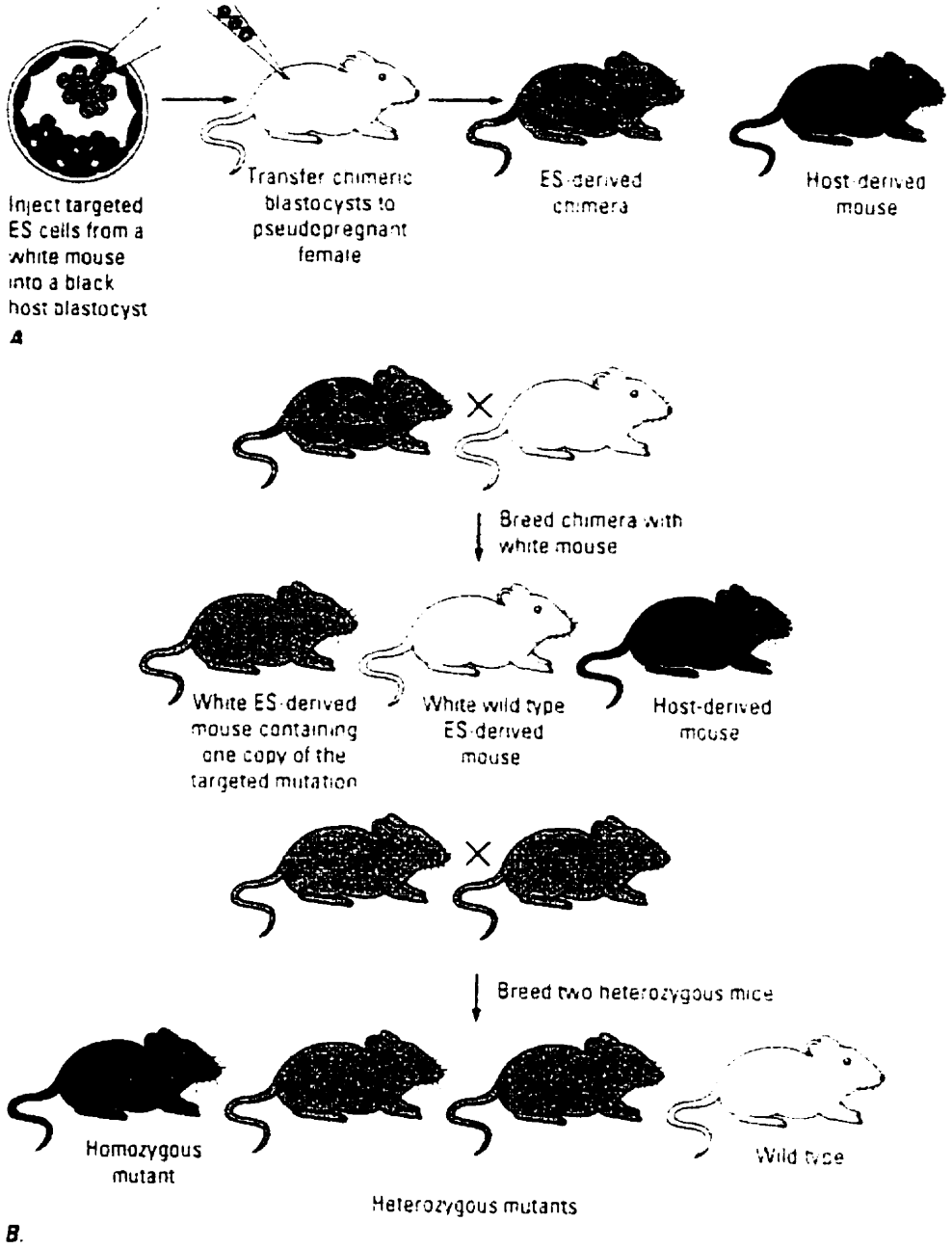


Figure 7. Strategy for obtaining mice homozygous for a targeted mutation from ES cells heterozygous for the mutation.

A. Production of ES cell derived chimeras. The targeted ES cells and their derivatives in mice are shown in light grey and the host blastocyst derived cells in black.

B. The male chimeras are bred to produce mice derived from the targeted ES cells (light grey and white). The ES cell derived mice are analyzed to determine which contain one copy of the mutation (light grey). The mice that are heterozygous for the mutation are bred, and one quarter of their offspring will be homozygous for the mutation (dark grey). (113)

simpler than preparing feeder cells, it has been found that unlike feeder cells, LIF does not completely satisfy all the growth requirements of ES cells (110,113,116).

The second factor influencing the pluripotency of ES cells, is the length of time in which they are grown in culture. With most ES cell lines, there exists an inverse correlation between the number of times the cells are passaged and the percentage of chimeras obtained that have an ES cell contribution to the germline. This is due to abnormal variants that accumulate in the cell population. Therefore, to minimize the number of times the ES cells are passaged, when newly established cell lines are obtained, they are expanded immediately to produce a large number of cells and then small aliquots are frozen down for future use.

And lastly, the third factor influencing the pluripotency of ES cells is the way in which these cells are handled on a day to day basis by the investigator. For example, ES cell cultures require the exclusive use of high quality reagents dissolved in ultra pure water. In addition, disposable pipettes are preferred over glass and if glass materials are to be used, they must be extensively rinsed because ES cells are extremely sensitive to traces of residual detergents.

A few other areas to be mentioned on ES cells is the fact that ES cell lines derived from male embryos are more often used. The reason for this is due to the karyotype of male cell lines being more stable (the XY complement), than those of female cell lines, (the XX complement), where one X chromosome is sometimes lost. Another reason for using the male embryos is due to the production of a greater number of germ line chimeras or rather, those with an ES cell contribution to the germline. Finally, it is more

efficient to breed males over females since they can be bred with numerous females during the period of time a female requires to produce a single litter (110).

The present method for germline gene targeting is making the directed gene mutation in ES cells and then transmitting the mutation into mice by making germline ES cell chimeras. The mutation is made in ES cells by introducing a gene targeting vector into ES cells and allowing the cells to undergo a homologous recombination event that replaces the normal endogenous gene with the introduced mutant copy.

The Targeting Vector

As would be expected, the first step in designing a targeting construct is the availability of a genomic clone containing the gene(s) of interest. If the exons which encode regions of a protein are defined, inactivation of the molecule can be achieved without further knowledge of the regulatory sequences controlling transcription of the gene(s) encoding the molecule. With the genomic DNA in hand, gene targeting is achieved by making the mutation in ES cells and then transmitting the mutation into mice through the generation of ES cell chimeras. The way in which the mutation is made, is by introducing a gene targeting vector into ES cells and allowing the cells to undergo homologous recombination, which replaces the normal endogenous gene with the introduced mutant copy.

There are a number of factors to consider when designing a homologous recombination experiment, the first being whether the gene to be targeted is expressed in ES cells. This in turn will dictate the type of targeting vector that is needed. In general, there are two types of vectors that are used for ES cell targeting, replacement vectors and insertion vectors (Fig.8). Each can be used for different purposes in specific situations. The

insertion construct contains a region of homology to the target gene cloned as a single continuous sequence and is linearized by cleavage of a unique restriction site within the region of homology. Through homologous recombination, the insertion construct is introduced into the homologous site of the target gene where interruption of the normal target gene structure is achieved by the addition of exogenous DNA sequences. In general, insertion vectors achieve higher targeting efficiency however (116,117,121), due to the duplication of target homology (through intrachromosomal recombination, which can regenerate the normal gene from the mutated targeted gene), some expression of the targeted gene has been observed as a result of aberrant transcription and / or translation.

The other construct, replacement construct, has two regions of homology to the target gene, which is located on either side of a mutation (usually a positive selectable marker such as **neo**^{r}). Here, homologous recombination proceeds through a double crossover event that replaces the target gene sequences with the replacement construct sequences. In other words, the deleted portion of a particular gene is replaced with a selectable marker (the neo^r). This type of construct is used more often since the normal gene cannot be regenerated hence there is no possibility of duplication of sequences (Fig.9). For a better understanding of sequence replacement vectors, Fig.10 shows the pPNT replacement vector containing both the neomycin resistance gene and the hsv-tk gene, as well as the unique restriction enzyme sites that flank either side of the neomycin resistance gene, that are used to clone genomic DNA fragments into the vector.

Factors Influencing Homologous Recombination Efficiency

There are a number of requirements that must be considered when designing a

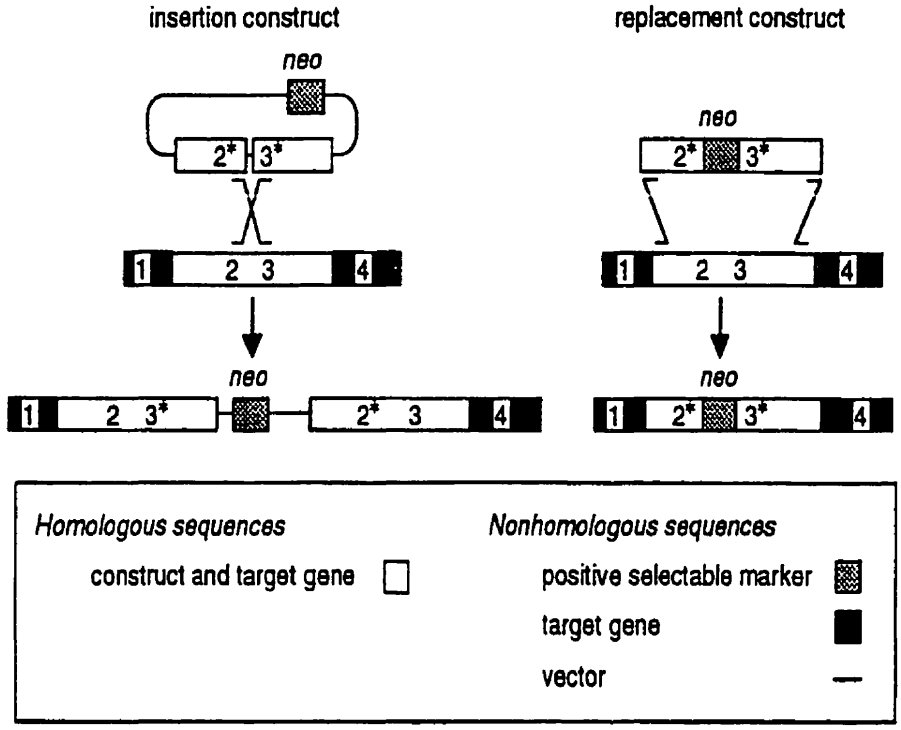


Figure 8. Two configurations of constructs used for homologous recombination . Numbers indicate target-gene sequences in the genome. An asterisk indicates homologous target-gene sequences in the constructs. Replacement constructs substitute their sequences (2*, neo, and 3*) for the endogenous target-gene sequences (2 and 3). Insertion constructs add their sequences (2*, neo, and 3*) to the endogenous target-gene, resulting in tandem duplication and disruption of the normal gene structure. (167)

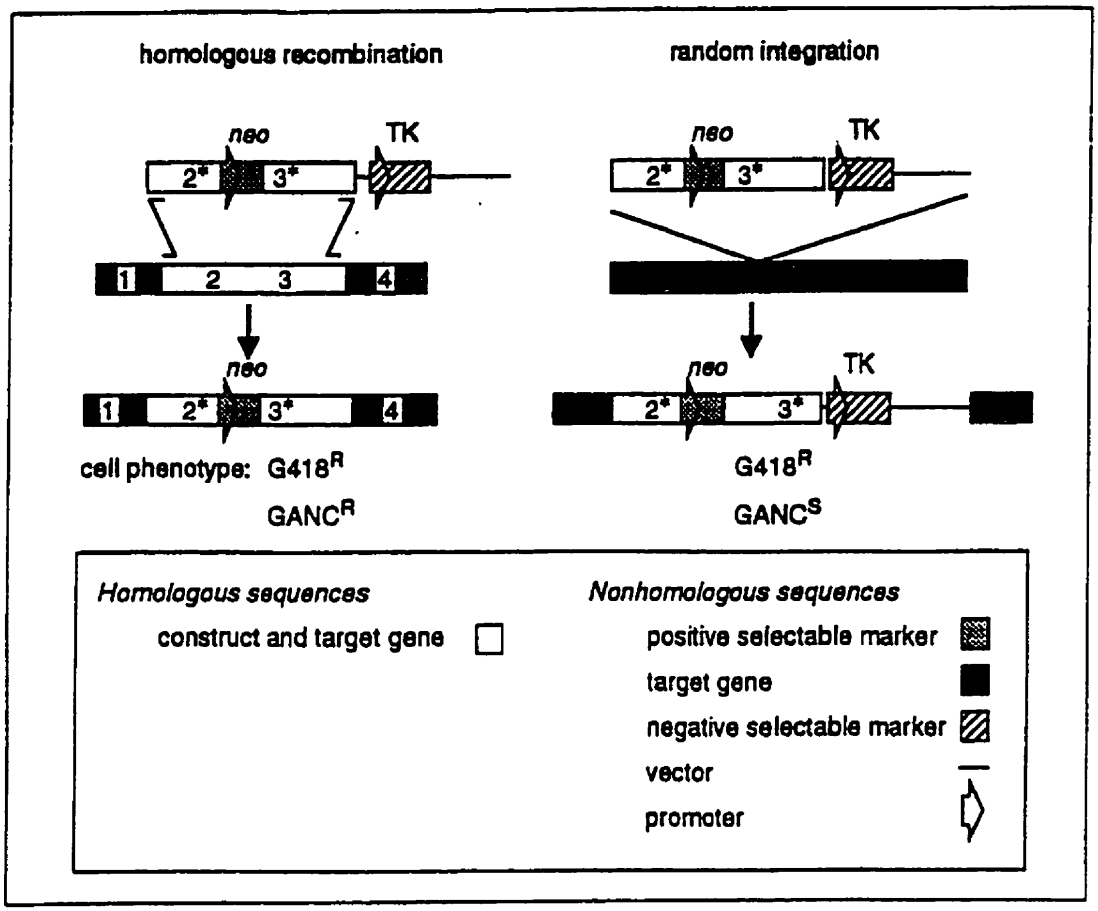
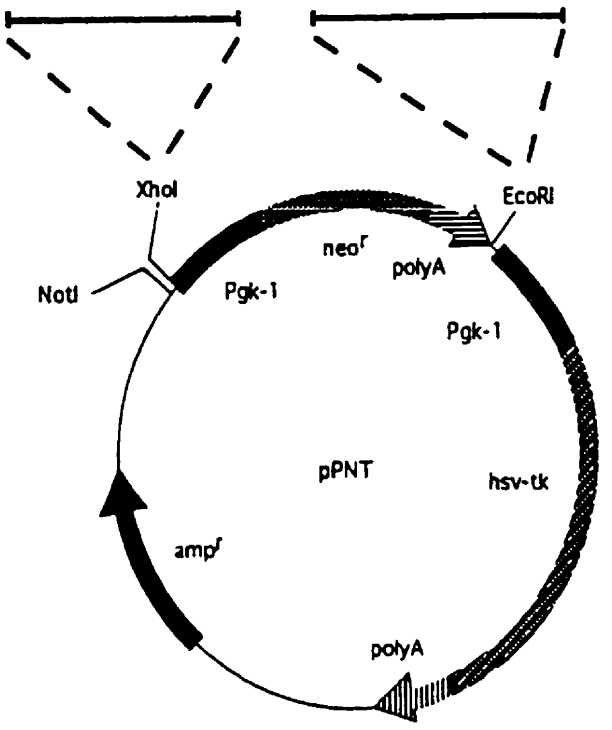


Figure 9. Enrichment for homologous recombinants by positive-negative selection using the TK gene. Homologous recombination involving cross-overs on either side of the *neo* gene results in the loss of the TK gene. Random integration tends to preserve the TK gene. The presence of the TK gene can be selected against because any cell expressing the gene will be killed by gancyclovir (GANC). Although both homologous recombinants and clones in which the construct integrated randomly are G418 resistant, only homologous recombinants are gancyclovir resistant. The construct is shown linearized so that the plasmid vector sequences remain attached to the TK gene. This configuration helps to preserve the integrity of the TK gene. The superscript R denotes resistance and the superscript S denotes sensitivity. (167)



Replacement Vector

Figure 10. Schematic representation of the pPNT replacement vector. Ampicillin resistance (amp^r); Phosphoglycerate kinase-1 (Pgk-1) gene promoter and poly A addition sequences. (110)

targeting vector in order to obtain the highest recombination frequency. One of them is the type of genomic clones used to flank either side of the replacement construct. In general, the DNA used should be isolated from a library that is isogenic with the embryonic stem cells used in the targeting experiment. In this way one helps to increase the homologous recombination frequency through the absence of mismatches between the vector and targeted sequences as well as by minimizing polymorphisms between the vector and targeted locus. These events are usually due to the divergent nature of intronic sequences between mouse strains even through the exons are highly homologous. Therefore such a trivial factor as using the same strain for construction of the targeting vector and ES cells can significantly improve the frequency of homologous recombination (106,111). For example, in one study it was demonstrated that the use of isogenic DNA (129Sv-derived) versus non-isogenic DNA (BALB/c-derived) resulted in a 20 fold increase in the targeting frequency when 129 ES cells were used (106).

Another factor to take into account is the length of homologous flanking sequences. By increasing the length of homology, one increases the recombination frequency. Although it has been shown that the length of individual flanking regions seems to be unimportant (114), in general, the best approach would be to include genomic clones containing 3-5 kb of genomic sequences homologous to the targeted gene on either side of the intended region of deletion (Fig. 11). Anything longer would probably increase the recombination frequency, but for handling purposes, this size of DNA would be much too fragile to use (110,111,121,134).

In addition, studies have shown that the length of DNA which is replaced by the selectable marker is also of minor importance (131). Lastly, one should choose the exons

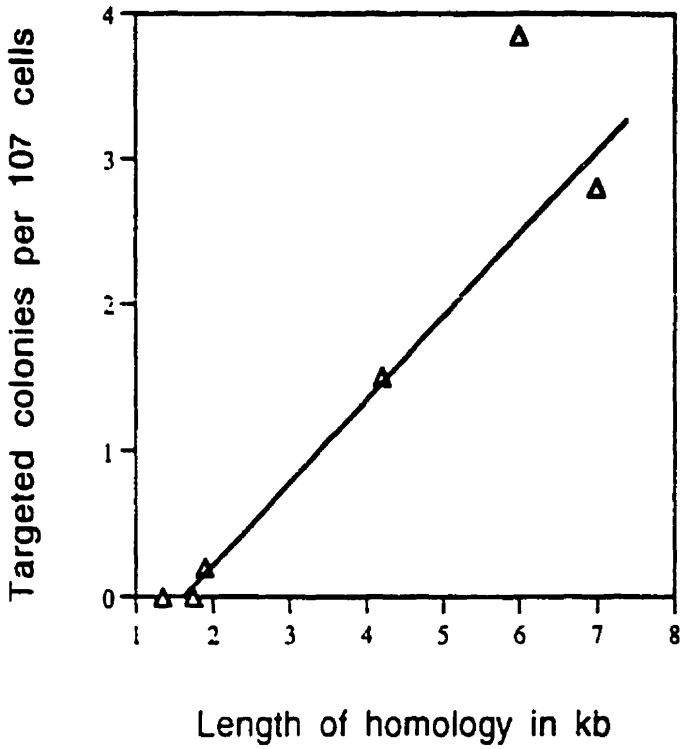


Figure 11. Relationship between the length of homologous sequences and targeting frequency. (110)

to be replaced so as to include mature protein sequences, thereby ensuring the complete functional disruption of the gene.

Enrichment of Yield

There are a variety of strategies that have been used to increase the number of clones positive for homologous recombination above the background of clones surviving selection due to non-homologous insertion (random integration) of the selectable marker (usually neomycin resistance) into the genome. Nearly all constructs used for homologous recombination rely on the positive selection of drug resistance, for example, neomycin resistance, neo^r. The neo^r gene encodes for the protein aminoglycoside phosphotransferase which confers resistance to the antibiotic Geneticin, G418. Use of these positive selection markers serves two functions. The first is to isolate those ES cells that have successfully integrated the transfected DNA. And second, they are used to disrupt and mutate the target gene through either insertion into the coding region (insertion vector) or replacing coding sequences (replacement vector). Therefore, by using G418 in the ES cell selection, one eliminates a great majority of cells that have not incorporated the construct.

However, a number of cells do survive this selection process that have not undergone homologous recombination. In some cells, random integration may have taken place thereby incorporating the drug resistance gene, but in an incorrect region.

Therefore, methods have been developed to enrich for homologous recombination, where the most widely used one uses a second negative selection step which was developed by Mansour and colleagues (123). This method, in combination with the positive selectable marker is known as positive-negative selection.

In this situation, aside from the positive selectable marker, these constructs contain a negative selectable marker such as the Herpes Simplex Virus Thymidine Kinase (HSV-TK), which is incorporated outside the region of homology to the target gene. All cells that have the TK gene are in turn sensitive to acyclovir and all its analogs (gancyclovir, GANC) as well as FIAU. In itself, expression of the protein is not toxic to mammalian cells, however, with the addition of gancyclovir or FIAU, the protein which is encoded by the HSV-TK gene will activate these drugs which then cause their incorporation into the growing DNA thereby resulting in chain termination and cell death (they interfere with DNA metabolism). Therefore, when homologous recombination takes place, the HSV-TK gene which resides outside the regions of homology is lost due to the crossover event. But when random integration occurs, this is not the case, rather, the gene is incorporated into the genome (Fig.12). And when one screens with FIAU or gancyclovir, those cells having undergone random integration will die.

In summary, when screening with G418, one isolates those cells having successfully taken up the transfected piece of DNA whereas with the addition of FIAU or gancyclovir, one isolates those cells having undergone homologous recombination rather than random integration (Fig.13). The positive-negative selection protocol normally results in 1-15% doubly resistant clones containing the appropriately targeted allele (110).

Transfection

The methods for introducing the targeting construct into the ES cells range from calcium phosphate DNA co-precipitation to retrovirus infection (129). Today, however,

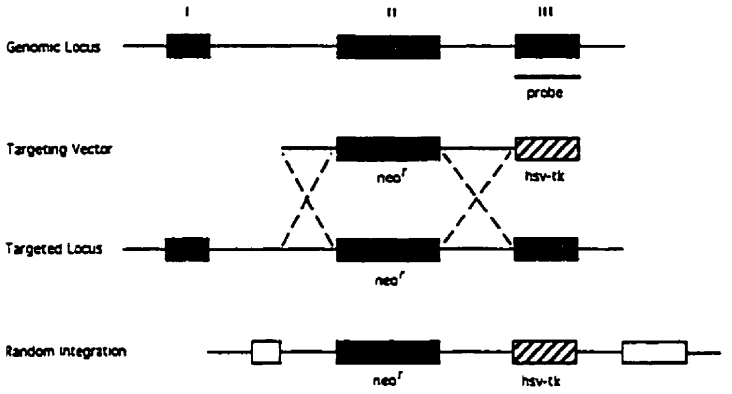


Figure 12. Gene disruption by sequence replacement vector in ES cells. A targeting construct is generated by cloning genomic DNA sequences flanking the gene section to be disrupted into the plasmid vector. Gene cassettes for neomycin resistance (*neo*^{r}) and herpes simplex thymidine kinase (*hsv-tk*) are included for positive-negative selection to enrich ES cells containing the targeted event. Homologous recombination (---) results in the linearized vector being inserted into the wild-type gene, resulting in the replacement of exonic sequences by the selectable marker (*neo*^{r}) and loss of *hsv-tk* sequences. In contrast, a nonhomologous insertion occurs through the ends of the linearized DNA and will include the *hsv-tk* gene (110).

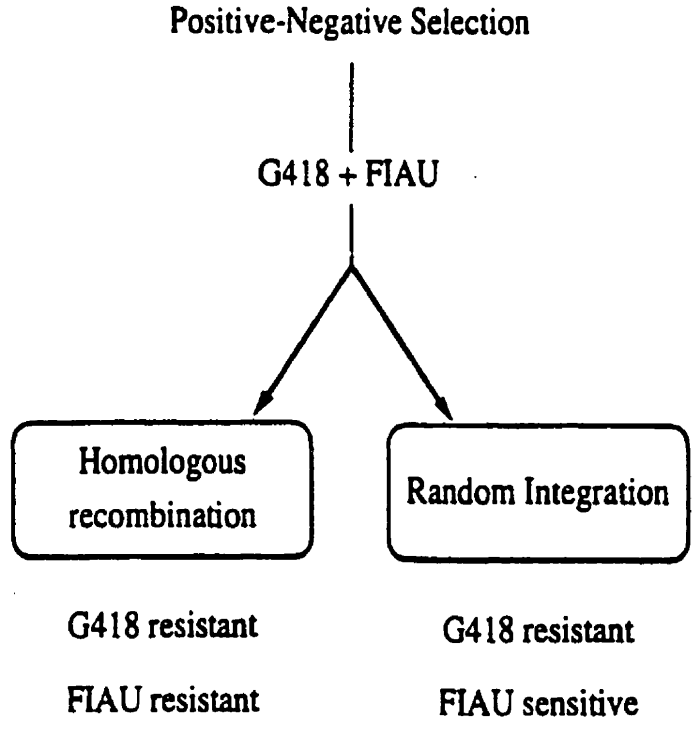


Figure 13. Positive-negative selection to enrich for targeted ES cells. Targeted clones will be neo^r positive but hsv-tk negative and will be resistant to both G418 and FIAU. In nonhomologous integration, clones will be neo^r and hsv-tk positive and therefore resistant to G418 but sensitive to FIAU. (110)

the method of choice is electroporation since it has been found to be both reliable and reproducible (134). A final requirement for the targeting vector is a unique restriction enzyme site to linearize before electroporation. An eight cutter restriction endonuclease site, such as NotI, is usually included during construction of the vector outside of the homologous sequences. The reason for the linearization requirement is to improve the recombination frequency. Studies have shown that recombination occurs at a much higher frequency when the exogenous piece of DNA is linearized as compared to when it is circular (126).

Screening

At this stage in the experiment, appropriate screening methods are required to differentiate between background random integration and the actual gene targeting event. Two methods, PCR and Southern blotting are commonly used to detect the replacement of the targeted gene.

When using PCR for the detection of positive clones, unlike Southern blotting, one can work with small amounts of low quality DNA and screen many colonies in a relatively short amount of time. The usual strategy requires one primer in the genomic DNA outside the targeting construct and one primer in the heterologous marker (eg., the neo^r gene) DNA. Here, amplification is not dependent on gene expression rather, a unique product is obtained if homologous recombination has occurred (133). However, studies have shown that reproducible results are sometimes difficult to obtain from a PCR consisting of only one set of primers when amplifying from small amounts of template DNA (128).

Southern Blotting on the other hand may be the alternative to PCR when long flanking regions to the neomycin resistance gene are present, (which decrease the efficiency of PCR amplification) or when there are repetitive sequences at the desired position of the primer. Although Southern Blotting is much more labor intensive, time consuming as well as requiring higher quality DNA as compared to PCR, it will produce clear and reproducible results. This, however, is dependent upon the presence of certain restriction enzyme(s) sites in the targeting construct, that will give a different product size if homologous recombination has taken place compared to genomic DNA cut with the same enzyme(s). In addition to the correct band size, another indication of successful homologous recombination is through examination of the intensity of the endogenous gene product after probe hybridization since it should be similar to that of the targeted gene, each representing the gene on one of the two chromosomes (Fig.14).

Once successfully targeted cells have been isolated, the next step is the production of chimeric mice through DNA microinjection. And once the correct chimeric mice are obtained, they are interbred to obtain mice homozygous for the mutation. It is at this point that studies can begin to determine the function of the gene in question.

Problems with Gene Targeting

For most studies, replacement vectors are used in the targeting experiments. However, as with all techniques there are shortcomings. With this method, large sequences of heterologous DNA (marker cassettes; ex: neo^r) that have functional promoter and enhancer sequences remain in the genome. Although this is of no consequence to the

generation of the null allele, the presence of transcriptionally active foreign marker DNA makes it simply impossible to study the effect of subtle mutations on genes and promoter / enhancer elements in situ. Where such studies are desired, a strategy must be devised whereby marker cassettes inserted into the target gene are integrated into the target sequence then removed subsequently leaving a subtle but sufficient mutation. Such " hit and run " experiments have been attempted (126), but with no success due to the intrinsic problem of a low frequency of successful recombination events.

A second limitation of the knockout technique is the difficulty of studying genes which are essential for murine development and therefore lethal if disrupted. However, this problem has recently been overcome through the development of a particular system in which such genes can be inactivated in a tissue or cell-type specific manner. This method is based on the Cre-LoxP recombination system of bacteriophage P1 (135). In short, Cre-recombinase is an enzyme which catalyzes the site specific recombination between 34 base pair motifs termed loxP sites. To use this in eukaryotic systems, a targeting vector is created by introducing three loxP sites to flank the gene segment of interest and the selectable marker gene cassettes. ES cells are screened for positive clones with use of the gene marker cassettes and are then transfected transiently with the gene encoding the Cre-recombinase enzyme, which has specificity for the introduced loxP sites. Three types of mutations result. The first has both the selectable markers and gene segment removed following recombination. The second, has the desired outcome, where the markers are deleted leaving the gene sequences flanked by two loxP sites. The third outcome which is lethal under selection pressure, has deletion of the gene segment as well as one selection marker.

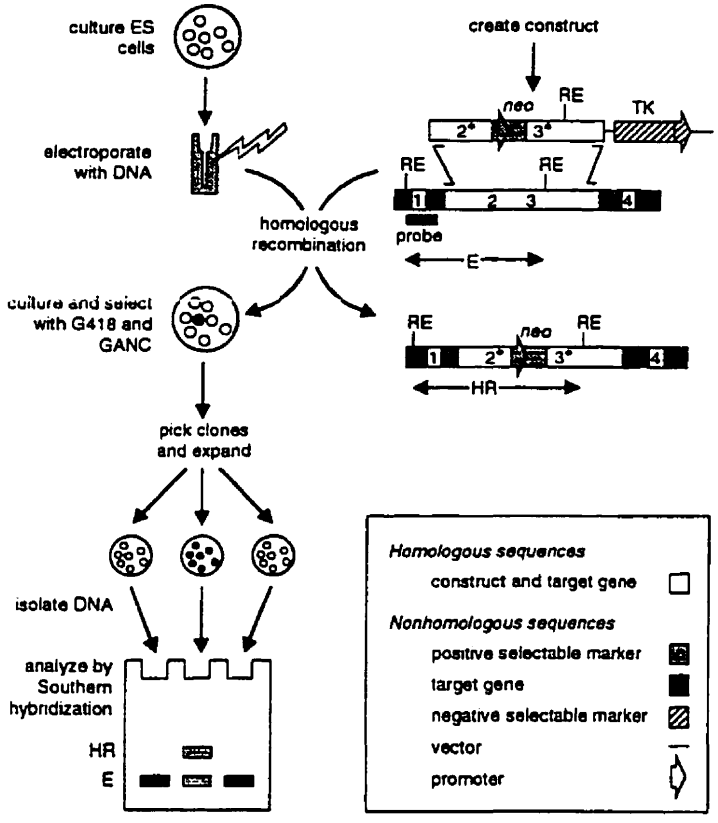


Figure 14. Production, selection and identification of targeted gene disruption by homologous recombination. An example of a restriction enzyme (RE) and hybridization probe that can be used to identify cells in which homologous recombination has occurred (shaded colony) is shown. The predicted size of the restriction fragment generated from an unaltered target gene (E) and a target gene that has undergone homologous recombination (HR) is shown. If equal amounts of DNA are present in the lanes of the Southern blot, the intensity of each of the two hybridizing fragments from the DNA of a homologous recombinant clone will be half of the intensity of the hybridizing fragment from unaltered clones. (167)

ES cells having the second outcome, are then used to generate chimeric mice carrying the modification which are bred until a homozygous offspring is produced. What is important to remember is that these mice still carry a functional gene. Therefore, in order to delete this particular gene, transgenic mice must be generated that carry the Cre-recombinase gene under a tissue specific promoter. These mice are then bred with the mice carrying the targeted gene flanked by the loxP sites. The result is viable progeny, which lack the gene of interest in the specific cell types where tissue specific promoter driven production of the Cre-recombinase is occurring.

Conclusion

To date, a number of gene knock-outs have been done. Over 50 have been produced which give significant insight into a number of immunological processes. Using either the sequence replacement vector or the sophisticated Cre-loxP method, one can now explore the function of genes through the absence of products for which they encode. There is little doubt that future knock-out mice will continue to offer more insights into the function and regulation of complex biological systems, which in turn will provide invaluable information into a number of human biological processes.

Part II

Experimental Section

Materials and Methods

Restriction Enzyme Reactions

All restriction enzyme reactions were performed under the conditions suggested by the supplier (Gibco/BRL or Pharmacia), using the appropriate reaction buffer. All reactions were incubated at the optimal temperature for a minimum of one hour in a total volume of 15 - 20µl.

Agarose Gel Electrophoresis

DNA was analyzed by agarose gel electrophoresis (162). DNA loading buffer (10X stock) was added to all DNA samples for a final concentration of 1X. Samples were loaded into 0.7-0.8% agarose gels and electrophoresed in 1X TBE Running Buffer at 70-100 volts, until the bromophenol blue dye ran off of the gel. All gels were stained by adding ethidium bromide at a concentration of 0.5µg/ml. Following electrophoresis, the gels were examined using a transilluminator and photographed with a Polaroid Camera using Polaroid type 57 instant sheet film.

Gel Band Purification

To purify particular fragments of DNA from an ethidium bromide stained agarose gel, the GENECLEAN kit II (BIO/101) was used. Following restriction digestion and electrophoresis, a piece of agarose gel containing the fragment of interest was excised with a scalpel and transferred to a sterile microfuge tube. A volume (4.5:1) of NaI stock solution was added followed by 0.5 volumes of TBE modifier. To dissociate the agarose

fragment, it was placed in a 55°C water bath for 5 minutes. Upon dissociation, 5µl of glassmilk (for DNA of 5µg or less) was added to the microfuge tube, mixed and placed on ice for a minimum of 5 minutes to allow the DNA to bind to the silica matrix. The microfuge was then centrifuged at 14 000rpm for 30 seconds to pellet the matrix with the bound DNA. The supernatant was removed and the pellet was washed twice (resuspended) with 50 volumes of ice cold New Wash (provided with the kit). Resuspension was achieved by pipetting back and forth while digging into the pellet with the pipette tip. After each wash, the microfuge tube was centrifuged at 14 000rpm for 5 seconds to pellet the matrix. The supernatant was removed and fresh New Wash is added. After all the New Wash was removed in the second wash, 15µl of TE buffer was added to elute the DNA from the matrix. After adding the TE buffer, the microfuge tube was placed at 55°C for 3 minutes, then pelleted by centrifugation for 3 minutes at 14 000rpm. The supernatant was then carefully removed and placed in a fresh microfuge tube. The eluted DNA could be used immediately for restriction digestion or other manipulations.

Southern Blot Analysis

Digested DNA samples were electrophoresed for 6-12 hours in a 0.8% agarose gel. After the picture was taken, the top left corner of the gel was cut in order to orient the gel during succeeding operations. The gel was then placed in 100 ml of a 0.25N HCl solution with constant, gentle agitation (rotary platform) for 10 minutes in order to depurinate the gel. The gel was then denatured in 100ml of a 0.5N NaOH/1.5M NaCl

solution and then neutralized in 100ml of a 1.0M TrisHCl pH8.0/1.5M NaCl solution, each time with gentle agitation for 2X15 minutes.

DNA was capillary transfered onto a supported nitrocellulose membrane (Hybond-C 0.2micron/Amersham). 3MM paper was presoaked for 2 minutes in 20XSSC and spread across the plastic support of the transfer apparatus, with no air bubbles present. Following the neutralizing step, the gel was placed upside down on the presoaked 3MM paper, with the edges of the gel surrounded by Saran Wrap. The nitrocellulose membrane (cut to the size of the gel) was pre-soaked in 20XSSC for 10 minutes and then placed on top of the gel. Air bubbles were removed by rolling a plastic pipette over the membrane a few times. Two sheets of 3MM paper, cut to the size of the gel and pre-wet in 20X SSC for 2 minutes, were placed directly on top of the nitrocellulose membrane. A stack of absorbent towels was then placed on top of the 3MM paper with a 0.5kg weight and left overnight. The next day, the blot apparatus was disassembled and the nitrocellulose membrane was placed in between two dry sheets of 3MM paper and baked at 80°C for 2.0 hours.

Hybridization was performed by placing the nitrocellulose membrane in 10ml hybridization buffer (1%BSA, 7% SDS, 0.5M Sodium Phosphate pH6.8 and 1mM 0.5M EDTA pH8.0), at the required temperature (54-59°C) and placed in a hybridization incubator (Robbins Scientific/model two). The following day, labelled probe was denatured by insertion in boiling water for 2 minutes, placed on ice for two minutes and then added to the prewarmed hybridization solution at a concentration of $1X10^6$ cpm/ml.

The membrane in the hybridization bottle (Tek*Star) was then returned to the incubator at the required temperature and left overnight.

Membranes were washed with wash A (0.5% BSA, 5% SDS, 40mM $NaPO_4$/pH6.8 and 1mM EDTA/pH8) 4X15 minutes and then with wash B (1% SDS, 40mM $NaPO_4$/pH6.8 and 1mM EDTA/pH8) for 2X15 minutes at the specified temperature. The membranes were then removed and vacuum sealed in a plastic bag (Seal-a-Meal Pouches). Autoradiography was performed by packing the wrapped membrane into an autoradiography cassette (Fisher Biotech/FBAC 1417) with two intensifying screens and a sheet of Kodak Scientific Imaging Film (Eastman Kodak Company). The cassette was left to expose overnight at 80°C. The film was developed using a Kodak RP X-OMAT developer.

Isolation and Radiolabelling of cDNA probe for CgA

To screen for the 3' end of the CgA gene, a probe was generated by restriction digestion of a pcDNA3.1(+) (Invitrogen) expression vector having the mouse CgA cDNA subcloned within the EcoRV restriction site of the vector. The mouse CgA cDNA was amplified from ATT-20 cells, a mouse pituitary cell line. The mouse CgA clone was digested with the restriction enzymes NcoI and XhoI. The NcoI restriction site is a unique site in the mCgA clone, found 117 nucleotide bases downstream from exon 7. The XhoI site is also a unique restriction site found in the multiple cloning site of the expression vector, 23 nucleotide bases downstream from the EcoRV restriction site. Upon digestion, a number of restriction fragments should be obtained including one of 480 nucleotide bases long. This fragment would contain part of exon 7, and part of exon

8 in addition to 23 nucleotides of the expression vector. Following digestion, the sample was resolved by electrophoresis and the 480 base fragment was purified using the Geneclean II kit (BIO/101).

In order to have a sufficient quantity of the cDNA probe, the 480 base fragment was gel purified and subcloned into the NcoI and SalI (SalI and XhoI have compatible cohesive ends) sites of the pGEM-T Vector (Invitrogen). Following the transformation and mini-prep screenings, a positive clone was grown in a 600ml culture and purified using the Qiagen Maxi-Prep kit.

Purified cDNA fragments were randomly labelled with α-^{32}P-dCTP using the Ready-to-Go Reaction Labelling Beads (Pharmacia). 25μg of the cDNA fragment was added to sterile water in a total volume of 45μl, boiled for 2 minutes (to denature the DNA) and then placed on ice for 2 minutes. To the mixture, 5μl of α-^{32}P-dCTP (50μCi/ICN) was added followed by a 15 minute incubation at 37°C. Following the incubation, unincorporated radioactivity was removed by passing the reaction mixture through a Ready-to-GO spin column. Prior to use, the spin column was centrifuged for 1 minute at 3000rpm to remove the buffer within the column. The reaction mixture was then added and spun again at 3000rpm for 2minutes. The flowthrough was recovered, and 1μl was removed for scintillation counting (1219 RACKBETA Liquid Scintillation Counter/LKB Wallac) after the addition of 10ml of Beta Max Liquid Scintillation Cocktail (ICN). Labelled probes were used immediately at a concentration of no less than $1x10^6$ cpm/ml.

Titering of Genomic Library

The mouse genomic library, which was a gift from Dr. Janet Rossant (University of Toronto), was prepared from a Sau3A partial digest of strain 120Sv female kidney DNA, which was size selected on a sucrose gradient and inserted into the BamHI site of the Lambda Dash II (Stratagene) vector. Phage were propagated in E.Coli LE392 which were grown overnight in 30ml Luria Broth containing 0.2% maltose and 0.7% $MgSO_4$. Growth in maltose induces production in E.Coli of the λ receptor (lamB protein), which is necessary for maltose transport. Mg^{++} ions also aid in phage adsorbtion. The bacterial cells were collected by centrifugation in a Sorvall RC5C centrifuge at 4000rpm for 15 minutes and then resuspended in 10mM $MgSO_4$ until the OD_{600} was between 0.7-1.0. To determine the titre, serial dilutions of the library were prepared in SM buffer, in a total volume of 100µl. 10µl of each dilution was added to 600µl of bacteria respectively, and then incubated at 37°C for 20 minutes with slight shaking at 50rpm. The infected cultures were then plated by adding 8ml of NZY top agar (allowed to cool to 48°C before plating) and poured onto NZY plates pre-warmed in a 37°C incubator for 1-2 hours. The top agar was allowed to solidify at room temperature and then the plates were placed inverted in a 37°C incubator overnight. The number of plaque forming units (pfu) per µl was determined by multiplying the number of plaques on the plate by the corresponding dilution factor and dividing by the plating factor (which represents the volume of phage used in the serial dilution).

Plaque Hybridization for the Mouse CgA Gene

In order to increase the frequency of homologous recombination, isolation of the isogenic CgA gene was required (106,111). The library was screened according to the method of Sambrook et al, 1989 (162). By using the isogenic DNA, was to increase the frequency of homologous recombination. Just as for titering, the library was plated on large NZY plates (150mm). The library titre was calculated to be $5.5x10^4$ pfu/μl. Therefore, 14μl of the library were added to SM buffer for a total volume of 250μl so that each plate would have approximately 30000 plaques. To 600μl of bacteria, 10μl of the diluted library was added and incubated at 37°C for 20 minutes. 8ml of melted NZY top agar was then added and poured onto the pre-warmed plates, for a total of 25 plates. The plates were incubated overnight (12-16 hours) at 37°C and then placed at 4°C the following day for 1 hour.

After the plates had cooled, each plate was marked for orientation by using a sterile pipette tip and removing 5 random pieces of agar. The pipette was placed directly through the agar and a small piece was sucked up. Circular nitrocellulose membranes (137mm, pore size 0.45 μm/Amersham) were placed on top of the agarose surface of each plate. This was done with five plates at a time. Each membrane was marked both with pencil and permanent marker on the orientation holes and left on the plates for 7 and 15 minutes respectively (primary and secondary lifts). With the aid of forceps, membranes were removed and placed agarose-exposed-side up in denaturing solution (0.5N NaOH/1.5M NaCl) for 10 minutes. The membranes were then placed in neutralizing solution (1.0M TrisHCl pH8.0/1.5M NaCl) for 5 minutes and then in 4xSET

washing solution for another 5 minutes. Membranes were allowed to air dry at room temperature for 1 hour and then were baked at 80°C for 2 hours.

Plaque hybridization was done by pre-wetting the membranes in warm (59°C) hybridization solution (1% BSA, 7% SDS, 0.5M $NaPO_4$/pH6.8, 1mM EDTA). This was done by laying each membrane, phage side down, in a circular plastic container and adding enough pre-warmed hybridization solution to cover the membrane entirely. This was repeated for all membranes that were to be hybridized.

The genomic library was screened by standard methods using a mouse CgA synthetic (36-mer) oligonucleotide (5'-AGTGTCCCCTTTTGTCATAGGGCTGTTCACAGGAAG-3') complementary to the rat CgA mRNA sequence encoding amino acids +1 - +12 (14). The oligonucleotide was 5'-endlabelled using [^{32}P] adenosine-5-triphosphate and T4 polynucleotide kinase. Labelled probe was denatured by boiling for 2 minutes, placed on ice for 2 minutes and then added to the plastic container containing the membranes and the hybridization solution to a concentration of about $1X10^6$ cpm/ml. The hybridization volume depended upon the number of membranes that were being screened. The hybridization period was between 18-26 hours.

Membranes were washed with wash A (0.5% BSA, 5% SDS, 1mM EDTA, 40mM $NaPO_4$/pH6.8) 4 times and then with wash B (1% SDS, 1mM EDTA, 40mM $NaPO_4$/pH6.8) 2 times, both at 59°C. The washing solutions were added so as to immerse the membranes entirely. The membranes were then placed on used x-ray film that was packaged in saran-wrap. Six membranes were placed on each x-ray film and then wrapped in saran-wrap. The membranes were then placed into autoradiography

cassettes (Fisher Biotech/FBAC 1417) with two intensifying screens and a sheet of Kodak Scientific Imaging Film (Eastman Kodak Company) and left to expose overnight at -80°C. The film was developed using a Kodak RP X-OMAT developer.

Positive clones were identified as discrete spots on the autoradiograph. The x-ray films with positive signals were matched up according to the orientation dots with their respective plates. A number of positive signals were picked using a sterile pipette tip and the positive plugs were placed in 1ml of SM buffer with 20µl of chloroform (to prevent bacterial growth) and left overnight at 4°C. These phage stocks were then replated at a lower density (200-500 pfu per plate) in NZY top agar as done previously. Positive plaques were isolated and then replated at a density of 10 pfu per plate. This was done until a single positive plaque was isolated to purity. These were then stored in 1ml of SM buffer with 2.0% chloroform.

Isolation of Lambda DNA

An aliquot of E.Coli strain LE392 was infected with a single positive phage clone [100µl phage/600µl bacteria (10^5-10^7pfu)] and plated with NZY top agar on NZY plates as described previously. The following day, 10ml of λ diluent were placed onto each plate and placed on a rocker at 4°C overnight.

The λ diluent was collected and then centrifuged at 6000g (Sorvall) for 10 minutes at 4°C to remove any bacterial debris. The supernatant was removed and then incubated with both RNAse A and DNAse I (final concentration of 1µg/ml) for 30 minutes at 37°C. Following this, an equal volume of a solution containing 20% w/v

polyethylene glycol (PEG 8000) and 2M NaCl in λ diluent was added, vortexed gently and incubated for 1 hour in ice water. Precipitated bacteriophage particles were recovered by centrifugation at 10,000g for 10 minutes at 4°C. The supernatant was removed by gentle aspiration and the tube was placed in an inverted position on a paper towel to allow all the fluid to drain away. 0.5ml of TE was then added and the bacteriophage particles were resuspended by vortexing. 25μl of a 10% SDS (pH7.2) solution was then added and the preparation was incubated at 68°C for 5 minutes. 10μl of a 5M NaCl solution was added and the bacteriophage DNA were then purified by extracting once with phenol:chloroform and once with chloroform alone. The aqueous phase was transferred to a fresh microfuge tube between extractions. An equal volume of isopropanol was added to the aqueous phase and mixed. The solution was then stored at -70°C for 15 minutes. The DNA was recovered by centrifugation at 12,000g for 15 minutes at 4°C in a microfuge. The pellet was washed with 70% ethanol and recentrifuged briefly. The supernatant was removed by gentle aspiration and the pellet was dried by centrifuging the sample in a speed vac for 3 minutes. The DNA was then dissolved in TE to a final concentration of 1μg/ul.

Subcloning of DNA fragments into Plasmid Vectors

Phage DNA, containing the gene of interest, was digested with a number of different enzymes, EcoRI, BamHI, NotI, SmaI, SalI, XhoI, and then run on a 0.8% agarose gel. Partial characterization of the mouse CgA gene from a mouse (strain AJ)

genomic DNA library was reported previously (Fig. 15). Based on the restriction pattern for the EcoRI digest and comparison to the restriction map previously described (Fig. 15), a number of EcoRI fragments were chosen to be cloned into a plasmid vector. All fragments were subcloned into the plasmid Bluescript II KS- (Stratagene). The plasmid was linearized with EcoRI, then treated with calf intestinal alkaline phosphatase (CIP/Pharmacia). The digestion with EcoRI was done for 2 hours at 37°C, then CIP was added (1/10 volume, following dilution in the provided buffer), and left at 37°C for 1 hour. The sample was then run on a 0.8% agarose gel and purified using the GENECLEAN II kit (Bio101).

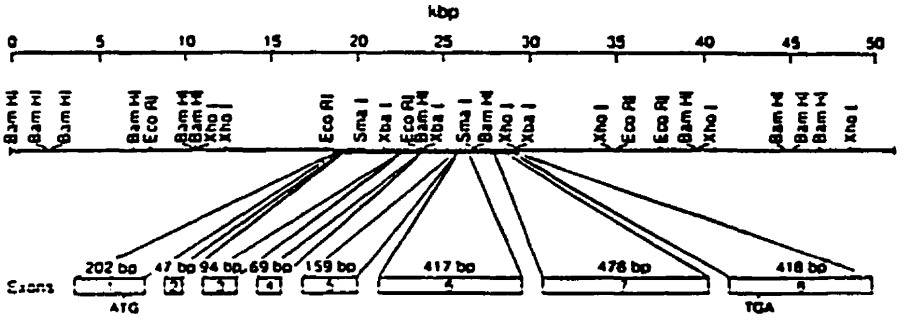


Figure 15. Partial restriction map of the mouse CgA gene. (12)

Ligations were done using the EcoRI digested lambda DNA with the CIP treated bluescript plasmid in a ratio of 1:4 (insert:vector). Insert and vector were combined in a total volume of 10µl (1µl insert, 4µl vector, 1µl T4 ligase, 2µl 5X Ligase Buffer, 2µl sterile H_2O) and left overnight at 14 - 16°C.

Transformations were done using either MAX Efficiency DH5α Competent Cells (Gibco/BRL) or Epicurian Coli SURE Competent Cells (Stratagene). Competent cells were stored in 100µl aliquots at -80°C. Competent cells were thawed on ice for 30 minutes. 2µl of ligation reaction was added to the cells, stirred gently and placed on ice for 30 minutes. Cells were then heat shocked for 45 seconds at 42°C, then immediately placed on ice for 2 minutes. SOC media or Luria Broth (0.9ml) were added to the cells and they were allowed to grow for 1 hour at 37°C. The cells were then plated on LB plates with ampicillin (100µg/ml). Plates were prepared 30 minutes prior to plating by adding 30µl X-Gal (25mg/ml) and 80µl IPTG (100mM) and spreading them evenly on the plate with a molded sterile glass pasteur pipette. Plates were incubated overnight at 37°C. White colonies were then picked the following day for minipreps and screened by restriction digestion.

Targeting Vector Construction

The first screening of the genomic library with the ^{32}P-labelled rCgA oligonucleotide probe successfully isolated 17.0kb of the CgA gene that includes 8kb of the 5′ untranslated region. This phage DNA was amplified, isolated and digested with EcoRI. The sample was electrophoresed on a 0.8% ethidium bromide stained agarose gel

and each fragment was sequentially subcloned and sequenced. Each fragment of DNA was isolated using the GENECLEAN II kit and ligated to the pBluescript vector in a total volume of 10μl. In one of the pBluescript vectors, a 5kb fragment was subcloned which contained exon 4 to exon 7 genomic sequence. From this, a 3.0kb XbaI and BamHI fragment containing exon 5 and 6 was isolated and subcloned into the pPNT vector, flanking the 3′ end of the neomycin resistance gene. The next insert to be subcloned into the pPNT plasmid was a 5.5kb fragment representing the 5′ untranslated region. This fragment was originally subcloned as a 7.5kb EcoRI insert into the pBluescript vector. An XhoI 5.5kb fragment was excised from within the insert and gel purified. Next, the pPNT plasmid with the 3.0kb insert described above was linearized with XhoI, CIP treated and gel purified. Ligation of the XhoI fragment and the pPNT plasmid was done in a total volume of 10μl. After transformation and mini-prep screening, a positive clone was isolated and used to inoculate a 600ml LB culture (100μg/ml ampicillin). Plasmid DNA was purified by the CsCl method.

The resulting targeting vector has a 5.5kb XhoI insert containing the 5′ flanking region of the CgA gene upstream of the neo^r gene and a 3.0kb insert containing exon 5, intron 5 and exon 6 of the CgA gene, flanking the 3′ end of the neo^r gene.

Plasmid DNA preparation

Miniprep protocol

Plasmid minipreps were done by the alkaline lysis method (162). After an overnight grow up of selected colonies in 5ml of LB with 100μg/ml ampicilin, 1.5ml of culture was placed into a microfuge tube and centrifuged at 12 000g for 30 seconds at

4°C. The medium was removed by aspiration and the bacterial pellet was resuspended in 100µl of ice cold sterile Solution I (50mM glucose, 25mM Tris-HCL pH8.0, 10mM EDTA pH8.0) by vigorous vortexing. 200µl of Solution II (0.2N NaOH, freshly diluted from a 10N stock, 1.0% SDS) was added to the tube, mixed and left on ice. 150µl of ice cold Solution III (CH_3COOK, glacial CH_3COOH, H_2O) was then added and the tube was vortexed for 10 seconds. The microfuge tube was left on ice for 5 minutes and then centrifuged at 12 000g for 5 minutes at 4°C. The supernatant was transferred to a fresh microfuge tube and the double stranded DNA was precipitated with 2 volumes of 100% ethanol at room temperature. The mixture was vortexed and allowed to stand at room temperature for a minimum of 2 minutes. The microfuge tube was centrifuged again at 12 000g for 5 minutes at 4°C, the supernatant was removed by gentle aspiration and the pellet was rinsed once with 75% ethanol (4°C). The pellet was dried by spinning in a speed-vac (SVC 1000/SAVANT) for 3 minutes and then dissolved in 30µl of TE containing DNAase-free pancreatic RNAase (20µg/ml).

Maxiprep Protocol

All maxipreparations of plasmid DNA were prepared using a Qiagen Plasmid Maxi kit. A 600ml preparation of LB media with 100µg/ml ampicillin was inoculated with 1ml of a minipreparation culture. The flask was incubated in a shaker (innOva 4000/New Brunswick Scientific) at 37°C / 225rpm overnight. The bacterial cells were harvested by centrifugation at 6000g for 15 minutes at 4°C. The bacterial pellet was then resuspended in 10ml of Buffer P1 (provided with the kit) by vigorous vortexing. 10ml of

Buffer P2 were then added, mixed gently by inverting the centrifuge bottle 4-6 times and allowed to incubate at room temperature for no more than 5 minutes. 10ml of chilled Buffer P3 were added, mixed immediately again by inverting 4-6 times and incubated on ice for 20 minutes. The sample was then centrifuged at 16 000g for 30 minutes at 4°C. Meanwhile, a Qiagen-tip 500 was equilibrated by adding 10ml of Buffer QBT (provided with the kit) and the column allowed to empty by gravity flow. After centrifugation, the supernatant which contained the plasmid DNA was removed promptly and added to the Qiagen-tip. The Qiagen-tip was washed twice with 30ml of Buffer QC (provided with the kit) and then the DNA eluted from the column with 15ml of Buffer QF (provided with the kit). The plasmid DNA was precipitated by adding 0.7 volumes of isopropanol at room temperature. The sample was mixed and centrifuged immediately at 16 000g for 30 minutes at 4°C. The supernantant was carefully removed and the DNA pellet was washed with 75% ethanol at room temperature. The DNA solution was recentrifuged at 16 000g for 10 minutes, the supernatant removed and the pellet allowed to air dry for 10 minutes. The DNA pellet was then dissolved in 1ml of sterile TE (pH8.0).

Equilibrium Centrifugation in CsCl-Ethidium Bromide Gradient

For electroporation of the targeting vector into embryonic stem cells, CsCl purified plasmid DNA was required. Following a plamid maxi-preparation, solid CsCl was added (1.0g for every ml of DNA solution), followed by ethidium bromide (0.8ml per 10ml DNA/CsCl solution). The resulting red solution was immediately mixed and transferred to Beckman Quick-Seal tubes that were balanced with a 1:1 TE/CsCl solution and sealed. The density gradients were centrifuged for 48 hours at 20°C in a Ti50 rotor at

45 000rpm. After centrifugation, two bands were clearly visible. A 21-gauge hypodermic needle was inserted into the top of the tube to allow air to enter. A second hypodermic needle, 18-gauge, was used to collect the chromosomal DNA band, in order to minimize contamination during subsequent collection of the plasmid DNA. Next, a third needle, 18-gauge, was used to remove the plasmid DNA. The ethidium bromide was removed from the DNA by extraction with isoamyl alcohol, an organic solvent. An equal volume of the organic solvent was added, vortexed and centrifuged at 1500rpm for 3 minutes at room temperature. The lower aqueous phase was then transferred to a clean plastic tube. The procedure was repeated several times, until the red color was gone. The CsCl was removed from the DNA by diluting 3 fold with water and then precipitating the DNA with 2 volumes of ethanol. The solution was allowed to stand for 15 minutes at 4°C and then centrifuged at 10 000g for 15 minutes at 4°C. The precipitated DNA was washed 2X with 75% ethanol, dried in a speed-vac and then dissolved in 1ml of sterile TE buffer.

Tissue Culture

Preparation of Inactivated Fibroblast Feeder Layer

Neomycin resistant fibroblasts, isolated from mouse embryos were plated in a T75 flask, fed every second day, and grown to confluency. The cells were trypsinized and replated in 2 X T175 flasks, (1:5) dilution, and grown to confluency. The cells were again split for a total of 10 X T175 flasks. Once confluent, mitomycin C (0.5mg/ml, 50X stock) was added to the media to a final concentration of 10μg/ml and incubated at 37°C,

5% CO_2 for 2 hours. The medium was then aspirated and the cells were washed profusely with 1XPBS (2 times) and then trypsinized. The cells were resuspended in media containing 10% DMSO (dimethyl sulfoxide). Each T175 flask was split into 6 freezing vials at 1.5ml each. A total of 60 inactivated fibroblast vials were stored at -85°C until needed.

Results

Genomic Library Screening

A mouse kidney genomic DNA library, constructed in Lambda DashII phage was screened by plaque hybridization with 1) a rat CgA oligonucleotide (36mer) complementary to the majority of exon 1 and 2) a cDNA probe containing exons 7 and 8 of the mouse CgA gene. Although no evidence has been provided to indicate that the CgA gene was isolated, through restriction enzyme analysis as well as sequencing data, it was confirmed that all clones isolated did indeed contain the CgA gene.

The first screening using the rat oligonucleotide as probe yielded a number of positive clones. These clones underwent secondary and tertiary screening until two positive clones were isolated and plaque purified. (Both clones subsequently proved to be identical and were desiganted as λmCgA.1) (see Fig. 16). Following amplification and DNA isolation, the lambda insert was digested with a number of restriction enzymes, either individually or a combination thereof, for Southern analysis with the rat CgA oligonucleotide as probe.

A restriction digest with the enzyme XhoI, revealed the size of the recombinant insert to be 17.0kb. Digestion of the lambda phage DNA with EcoRI yielded DNA fragments of 5.1kb, 7.5kb, 9.6kb and 20kb. The first two bands represent segments of the recombinant insert, whereas the last two are the phage arms. The reason the two bands from within the insert do not add up to the overall insert size was because there are two EcoRI restriction fragments of equal size which are not resolved by the gel electrophoresis. However, upon digestion with EcoRI and BamHI, the intensity of the

5.1kb fragment was reduced and two additional bands appeared, one at approximately 3.1kb and one at 1.4kb. With these fragments now visible, the overall insert size was confirmed to be 17.0kb.

Due to this ambiguity, the first subcloned fragment sent to be sequenced resulted in data containing exon 4 through intron 7 and not the presumed sequence of exon one through exon three, the region that was targeted with the rat oligonucleotide. This was due to having chosen the incorrect clone. When this was finally understood, efforts were made to subclone the 5.1kb fragment following the digestion of the phage arm with EcoRI and BamHI. Sequence analysis of this fragment confirmed that it corresponded to the 5′ portion of the gene.

The next fragment to be subcloned contained the 5′ flanking region of the mouse CgA gene. Based on the EcoRI restriction digest, two other larger DNA fragments were present in addition to the 5.1kb fragment. From the data obtained so far, a partial restriction map of the CgA gene was generated (Fig. 16). Based on this map, the CgA gene seemed to contain another EcoRI site far upstream from the ATG initiation codon, approximately 7.5kb from the EcoRI site just upstream of exon 1. The size of this fragment seemed to correspond with the 7.5kb band seen in the restriction pattern of the EcoRI digest of the phage clone (Fig. 17 A&B, Fig. 18). This fragment was subcloned and sequenced which confirmed that it corresponded to 5′ flanking region of the CgA gene.

The second screening of the genomic library was done in order to clone the 3' end of the gene, since it was assumed that the previous clone ended somewhere in intron 7.

Isolation of the 3' flanking region would allow for characterization of the intronic sequences of the CgA gene. The library had to be rescreened using a different probe, one that targeted the 3' end of the gene. Screening was carried out with a cDNA probe having exons 7 and 8 of the mouse CgA gene. It was obtained from a plasmid which had been constructed by cloning a mouse CgA cDNA into the mammalian expression vector, pcDNA3. From this plasmid, part of exon 7 and all of the exon 8 were removed and subcloned into the pGEM vector. The primary screening yielded a number of positive signals, 10 of which were carried further to the secondary and tertiary screening. In the end, three positive clones were isolated and plaque purified. All three clones were amplified, the DNA isolated and then digested with a number of enzymes. (All three clones subsequently proved to be identical and were designated λmCgA.2) (see Fig.16). The restriction pattern revealed a similar banding pattern to λmCgA.1 isolated with the rat oligonucleotide probe. A Southern analysis of the λmCgA.2 clone digested with EcoRI and using the exon 7/ exon 8 probe resulted in a signal at 10.0kb. This fragment will be subcloned and sent to be sequenced.

Construction of the Targeting Vector for Homologous Recombination

The targeting vector was constructed by inserting a 5' and 3' homologous region into the cloning sites of the pPNT vector which are separated by the neo^r gene. A large portion of the 5' untranslated region of the mouse CgA gene, 5.5kb, was cloned into the XhoI site of the vector (Fig. 17 C&D, Fig. 18). A 3.0kb exonic/intronic fragment having exons 5 and 6 of the mouse CgA gene was cloned into the XbaI and BamHI site of the

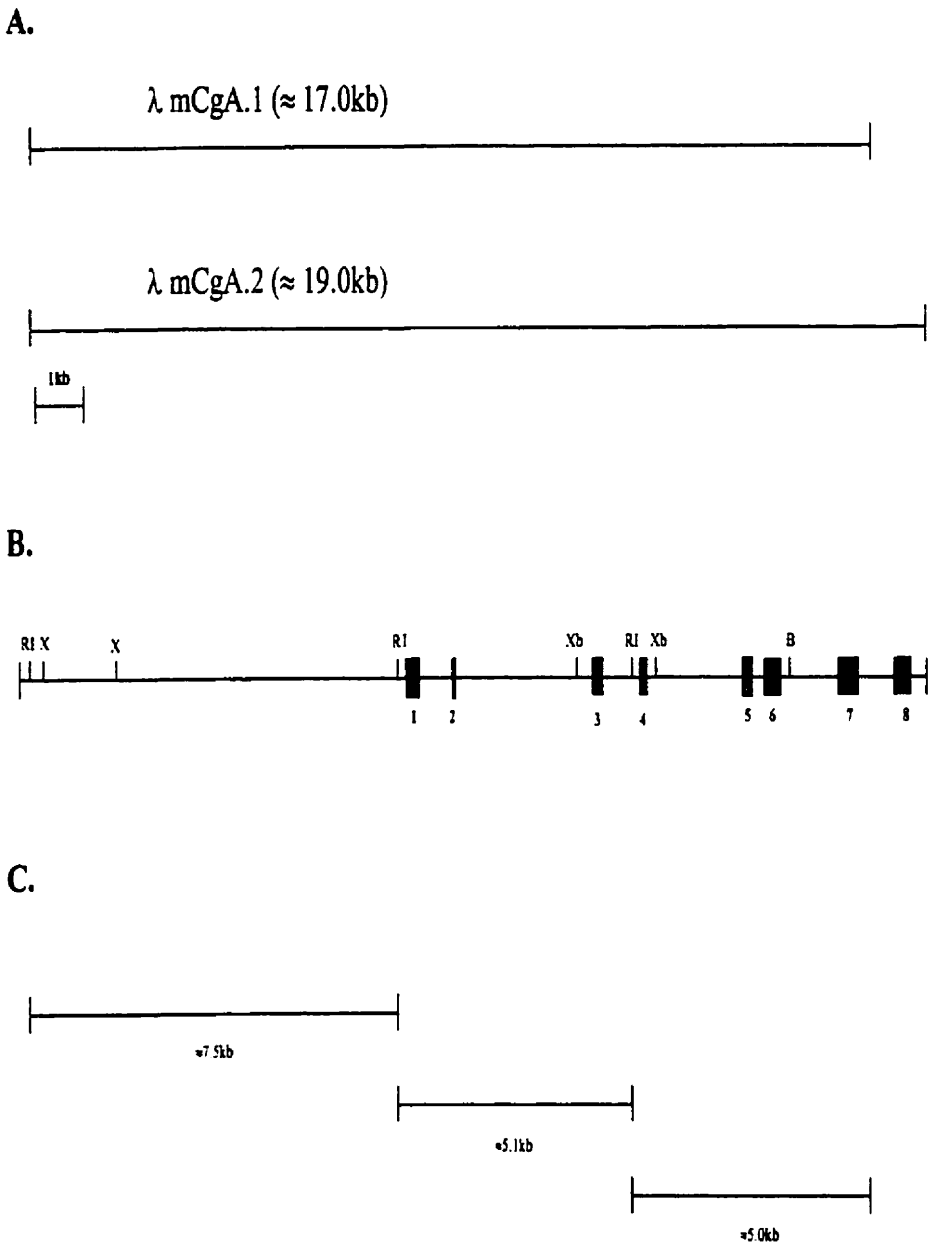


Figure 16. Physical map of mouse DNA encoding the CgA gene. Panel A, two independent bacteriophage recombinant clones, λmCgA.1 and λmCgA.2, were isolated after screening a mouse kidney genomic DNA library as described under "Experimental Methods". Panel B, restriction map of the mouse CgA gene. Restriction sites are: B, BamHI; E, EcoRI; Xb, XbaI; X, XhoI. Solid boxes denote exons. Panel C, the three restriction fragments cloned into pBluescript SK vector used for restriction mapping and nucleotide sequence analysis.

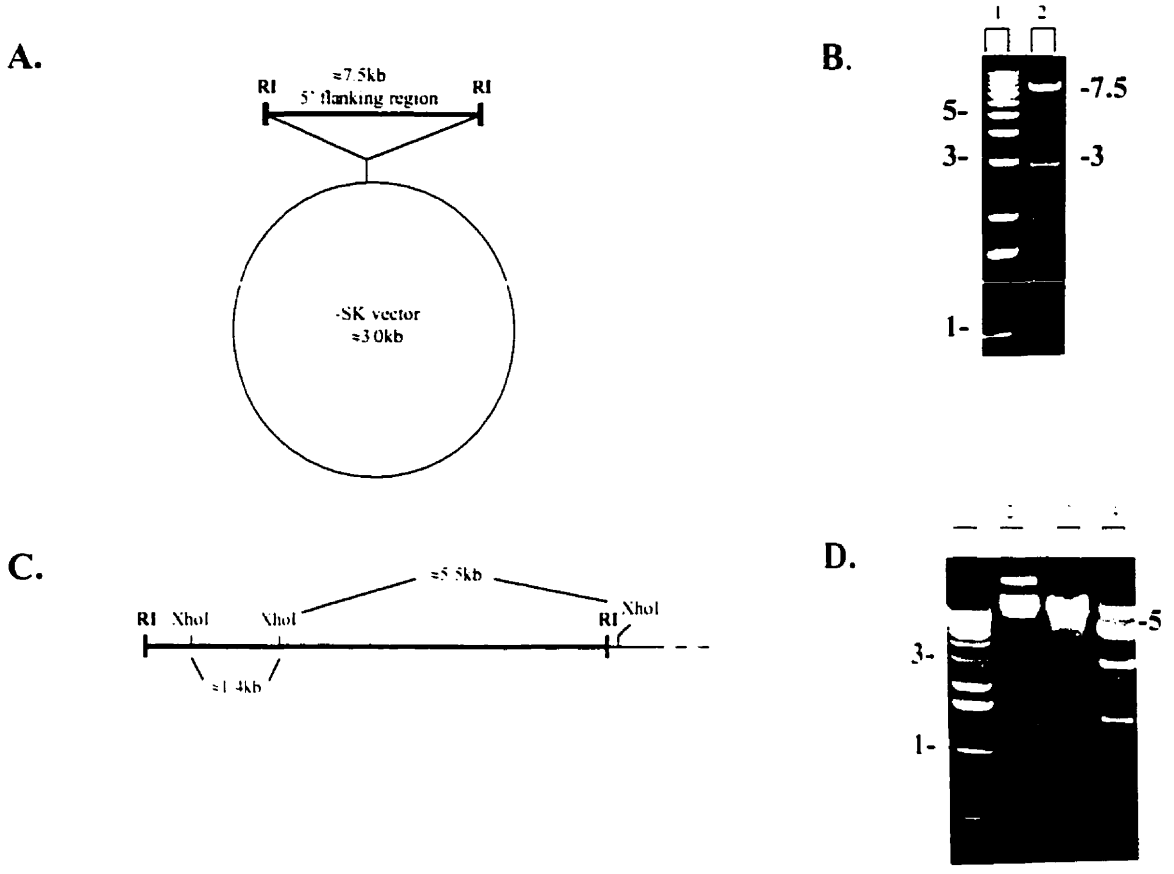


Figure 17. Cloning of mCgA gene 5' flanking region into pPNT targeting vector (step 1)

A. SK plasmid containing the 7.5 kb insert within the EcoRI restriction site.

B. Digestion of the plasmid/insert: Lane 1: 1kb ladder; Lane 2: digestion of the plasmid/insert with EcoRI. Two bands appear; one at ≈3.0kb, which represents the vector, and the other at ≈7.5 kb, representing the insert.

C. Schematic representation of the 7.5kb insert. Two XhoI sites are present at the 5' end and one is present immediately following the vector EcoRI restriction site at the 3' end of the insert.

D. Digestion of the plasmid/insert: Lane 1: 1kb ladder; Lane 2: undigested plasmid/insert. Lane 3: linearized version of the plasmid/insert. Lane 4: XhoI digested insert. Four bands are visible: one at 5.5kb, 3kb and 1.4kb. The band above the 5kb mark is undigested plasmid.

Restriction sites are: RI, EcoRI; XhoI.

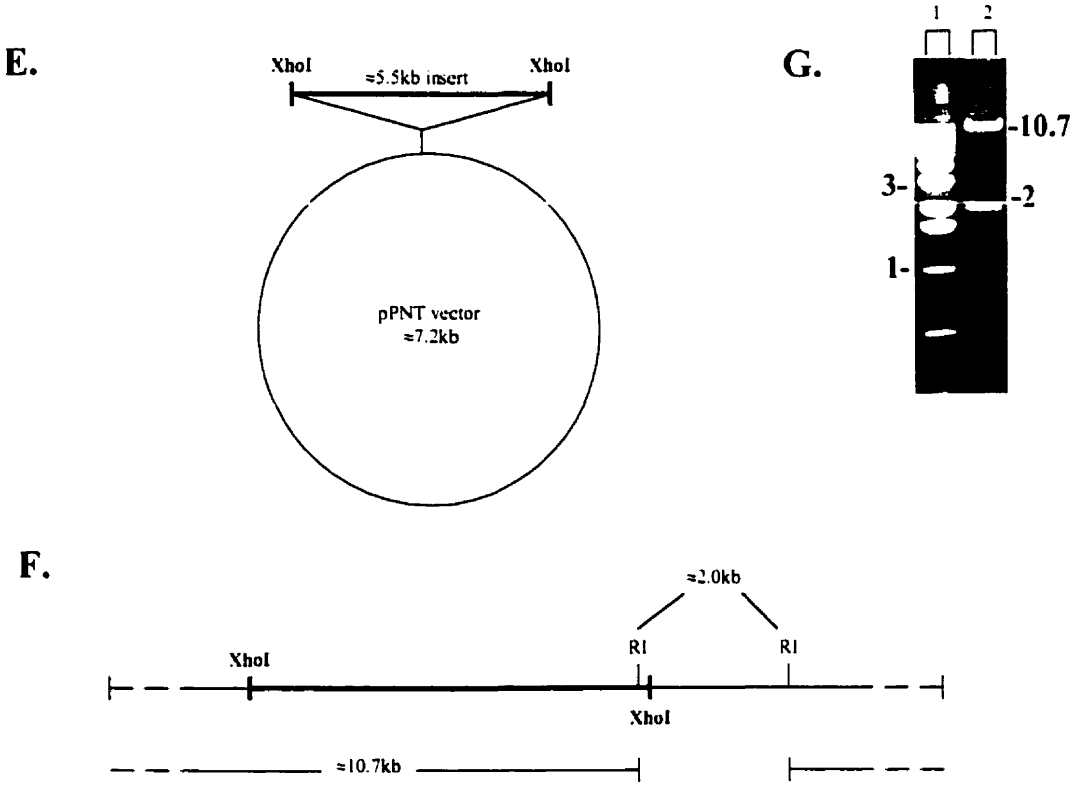


Figure 18. Cloning of mCgA gene 5' flanking region into pPNT targeting vector (step 2)

E. pPNT plasmid having the 5.5kb insert within its XhoI restriction site.

F. Schematic representation of the 5.5kb insert in the pPNT plasmid. Note the EcoRI restriction sites; one present at the 3'end of the insert and at approximately 2kb downstream of the XhoI restriction site at the 3' end of the insert. Digestion with EcoRI will yield two DNA fragments as shown.;one of ≈10.7kb, and another one at ≈2kb.

G. Digestion of the pPNT plasmid/insert. Lane 1: 1kb ladder; Lane 2: digested pPNT plasmid/insert with XhoI.

Restriction sites are: RI, EcoRI; XhoI.

vector (Fig. 19). Total vector size is 15.7kb. This can be seen in Fig. 20. Proper orientation of both inserts were verified by restriction enzyme digestion. Vector construction in this manner results in the replacement of 6.5kb of exonic and intronic sequences with the neo^r gene following homologous recombination. This construct therefore, deletes the transcriptional start site as well as exons 1, 2 and 3.

Screening for the targeted recombination event would then be done by a Southern blot. The DNA probe to be used for the Southern blot would hybridize to a region not present in the targeting vector (outside the region of homology). In this case, the cDNA probe used in the library screening for the 3' end of the CgA gene would be sufficient. Successfully targeted doubly resistant ES clones when digested with the restriction enzyme XhoI, would give two signals upon hybridization with the cDNA probe. The normal wildtype allele would give a signal that is approximately 17.0kb since the two immediate XhoI sites in the CgA gene are present far upstream of exon 1 and directly downstrean of exon 8 (see Fig. 15). On the other hand, the targeted allele has an additional XhoI site just upstream of the neo^r gene that came about from the subcloning of the insert into the pPNT vector. Therefore, when digested with XhoI a signal of approximately 6.7kb would be obtained (Fig. 21). This would then indicate that the allele has been successfully targeted. Once identified, these mutant ES cells will be microinjected into a normal blastocyst to produce chimeric mice. Wild type litter mates and fetuses heterozygous for the mutation will be identified again by Southern blot analysis and their neuroendocrine function will be tested.

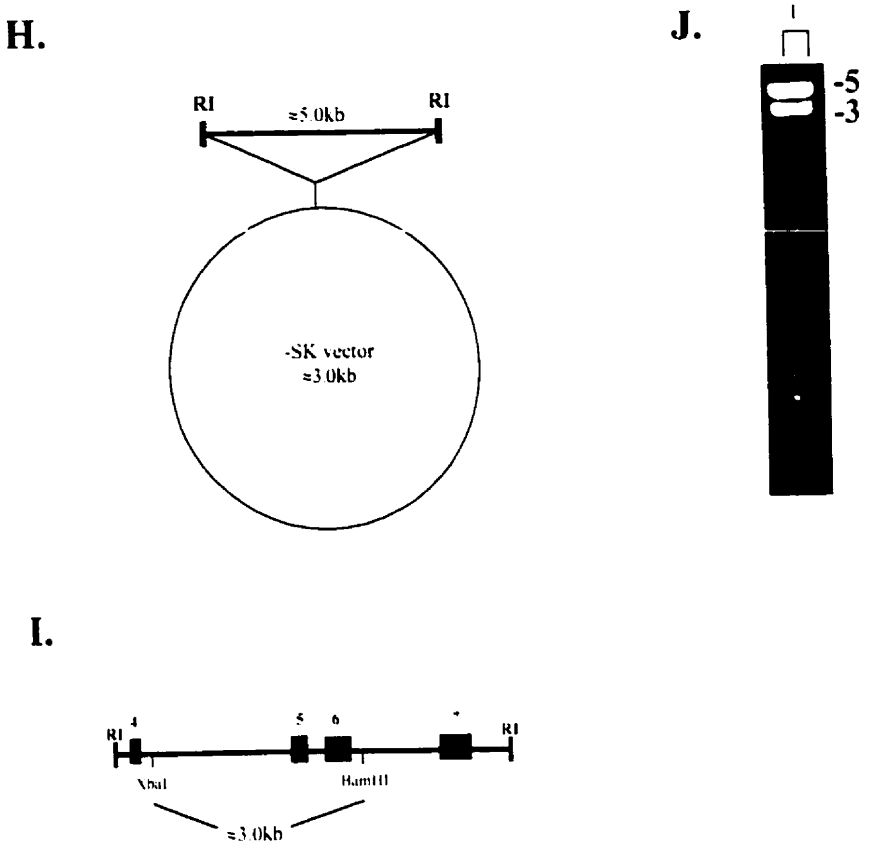


Figure 19. Map of SK plasmid containing the 3.0kb XbaI/BamHI insert

H. SK plasmid containing the coding regions for exons 5 and 6.

I. Schematic representation of the plasmid/insert.

J. Digestion of the plasmid/insert. Lane 1: plasmid/insert digested with XbaI and BamHI. Two bands are present: one at ≈5kb which represents the plasmid and the rest of the insert; and one at ≈3kb representing a genomic fragment from exons 5 to 6 of the mCgA gene.

Restriction sites are: RI, EcoRI; XbaI; BamHI.

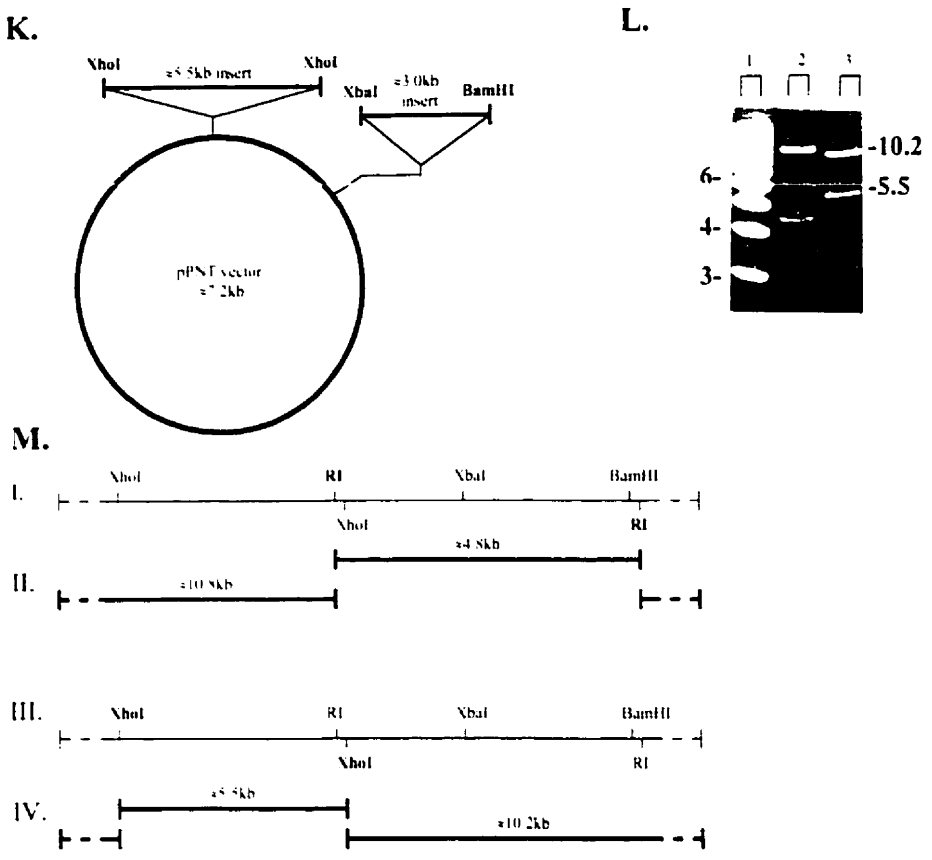


Figure 20. Map of completed targeting vector.

K. pPNT targeting vector with both genomic sequences cloned into their respective sites.

L. Digestion of the targeting vector. Lane 1: 1kb ladder. Lane 2: vector digested with EcoRI. Two fragments are obtained. One at ≈10.8kb and the other at ≈4.8kb. (see schematic drawing, MI and MII) Lane 2: XhoI digested vector. Two fragments are obtained, one at ≈10.2kb and the other at ≈5.5kb. (see schematic drawing MIII and MIV).

M. Schematic representation of the targeting vector. MI and MII depict digestion with EcoRI giving DNA fragments of ≈10.8kb and ≈4.8kb. MIII and MIV depict digestion with XhoI giving DNA fragments of ≈10.2kb and ≈5.5kb.

Restriction sites are: RI, EcoRI; XhoI; XbaI; Bam HI.

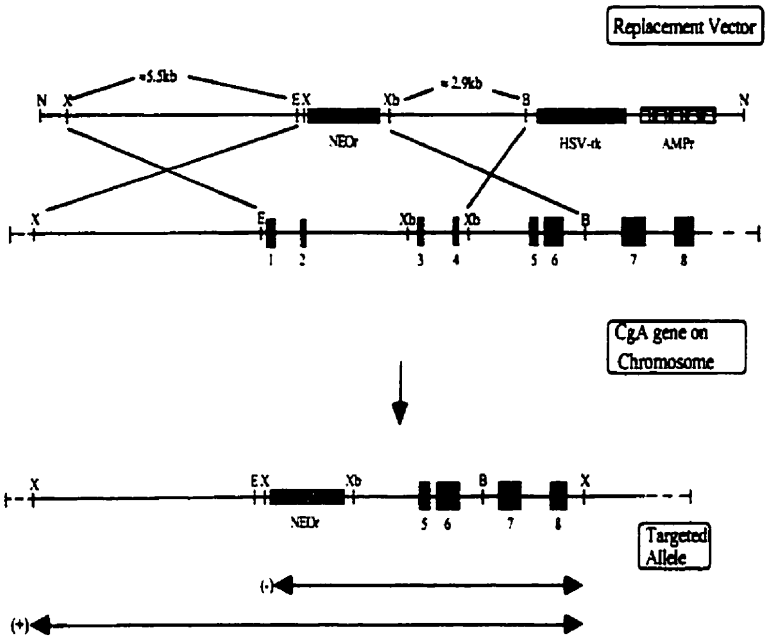


Figure 21. Homologous recombination event and targeted allele. Top line represents the linearized incoming targeting vector with CgA inserts designed to undergo homologous recombination with the CgA gene (shown in middle). Bottom line represents the ablated CgA gene after recombination. The sizes of the genomic DNA fragments expected from the wildtype (+) and disrupted (-) alleles following digestion with XhoI are indicated.
Restriction sites are: N, NotI; E, EcoRI; X, XhoI; Xb, XbaI; B, BamHI.

Part III

Discussion and Conclusion

Discussion

Chromogranin A (CgA) is an acidic glycoprotein widely expressed in endocrine and neuroendocrine cells. CgA was first identified in chromaffin granules of the adrenal medulla (1) and is the major member of the chromogranin/secretogranin family of proteins. CgA is thought to have multiple important roles in the process of regulated hormone secretion. Intracellularly, it is costored within secretory granules and cosecreted with the resident hormone. Extracellularly, peptides formed as a result of proteolytic processing of granins, are thought to regulate hormone secretion in an autocrine manner. Although a number of functions have been postulated for CgA, its major function is as yet unknown.

Our aim was to isolate the mouse CgA gene and disrupt one copy of the CgA gene in ES cells, which would then be used in the generation of a "knock out" mouse. Following the targeted disruption of the gene, all phenotypic changes within the mouse would be characterized which in turn, would help to reveal the exact function(s) that CgA has in the endocrine system.

To date, a genomic DNA library of mouse strain SVJ129 was screened with a mouse CgA synthetic oligonucleotide and a number of positive clones were detected. Restriction enzyme analysis of the phage DNA in addition to sequencing data, confirmed isolation of the CgA gene. Of the known eight exons of the mouse CgA gene, the first seven were present in the phage DNA including an eight kilobase 5' flanking region. Through the sequence information obtained from the above regions, a targeting vector was constructed containing approximately six kilobases to the left of the neomycin gene

and three kilobases to the right of the neomycin gene (see Fig. 21). The targeting vector was constructed in such a way, so as to disrupt the transcriptional start site of the mouse CgA gene, by inclusion of the neomycin gene in its place. The genomic sequences included within the targeting vector contain the upstream 5' flanking region of the mouse CgA gene in addition to exons one through four. Although the entire protein coding region of the CgA gene was not included within the construct, with removal of the initiation site, no protein would be generated. In addition, analysis of the sequencing data did not reveal any additional transcriptional start site downstream within the gene. However, if exons five through eight are still transcribed (for whatever reason), it would be of no consequence since a non-functional protein would be generated. Lastly, through both restriction enzyme analysis and sequencing data, a partial restriction map of the mouse CgA gene was generated (see Fig. 16). Intronic sizes were assigned based upon sequencing data obtained from the isolated phage clones. In addition, upon comparison with published sequence information from the same region of the mouse CgA gene, no discrepancies or differences were detected both with regard to intron sizes and enzyme restriction sites within the mouse CgA gene.

Gene targeting through homologous recombination is by far one of the most powerful tools that scientists have today. It allows for the selective removal of a specific single gene. Most often, this technique is used in the generation of null mutations; that is, a selected gene is silenced through targeted disruption. It is through this disruption that one is able to determine what the functional importance a particular gene has in the animal.

In any gene targeting ablation experiment, there are three possible outcomes. Inactivation of both alleles of a gene may result in effects that are lethal and cause the animal to die. On the other hand, the experiment may give rise to an animal with a variety of phenotypic changes. And lastly, although the gene has been inactivated, removal of the gene produces no effects in the animal.

Lethality

In some instances, targeted ablation experiments on certain genes resulted in effects that were lethal to the animal resulting in premature death. One recent example is the targeted disruption of the parathyroid hormone related peptide (PTHrP) gene (163). Mice that were targeted died postnatally. Results such as these make it somewhat difficult for the researcher to determine the functionality of a gene. He or she must therefore examine the animal during the gestation period and determine when expression of the particular gene becomes critical for continued correct development by assessing when lack of its expression results in aborted fetuses. For example, targeted disruption of the PTHrP gene gave rise to progeny that when analyzed three weeks after birth, showed no homozygous PTRrP mutants were present. This result suggested that PTHrP absence was lethal. Although gene lethality makes this research slightly more complicated, it provides a direct indication of the critical role this particular gene has within the animal.

To further characterize the time point of lethality in the PTHrP homozygous mice, fetuses were examined at day 18.5 of gestation. Although gene disruption of PTHrP

provided no viable progeny, examination of the fetuses during this period provided valuable information on the importance of PTHrP in fetal development.

Offspring that were analyzed both after birth and by cesarean section near term were found to be phenotypically distinct. These animals were hypotonic and made feeble attempts to breath. Their color remained blue gray until they died within minutes after birth. From this data, it was concluded that PTHrP homozygous mice survived till birth but died immediately after delivery due to respiratory failure. Additional phenotypic features included a domed skull, short snout and mandible, protruding tongue and disproportionately short limbs. These features define a form of osteochondrodysplasia. These findings indicated that PTHrP was needed for normal development of chondrocytes, whereby its absence resulted in premature chondrocyte differentiation. This resulted in a reduced number of proliferating chondrocytes, early hypertrophy and subsequent death of chondrocytes, and associated premature bone formation. In addition, accelerated endochondral ossification of the mutant skeleton was also observed.

To summarize, targeted disruption of the PTHrP gene provided direct evidence implicating PTHrP in normal skeletal development. Lack of the functional gene caused premature death postnatally due to respiratory failure, which is surmized to be the direct result of the observed skeletal abnormalities.

In the targeted disruption of the CgA gene, it is possible that lethality will not be a problem due to the partial homology that exists between CgA and the other granins. One example is the homology between exons II, III and VIII of the mouse CgA gene and exons II, III and V of the mouse CgB gene. Such a relationship may indicate that the two genes are functionally redundant. In other words, if mCgA is ablated, then to a certain

degree its function may be carried out by mCgB. It should be emphasized, however, that the degree of homology between CgA and CgB is not great, even within the sequences specified.

However, if the targeted disruption of CgA does result in premature death, then just as in the PTHrP experiment, characterization of the time point of lethality would be needed to determine what is/are the function(s) of the CgA gene.

Recent immunhistochemical studies have shown early ontogenic expression of CgA and its derived peptides (164). Analysis of both fetal and neonatal specimens from 12.5 day embryos (12.5E) to 42 day postnatal rats have detected CgA immunostaining in the endocrine cells of the pancreas, stomach, intestine, adrenal gland and thyroid at E13.5, E14.5, E15.5, E15.5 and E18.5 respectively. Such early expression of the gene seems to indicate that CgA has some function in fetal development. In addition, if the targeted disruption of CgA does turn out to be lethal, then the above information provides critical insight on when and where CgA is expressed which in turn would allow for characterization of its lethality.

Phenotypic changes / No changes

If the targeted disruption is not lethal, two possibilities exist: the first includes any and all phenotypic changes that arise. These effects can be either mild or very pronounced, both of which depend on the function that the particular gene has. One example involves the targeted disruption of the calcium sensing receptor (CaSR) in mice (165). These mice were created in order to examine what exactly the receptor's role in

calcium homeostasis was, in addition to determining the mechanism by which inherited human Casr gene defects cause diseases.

Heterozygous mice for the targeted disruption, $Casr^{+/-}$, exhibited similar symptoms to those of humans with familial hypocalciuric hypocalcemia. Mildly elevated levels of calcium, magnesium and inappropriately normal PTH levels in serum were observed in addition to hypocalciuria.

On the other hand, mice homozygous for the deletion, $Casr^{-/-}$, exhibited symptoms of severe hyperparathyroidism. Highly elevated serum calcium and PTH levels were observed in addition to parathyroid hyperplasia, bone abnormalities, retarded growth and premature death. Based on this information derived from these particular mouse models, it could be concluded that the corresponding human disorders are the direct result of a reduced number of functional Casr receptor molecules on the cell surface of parathyroid and kidney cells.

Successful disruption of the CgA gene will hopefully generate a number of phenotypic changes that will help to elucidate whether CgA's primary function is intracellular, playing a role in targeting peptide hormones and neurotransmitters to granules of the regulated pathway, or extracellular, whereby peptides formed as a result of proteolytic processing of CgA regulate hormone secretion.

If CgA's primary function is determined to be primarily intracellular, the targeted mice would most likely exhibit elevated levels of a number of peptide hormones and neurotransmitters due to an inability of these substances to aggregate and package into a secretory granule. For example, it is well known that PTH and CgA are the two major

proteins secreted by the parathyroid gland in response to hypocalcemic stimulation (13). Upon secretion, PTH causes restoration of serum calcium through actions that are primarily on bone and kidney, and therefore, it plays a key role in calcium homeostasis. If however, the targeted disruption of CgA prevents the sorting of PTH from other constitutively secreted proteins within the secretory pathway of the parathyroid cell then one might speculate that elevated levels of PTH in the circulation would be found since it will now be constitutively secreted as well. As a result, elevated levels of PTH will in turn cause an increase in the level of blood calcium, a symptom which is characteristically seen in hyperparathyroidism. More specifically, since PTH exerts direct effects on bone and kidney (and indirectly influences the gastrointestinal tract), excess circulating PTH can lead to altered function of bone cells, renal tubules and gastrointestinal mucosa. This in turn can result in kidney stones and calcium deposits in renal tubules due to tubule fluid (urine) becoming highly supersaturated with calcium salts such as calcium oxalate in addition to decalcification of bone, which in humans result in bone pain and tenderness and spontaneous fractures.

However, it should be mentioned that the pathophysiology of hyperparathyroidism relates to the *loss* of normal feedback control of PTH by extracellular calcium, whereby under normal hypercalcemic conditions, the parathyroid gland is suppressed thereby inhibiting PTH secretion. In the CgA knockout mice however, no modification of the feedback control mechanism would have occured, therefore, the high levels of PTH would result in the suppression of the parathyroid gland thereby inhibiting PTH secretion. As a result, subsequent PTH measurements would indicate less than normal PTH levels. If this were the case, then the mice would exhibit

symptoms characteristically seen in hypoparathyroidism. For example, a deficiency in PTH secretion is usually associated with hypocalcemia and hyperphosphatemia. In humans, these conditions give rise to symptoms that include cataracts (due to soft tissue calcification), dry rough skin, coarse brittle hair, alopecia and abnormal dentition (109).

Although both scenarios are feasible, one could imagine a CgA targeted mouse that combines characteristics from both of them. For example, since CgA is expressed early on in fetal life, ablation of the gene would result in elevated levels of PTH. However, as the endocrine system develops, the feedback inhibitory mechanisms develop as well. As a result, during fetal development, a drop in total PTH secreted would be observed, due to inhibition of the parathroid gland by excess PTH.

Since CgA is found throughout the endocrine / neuroendocrine system packaged in secretory granules together with the resident hormone, and one assumes that another gene cannot take over all its functions, one can certainly expect to find widespread effects associated with the targeted disruption of CgA in the pituitary, adrenal, thyroid, pancreas and thymus glands. CgA is highly expressed in sympathetic neurons and it would be of interest to consider what ablation of CgA would do to their function.

Lastly, evidence from a number of studies have shown that specific granins promote the formation of particular types of secretory granule (small versus large, for example). Lack of CgA would be anticipated to lead to a decrease in large secretory granules in certain pituitary cells.

On the other hand, the major function of CgA may turn out to be primarily extracellular, whereby CgA derived peptides have been shown to act as modulators of endocrine cell secretory activity. For instance, ablation of the CgA gene would eliminate peptides such as: pancreastatin, β-granin and vasostatin, all of which are derived from CgA. Each of these peptides function in one way or another by inhibiting exocytosis. For example, pancreastatin inhibits secretion of insulin by the pancreas as well as secretion of PTH from the parathyroid (23,43,44,45). Chromostatin blocks stimulated secretion of catecholamines by chromaffin cells (52,53). Vasostatin inhibits arterial smooth muscle contraction and β-granin has been shown to inhibit parathyroid cell secretion (50). Therefore, once CgA is selectively removed from the mouse genome, none of the above peptide fragments would be generated hence none of their biological effects would be observed. For instance, elevated levels of insulin would have drastic effects on bone matrix synthesis and cartilage formation, due to the effects of insulin on bone forming cells, the osteoblasts, which may lead to a number of skeletal abnormalities within the mice (109). In addition, elevated levels of catecholamines such as epinephrine and norepinephrine would give rise to an increase in the basal metabolic rate of the animal. This would be manifested for example, by increased heart beat and constricted/dilated blood vessels (109).

Finally, the last outcome of a gene disruption experiment may be no phenotypic change at all. This can imply one of two things; the particular gene has no significant function within the animal, or that functional redundancy exists between the gene that has been disrupted and other genes within the genome. In the case of CgA, although

sequence homology to the other granins is quite weak, this does not mean that functional homology is not strong. Therefore if CgA is ablated, its function within the endocrine system could be taken over by either CgB, CgC or another of the granins. In addition, recent colocalization studies have indicated that not all types of neuroendocrine cells contain CgA, but that CgB and/or CgC may be present (166). These differences in granin expression, indicate one of two possible functions. The first, is that these granins do indeed have different functions, or second, that granin family members are capable of carrying out certain common functions and therefore are functionally redundant. And so, not all granin family members need to be equally expressed in all neuroendocrine cells. In addition, studies have shown that the distribution of the chromogranins, specifically CgA and CgB, vary from tissue to tissue: however, in general, both proteins are found to be co-stored. Although their levels of expression may be different, this again may indicate that equal expression is not necessary for one granin to function adequately in the absence of the other (10).

The Importance of Null Mutations (113)

Why are null mutations important? At the simplest level they provide a base line for the development or functioning of an organism in the absence of a particular gene. This will answer the question, Is the gene essential for life? If it is essential, then experiments can proceed to determine what process is interrupted in the absence of the gene. If the gene is not essential, then the question becomes, Why Not? Does the gene

not function the way that was predicted from the biochemical analysis and from its expression pattern, or are there other backup systems that can partially compensate for the loss of the gene? If the latter is the case, then other genes must be removed to test the combined function of the genes (113).

Due to the complexity of gene function, it is necessary to make animals with different kinds of mutations, and gene targeting offers the only method for making predetermined mutations in which the maximum number of potential complicating factors can be controlled. To date, the targeting approaches that have been used extensively in ES cells include insertion of a selectable gene into the gene of interest. This insertion type of mutation has been very successful in the production of null mutations and dominant mutations involving altering the expression of a gene.

However in a number of instances, independent disruption of a particular gene produced minimal effects. In these situations, a second disruption was necessary in order to produce phenotypic changes within the animal (106). This has led to support the theory of gene function redundancy. For example, in the granin family, a number of members have a great deal of similarity to each other, which would lead one to believe in functional redundancy. If this is the case, then perhaps the targeted ablation of one granin would result in the signaling of another granin(s) to substitute for its function. Nevertheless, whatever the phenotypic changes that occur within the CgA knock out mouse, our fundamental understanding of the function(s) of CgA will undoubtedly be enhanced.

Yeast Genome Search

To determine how well the granins are conserved throughout evolution, an attempt was made to identify granin sequences within the yeast genome. Although a lower eukaryotic life form, a number of proteins within higher eukaryotes have been found to be well conserved in Saccharomyces cerevisiae. One such example is the Kex2 endoprotease, a protein that functions as a membrane bound processing enzyme (136,137). To date, a number of mammalian proteins that resemble Kex2 have been identified. One such example is the fur (fes/fps upstream region) gene and its product furin or PACE (138,139,140). In addition, through PCR amplification, more Kex2 related genes have been identified. These include, PC1/PC3 and PC2 (141,142,143), PC4 (144,145), PACE 4 (146), PC5/PC6A/PC6B (147,148,149) and PC7/PC8/LPC (150). Although these endoproteases were found in higher vertebrates, homologous convertases have also been found in molluscs (151), Xenopus laevis (152), Drosophila melanogaster (153) as well as Hydra vulgaris (154). These convertases have been very well conserved throughout evolution.

CgA immunoreactivity has been detected in a single celled eukaryotic organism, the protozoan, Paramecium tetraurelia (155), and therefore it is of interest to know whether granin sequences are present within the yeast genome. To accomplish this task, stretches of amino acid sequences that correspond to the chromogranin proteins, and that are highly conserved among mammalian species (Table 3), were cross-referenced with the yeast genome database. No significant matches were found.

Even though no sequence homology was detected, this result provides additional information concerning the secretory pathway of yeast. Since the granins function in

mammalian species as modulators of cell secretory activity, by targeting peptide hormones and neurotransmitters to granules of the regulated pathway, there should be no need for such a chaperone in a species which does not require such a sophisticated system. Saccharomyces cerevisiae for instance, appears to exhibit only a constitutive secretory pathway (156,157). It transports proteins from the endoplasmic reticulum to the cell surface in an extremely rapid fashion, which is due in part to the high density of cytoplasmic ribosomes that it possesses. Furthermore, unlike PC1 and PC2, endoproteases which are present only in cells with a regulated pathway (150), PC7 is not present within secretory granules, yet it is the convertase which shows the greatest homology to the yeast Kex2 endoprotease (150). Therefore, it would have been surprising if significant sequence homology was detected within the yeast genome database to any of the granins.

On the other hand, one should not completely discount the possibility of the existence of such a mechanism within yeast. Recent findings have shown that factors, which promote secretory vesicle release from the trans-Golgi, are conserved between yeast and mammalian cells (157). In this study, permeabilized pituitary GH3 cells were treated with high salt to remove endogenous budding factors. As a result of this treatment, the cell preparation required exogenous cytosol and energy to promote secretory vesicle binding. When a salt extract of membranes from Saccharomyces cerevisiae was used, secretory vesicle budding was stimulated. Hence, the fact that yeast does not employ the use of a regulated system may simply mean that it functions adequately without one. However, if a regulated pathway does exist in yeast, the fact that no granins were detected within the genome may suggest that a different type of modified sorting

Table 3. Species specific CgA amino acid sequences used for yeast genome search

Species	Amino acids	NH_2/COOH terminus	Sequence	Length (a.a)
Bovine	+1 ← +75	NH_2	LPVNSPMNKGDTEVMKCIV EVISDTLSKPSPMPVSKECF ETLRGDERILSILRHQNLLK ELQDLALQGAKERTHQ	75
Bovine	+79 ← +92	NH_2	HSSYEDELSEVLEK	14
Human	+79 ← +92	NH_2	HSGFEDELSEVLEN	14
Pig	+79 ← +92	NH_2	QSSYEDELSEVLEK	14
Rat	+94 ← +107	NH_2	HSSFEDELSEVFEN	14
Mouse	+99 ← +112	NH_2	HSSFEDELSEVFEN	14
Bovine	+314 ← +376	COOH	KRWSKMDQLAKELTAEKR LEGEEEEEEDPDRSMRLSFR ARGYGFRGPGLQLRRQWR PNSRED	62
Bovine/ Human/ Pig/Rat/ Mouse	+409 ← +418 Bovine +417 ← +426 Human +408 ← +417 Pig +426 ← +435 Rat +423 ← +432 Mouse	COOH	ESLSAIEAEL	10
Bovine	+391 ← +441	COOH	EKKEEEGSANRRPEDQELE SLSAIEAELEKVAHQLEELR RG	41

mechanism is used by yeast. For example, proteins may possess a "tag" or "sorting signal", which direct it to secretory granules without the aid of the granins.

For instance, one study has shown that the N-terminal disulfide bond plays a role in the control of prolactin intracellular storage and transit in mammalian cells (158). When the N-terminal disulfide bond was removed through mutation, constitutive secretion of prolactin increased by 50%. Another such example is that of pro-opiomelanocorticotrophic hormone (POMC). When mutants having amino acid deletions at the amino terminal of the proregion (2-26 or 8-20) were analyzed, the hormone was found to be constitutively secreted (159,160).

If such a system does exist in yeast, these sorting signals may function by directing the protein to specific sorting receptors, which then promote budding and secretory granule formation. Such a system has been recently identified in the regulated pathway of mammalian cells. When regulated secretory proteins exit from the TGN into immature secretory granules, they interact with specific components in the TGN membrane. One such component has been identified as being a membrane bound form of carboxypeptidase-E, which acted as a sorting receptor that recognized regulated secretory proteins in the TGN (161). When mice were generated lacking this particular receptor, the pituitary prohormone, POMC, was missorted to the constitutive pathway and secreted in an unregulated manner.

And lastly, a search was also conducted in both the C.Elegans and D.Melanogaster genome databases using the same stretches of amino acid sequences used for the yeast genome database search. Again, no significant matches were found. As a control, the protein sequence of furin, a membrane bound processing enzyme that is

highly conserved throughout evolution, was used in both database searches. A significant match was detected in the C.Elegans database yet non in the D.Melanogaster database. Although the drosophila database search is inconclusive (the genome has not been fully sequenced), the results obtained in the C.Elegans search seem to indicate that this species does not depend on regulated secretory proteins but, just as in yeast, has a different sorting mechanism available.

All this information seems to point to two classes of regulated secretory proteins. Those that contain all the structural information necessary for secretory granule sorting and therefore are the only proteins present in the TGN, and those which lack a sorting signal, but are competent for aggregation, yet depend on a class of regulated secretory proteins such as the granins. Once again, if a regulated secretory pathway does exist in yeast, it would seem to function through the former mechanism, and not the latter.

Part IV

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