THE CALCITONIN GENES; THEIR STRUCTURE, EXPRESSION AND ROLE IN DISEASE

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ABSTRACT

A detailed structural analysis of the calcitonin (CT) gene of a young male osteoporotic patient who is completely CT deficient is presented. Genomic Southern blot analysis revealed no gross abnormality. A genomic library was subsequently constructed and the patient's CT/CGRP gene was isolated. The nucleotide sequence of the coding regions of the gene was normal. The only abnormality identified was a single base insertion in one of the introns of the gene, in a position which might interfere with correct processing of the precursor RNA. This was originally suggested as a probable cause for his condition. Further experiments however showed that this change probably represents a neutral polymorphism as it is present in normal individuals as well. No explanation can therefore be offered as to why this patient cannot produce CT.

The CT gene also codes for a neuropeptide, Calcitonin Gene Related Peptide - CGRP. As there is a second (beta) gene for CGRP, and there are reports that more than one CT circulates in man, the possibility was investigated that further CT related sequences might exist in the human genome. Two genomic loci were identified. One is an area of the beta CGRP gene related to the CT specific exon of the alpha gene. This has the potential to code for a 32 amino acid peptide which has homology with CT. Certain structural characteristics in this sequence suggest that it is not expressed in present day man. The other genomic locus which was identified - the "gamma" sequence - has strong homology with the region of the α and β genes which codes for the signal and N-terminal peptides. No CGRP or CT related sequences were identified in the vicinity of the "gamma" sequence even under very low stringency hybridisation conditions. It is suggested that these loci have evolved as a result of sequence duplication events.

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LIST OF ABBREVIATIONS

BCIG	BROMO-CHLORO-INDOLYL-GALACTOSIDE
BCR	BREAKPOINT CLUSTER REGION
BSA	BOVINE SERUM ALBUMIN
CT	CALCITONIN
CGRP	CALCITONIN GENE RELATED PEPTIDE
DTT	DITHIOTHREITOL
EDTA	ETHYLENE DIAMINE TETRA ACETIC ACID
GDW	GLASS DISTILLED WATER
GH	GROWTH HORMONE
GTE	GLUCOSE / TRIS / EDTA SOLUTION
IPTG	ISO-PROPYL-THIO GALACTOSIDE
KB	KILO BASES (OF DNA)
LHRH	LUTEINISING HORMONE RELEASING HORMONE
LBM	LURIA BROTH WITH MAGNESIUM
MOPS	MORPHOLINO PROPANE SULPHONIC ACID
MTC	MEDULLARY THYROID CARCINOMA
O/N	OVERNIGHT
ORF	OPEN READING FRAME
PBS	PHOSPHATE BUFFERED SALINE
PCR	POLYMERASE CHAIN REACTION
PFU	PLAQUE FORMING UNITS (OF VIRUS)
POMC	PRO-OPIO-MELANO-CORTIN
PRL	PROLACTIN
PEG	POLYETHYLENE GLYCOL
PTH	PARATHYROID HORMONE
SDS	SODIUM DODECYL SULPHATE
SM	PHAGE DILUENT
SSC	STANDARD SALINE CITRATE
SSPE	AS SSC WITH PHOSPHATE & EDTA REPLACING CITRATE
TBE	TRIS / BORATE GEL BUFFER
TE	TRIS / EDTA BUFFER
TSE	TRIS / SODIUM / EDTA BUFFER

CHAPTER I

INTRODUCTION

CONTENTS:

The work described in this thesis involves the use of Molecular Biology techniques in the study of calcitonin related peptides and osteoporosis. The contribution of Molecular Biology techniques to the field of Endocrinology will therefore be briefly discussed in the first section of this chapter. The following sections deal with calcitonin and its possible role in osteoporosis, ending with an overview of current knowledge about the Molecular Biology of this hormone.

I.A. THE USE OF MOLECULAR BIOLOGY TECHNIQUES IN MODERN ENDOCRINE RESEARCH

The application of Molecular Biology techniques to medical research has profoundly affected Endocrinology. New regulatory peptides have been identified and the primary structures of larger protein hormones such as the humoral hypercalcaemia of cancer hormone, which had for years been impossible to sequence, were characterised in a few months. Much has also been learned about pathophysiological mechanisms through the analysis of the expression of genes in endocrine tissues. The contribution of Molecular Biology techniques to research in Endocrinology will be briefly reviewed in this section and the commonly used techniques will be presented. The use of recombinant DNA techniques for the production of hormones lies outside the scope of this thesis and will not be further discussed.

I.A.1. DNA STUDIES

The now classical way of studying DNA is by Southern blot analysis. DNA is extracted from a tissue, usually peripheral lymphocytes, and is then cut into specific small fragments. This is achieved by digestion with restriction enzymes, which are bacterial enzymes, which recognize and cleave defined nucleotide sequences. DNA fragments are then size separated by electrophoresis in an agarose gel and transferred onto a nitrocellulose or other membrane by capillary action (for details see chapter III and fig. 3.4). The membrane can then be hybridised to a radioactive probe specific for the gene under study and one can then see from the autoradiograph whether the gene is present or not, whether it has the correct size and if it exists in one or multiple copies in the genome.

In the human genome, there are naturally occurring neutral polymorphisms affecting the recognition sites of restriction enzymes. These can be identified by Southern blot analysis as extra bands hybridising to the probe. They are inherited in a Mendelian fashion and can therefore be used to trace the inheritance of the affected allele. When a polymorphic restriction site is located close to a particular gene, then the two tend to be inherited together and the shorter the physical distance, the lower the frequency of recombination between the two loci. This kind of analysis is called RFLP (restriction fragment length polymorphism) analysis (Kan and Dozy, 1978). RFLPs may also result from a variation in the number of tandem repeat sequences present between two restriction sites.

It is now possible to apply these techniques to the smallest of tissue samples. A DNA amplification method known as the polymerase chain reaction (PCR) has been developed (Saiki et al, 1985, Saiki et al, 1988), which has revolutionised the field allowing analysis even of single molecules. In this technique, a pair of oligonucleotide primers flanking the region to be amplified are used together with a thermostable DNA polymerase. There are three steps to the reaction. The DNA is rendered single stranded by heating, the primers are left to anneal to the DNA and the DNA polymerase then acts to extend the primers and synthesise new strands of DNA. The three cycles of the reaction (denaturation, annealing, extension) are then repeated , the newly synthesised DNA strands themselves serving as templates for further DNA synthesis and the reaction progresses in an exponential fashion amplifying the DNA between the two primers (fig I.1).

Southern blot analysis has been used in Endocrinology in studies of a number of conditions. For instance, growth hormone (GH) gene deletions in some forms of hereditary pituitary dwarfism were detected by Phillips et al (1981)

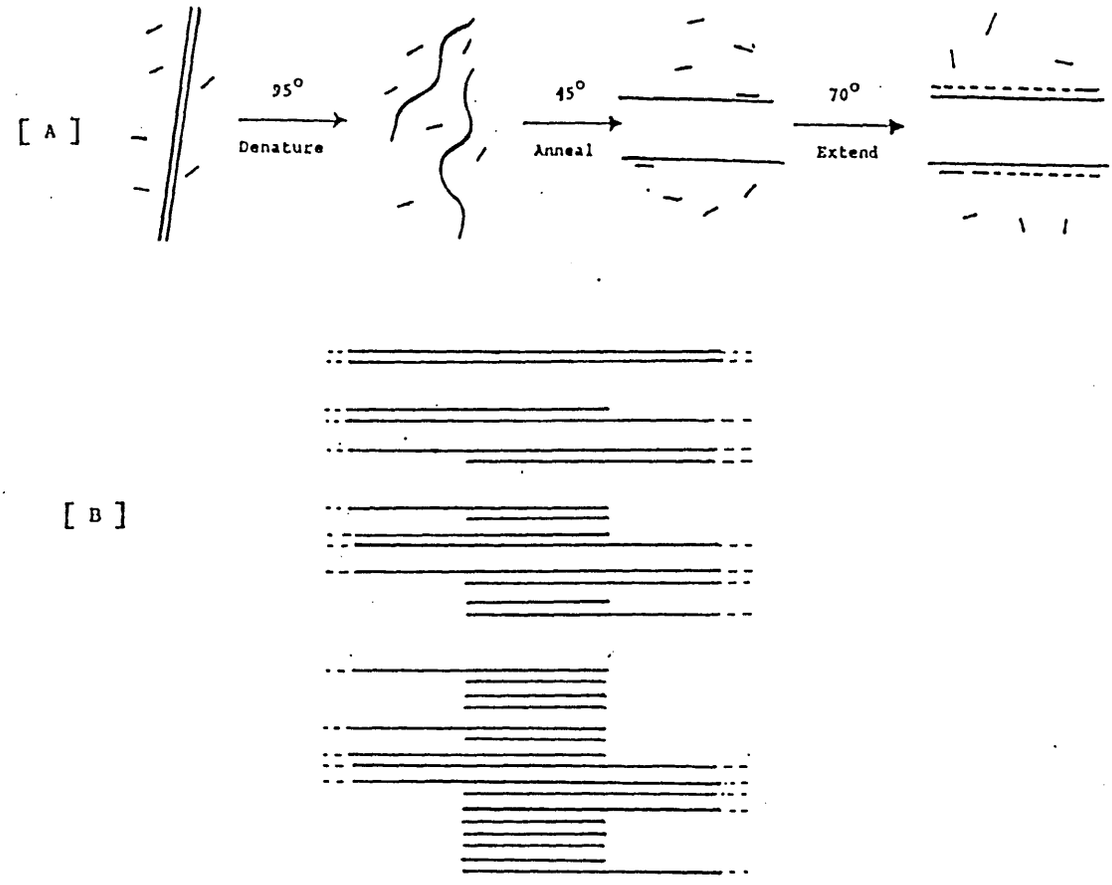

fig 1.1. Polymerase Chain Reaction.
[A]: first cycle [B]: multiple cycles

and Vnencak-Jones et al (1988). Southern blotting was also used to identify a highly polymorphic region at the 5' end of the insulin gene in the vicinity of the regulatory elements of the gene. This has been extensively studied in both type I and type II diabetes and a particular polymorphic fragment was found to be associated with the development of type I diabetes mellitus although the linkage did not prove to be very strong (Bell et al, 1987). The molecular basis for certain forms of 21 hydroxylase deficiency, which is the commonest form of congenital adrenal hyperplasia, has also been characterised using Southern blot analysis (White et al, 1987).

Chromosomal abnormalities which could not be detected using classical cytogenetics have been revealed by Molecular Biology techniques. An example is the small interstitial deletions of the X chromosome leading to amenorroea or premature menopause (Krauss et al, 1987). Chromosomal marker probes have also been used to identify loss of genetic material from tumour tissues such as insulinomas and parathyroid adenomas in cases of Multiple Endocrine Neoplasia (MEN) type I (Larsson et al, 1988, Friedman et al,1989, Thakker et al, 1989). This kind of analysis may lead to clarification of the pathogenetic mechanism of tumorigenesis in these cases.

RFLPs have been extensively used in the study of the MEN type IIa syndrome and have led to the identification of the genomic locus which is, in all probability, responsible for the inheritance of this disorder (Mathew et al, 1987, Simpson et al, 1987). Results from family screening have already been published (Sobol et al, 1989). More recently, the genomic locus for MEN type I has also been identified (Larsson et al, 1988, Thakker et al, 1989). The most commonly inherited endocrine disorder, type II diabetes mellitus, has also been studied by RFLP analysis, although without much success probably because it is a heterogeneous and multifactorial disorder (Bell et al, 1987). This kind of analysis has also led to the identification of the

genomic locus for hereditary hypophosphataemic rickets (Read et al, 1986, Thakker et al, 1987).

PCR can be used to amplify polymorphic restriction sites and the DNA can be subsequently assayed by gel electrophoresis and staining without Southern blotting (Feldman et al, 1988). However, in my view, the main contribution of PCR to Endocrinology is the possibility of amplifying specific regions of DNA (or cDNA) for sequence analysis or cloning. Insulin receptor gene defects have been identified in a case of acanthosis nigricans associated with diabetes mellitus, insulin resistance and the polycystic ovary syndrome (Moller and Flier, 1988). It has also been used successfully to type the polymorphic HLA DQ beta region, which is highly linked to the predisposition to type I diabetes mellitus (Todd et al, 1987).

I.A.2. RNA STUDIES.

The methods commonly used for RNA studies are Northern blot analysis or dot blot analysis which are both based on hybridisation to a radiolabelled probe and reveal the presence and level of expression of a particular gene. Northern blot analysis also indicates the size of the RNA molecules and is technically similar to Southern blotting (see Chapter II). For more detailed studies RNA molecules are copied into cDNA using the enzyme reverse transcriptase and the DNA can then be studied by the methods which were referred to in the previous paragraphs.

Study of endocrine gene expression by RNA analysis gives a better indication of the activity of a gene than peptide analysis as the latter reflects the released or the stored forms and not the immediate production. As the epitopes recognised by antibodies may be only a few amino acids, they may on the one hand fail to recognise closely related species, whilst on the other hand they may cross-react with

other proteins which are not otherwise related. By contrast, nucleic acid probes interact through more extensive sequences and when cross-hybridisation is seen it tends to indicate a significant homology provided hybridisation conditions are sufficiently stringent. Molecular hybridisation can also lead to the recognition of different but related mRNA species. A classical example is CGRP, a product of the CT gene produced through an alternative splicing mechanism, which will be discussed later.

A route to the identification of new peptides is illustrated by the POMC gene - sequencing of the complete mRNA revealed the presence of several new peptides within the precursor protein which had not previously been characterised (Nakanishi et al, 1979, Takahashi et al, 1983). Very often study of the peptide is combined with molecular biology. Initial studies provide limited amino acid sequence information which is sufficient to permit isolation of cDNA clones and hence prediction of the complete peptide sequence. An important illustration of this is provided by the characterisation of the humoral hypercalcaemia of cancer factor, which had long been known to exist but had proved very difficult to characterise (Suva et al, 1987, Broadus et al, 1988).

Sequence determination often leads to the identification of relationships between genes. For instance, sequencing of the cDNA for various steroid hormone receptors revealed similarities with the thyroid hormone and retinoic acid receptor. An interesting super-family of genes was thus identified (Evans 1988).

Finally, there are examples in which molecular studies have been useful in explaining clinical observations. For instance, ectopic expression of the insulin-like growth factor II gene in non-insulinoma tumours explains the hypoglycaemic episodes associated with this condition (Daughaday et al, 1988).

I.B. CALCITONIN AS A HORMONE

I B.1. Discovery and isolation.

In 1962 a new hypocalcaemic factor was discovered by Copp and colleagues, which was originally considered to be of parathyroid origin (Copp and Cheney, 1962). They showed that perfusion of the thyroid-parathyroid complex of the dog with hypercalcaemic blood caused a fall in systemic blood calcium, which occurred earlier and was of greater magnitude than that produced by total thyro-parathyroidectomy. Since hypercalcaemic perfusion of the whole thyroid alone had no effect, they concluded that the source of this factor must be the parathyroid glands (Copp and Cheney 1962, Copp et al 1962). Later work by Foster et al disproved this latter finding, which was apparently due to some technical problem (Foster et al 1964a). Copp gave this factor the name calcitonin (CT). Kumar et al soon confirmed the existence of CT but expressed some reservations about its origin, because they noted that perfusing the thyroid separately from the parathyroids was technically very difficult in the dog (Kumar et al 1963).

Hirsh et al independently showed that a similar hypocalcaemic factor probably originated from the thyroid in rats. They noticed that parathyroidectomy by excision had a less dramatic hypocalcaemic effect than parathyroidectomy by cautery, during which the thyroid itself gets injured as well. They suggested that this hypocalcaemic agent was probably released from the thyroid during the injury (Hirsh et al, 1963).

The thyroid origin of CT was definitively demonstrated by Foster et al (1964a).

Calcitonin was first isolated from pig thyroid by Baghdiantz et al in 1964. Subsequently, calcitonins from

various species have been isolated and sequenced (for review see MacIntyre 1978, Lasmoles et al, 1985a). The human form of the hormone was isolated from medullary carcinoma of the mediastinum (Riniker et al, 1968) and subsequently sequenced (Neher et al, 1968).

I.B.2. Biochemistry.

CT is a 32 amino acid (a/a) peptide hormone which has a 1-7 disulphide bridge at its aminoterminus and a prolinamide at its carboxy terminus. The structure of CTs from several species is now known both from mammals as well as from submammalian species (figure I.2). They display a similar characteristic a/a sequence. They all are 32 a/a long, have a highly conserved region at the N-terminus, in which the 1-7 disulphide bridge is included, and a carboxyterminal prolinamide.

There is marked variability in the mid portion of the molecule. This was a striking observation when the first calcitonins were sequenced as it was noticed that for instance, between the porcine CT and the human CT, there is a total of 18 substitutions (<50% homology), which is very high for two relatively closely related species (Neher et al 1968). The middle part of the molecule probably controls duration of action and potency. The C terminal tetrapeptide does not seem to be of great importance in this respect (Pless et al , 1971).

The close homology concerns only 8 out of the 32 amino acids (positions 1, 4, 5, 6, 7, 9, 28 and 32). The conserved (invariant) amino acids seem to be responsible for the common properties of the CTs.

Among the CTs from various species one can distinguish three types, which share structural features: The teleost (bony fish) type, the artiodactyle (bovine) type and the human/rat type (fig I.2). There is crossreaction between assays for peptides in the same group but no crossreaction between groups.

<u>COMPARISON OF CALCITONIN PEPTIDES</u>

	*			*	*	*	*		*																			*				*	*
Man	C	G	N	L	S	T	C	M	L	G	T	Y	T	Q	D	F	N	K	F	H	T	F	P	Q	T	A	I	G	V	G	A	P	G
Rat	C	G	N	L	S	T	C	M	L	G	T	Y	T	Q	D	L	N	K	F	H	T	F	P	Q	T	S	I	G	V	G	A	P	G
Pig	C	S	N	L	S	T	C	V	L	S	A	Y	W	R	N	L	N	N	F	H	R	F	S	G	M	G	F	G	P	E	T	P	G
Cow	C	S	N	L	S	T	C	V	L	S	A	Y	W	K	D	L	N	N	Y	H	R	F	S	G	M	G	F	G	P	E	T	P	G
Sheep	C	S	N	L	S	T	C	V	L	S	A	Y	W	K	D	L	N	N	Y	H	R	Y	P	G	M	G	F	G	P	E	T	P	G
Salmon 1	C	S	N	L	S	T	C	V	L	G	K	L	S	Q	E	L	H	K	L	Q	T	Y	P	R	T	N	T	G	S	G	T	P	G
Salmon 2	C	S	N	L	S	T	C	M	L	G	K	L	S	Q	D	L	H	K	L	Q	T	F	P	R	T	N	T	G	A	G	V	P	G
Salmon 3	C	S	N	L	S	T	C	V	L	G	K	L	S	Q	D	L	H	K	L	Q	T	F	P	R	T	N	T	G	A	G	V	P	G
Eel	C	S	N	L	S	T	C	V	L	G	K	L	S	Q	E	L	H	K	L	Q	T	Y	P	R	T	D	V	G	A	G	T	P	G
Chicken	C	A	S	L	S	T	C	V	L	G	K	L	S	Q	E	L	H	K	L	Q	T	Y	S	R	T	D	V	G	A	E	T	P	G

fig 1.2. Structure of CT peptides from various species.

The highly conserved a/a are marked with asterisks. The three types of CTs are grouped in the order: human/rat, artiodactyle and fish type. The C-terminal glycine is included to maximise homologies but this is eliminated during processing to leave only an amide group in the secreted peptide.

One of the striking findings about the activity of the various CTs was the fact that the submammalian CTs (also called ultimobranchial CTs), and in particular salmon CT, were found to be more potent in their hypocalcaemic effect than mammalian CTs, as tested by the in vivo rat hypocalcaemic assay. It appears that the increased stability of the fish molecule and its higher affinity for its receptor are responsible for this difference in biological potency (Marx et al, 1972, Habener et al, 1971, deLuise et al, 1970). When a human CT molecule was changed by two amino acid substitutions to resemble salmon CT, it was observed that its potency increased (Maier et al, 1974).

Modifications in molecular structure of human CT, which were followed by reduction in the hypocalcaemic activity, include deletion of the C-terminal amide group and opening of the disulphide bridge (Rittel et al, 1976). However, opening of the disulphide group in the salmon molecule and replacement by an S acetamylo-methyl-cysteinyl linkage did not abolish biological activity (Orlowski et al, 1987).

Various attempts have been made to construct CT analogues more potent than salmon CT - some of them successful. In a model suggested by Eppand et al, substitution of valine with glycine at position 8 of the salmon variant as well as leucine with alanine at position 16 results in increased potency (Epand et al, 1986). These latter changes are supposed to offer increased flexibility to the molecule as the replacing a/a have less bulky side chains. Increased flexibility probably allows the molecule to attain a large number of conformations when it binds to receptor.

Synthetic analogues with many amino acid substitutions in positions 8-22 retaining the structural characteristics of the putative amphiphilic α-helical region have also proved to be more potent than salmon CT, although they have a/a sequence with minimal homology with any of the naturally occurring CTs (Kaiser and Kezdy, 1984, Green et al, 1987). These authors suggest that it is the amphiphilicity of the putative α helix that confers this increased biological

potency to the molecule.

I.B.3. Cell of origin.

In submammalian species CT is produced in a distinct organ, the ultimobranchial body which is rich in cells producing the hormone. In eel it has been isolated from the pericardium and oesophagus (in Morikawa et al, 1976). In mammals it is found mainly in the parafollicular cells of the thyroid which originate from the neural crest. Parafollicular cells were identified as the source of CT in 1964 by Foster, MacIntyre and Pearse (Foster et al, 1964b). They were subsequently called C-cells. Early in embryogenesis most of these cells migrate to the thyroid. Although the C-cells can be found everywhere in the thyroid they are not uniformly distributed. They are mostly concentrated in the central portions of the lateral lobes at the junction of the upper and middle part of each lobe (McMillan et al, 1974, Wolfe et al, 1974). This is where the neoplasia which arises from the C-cells of the thyroid is localised.

This neoplasia of the C-cells is called Medullary Thyroid Carcinoma (MTC) and occurs in a familial form in 20% of the cases. The familial form of MTC (usually part of the Multiple Endocrine Neoplasia II -MEN II- syndrome) is an interesting example of malignant neoplasia which is preceded by a relatively benign hyperplasia. Basal or stimulated CT levels can be found elevated at these early stages and CT thus serves as a marker for the disease. Familial MTC is one example of a malignancy which can be diagnosed and cured in a pre-symptomatic stage (Ponder et al, 1988, Gagel et al, 1988). Raised CT levels may be found in non-C cell malignancies such as small cell carcinoma of the lung, which are known to have neuroendocrine features (Coombes et al, 1974).

Under normal conditions, apart from the C-cells small amounts of CT have been localised in other tissues as well by use of immunohistochemical techniques. In the rat CT-

like immunoreactive material has been localised in both the anterior and intermediate lobes of the pituitary (Deftos et al, 1978). However, Jacobs et al failed to detect mRNA for CT in the rat pituitary (Jacobs et al, 1982). CT has also been extracted from the lung, gut, skeletal muscle, bladder, liver and thymus in man (Becker et al, 1979). However, the actual peptide has not been isolated from any tissue other than tumours of the C-cells in man.

I.B.4. Secretion.

A number of secretagogues have been described for CT, the first recognised one is ionised calcium. CT secretion is stimulated by an increase in the concentration of ionised calcium after calcium infusion and reduced to undetectable levels during hypocalcaemia (Copp and Cheney ,1962, Kumar et al, 1963, Potts et al, 1969, Gundmundsson et al, 1969). Haller-Brem et al have recently reported that in a rat MTC cell line, increase in intracellular calcium after the administration of ionomycin, which is a calcium ionophore, was followed by an increase in CT levels. The secretion of CT from the C-cells is thus modulated at least in part by intracellular calcium levels (Haller-Brem et al, 1987).

Abnormally high levels of magnesium also stimulate CT secretion. An increase in CT levels is also caused by a number of gastrointestinal hormones such as gastrin, cholecystokinin and glucagon (Cooper et al, 1971, Care et al, 1971). Of these, gastrin is the most potent and the synthetic pentapeptide pentagastrin -which is widely used in everyday practice as a stimulation test for CT- contains the carboxyterminal sequence of the gastrin molecule which is responsible for this activity. The stimulation of secretion induced by calcium infusion is augmented by concomitant administration of pentagastrin (Cooper et al, 1978). CT secretion is also increased by β adrenergic agents such as isoproterenol (Vora et al, 1978) and also by alcohol ingestion (Dymling et al, 1976). Of all these fac-

tors, plasma calcium seems to be the most important for human physiology (Austin et al, 1979). The plasma half life of CT is 4-15 minutes (Potts et al, 1969). It is mainly metabolised and excreted by the kidney, although it is barely detectable in urine in normal subjects (Voelkel and Tashjian, 1971).

I.B.5. Heterogeneity of circulating forms.

The molecular weight of the CT monomer is 3400. CT exists in multiple forms in plasma. Some of them are dimers or possibly polymeric forms of the hormone. It has been suggested that it is the reactivity of the disulphide linkage of the CT molecule that can give rise to a dimeric form of the hormone. Dimers and possibly polymeric forms are secreted by MTC cells in vitro (Goltzman and Tischler, 1978). These large polymeric forms appear to be biologically inactive (Goltzman and Tischler, 1978). In patients with MTC one can get as many as 4 immunoreactive peaks by gel filtration (Body and Heath, 1983). In MTC, as well as in ectopic CT production from other malignancies, the circulating forms are larger, incompletely processed forms of the CT precursor probably due to defective proteolytic processing by the neoplastic cell (Zajac et al, 1985, Riley et al 1986). However, CT is also heterogeneous in normal subjects (Girgis 1980).

The CT assays currently used are generally much more sensitive than the earlier ones as they can detect CT in unextracted plasma at concentrations in the range of 10-25 pg/ml. With such assays plasma levels of CT can be detected in 90% of individuals (Parthemore and Deftos, 1978). When an extraction method was applied for determining monomeric CT, it was found that the levels of circulating CT were much lower using this method (Body and Heath, 1983). Recently, a two site double monoclonal assay was developed (Seth et al, 1988). This assay, which is an overnight one, was found to be more sensitive than a seven

day incubation radioimmunoassay.

The commonly used bioassay for CT used to be the hypocalcaemic assay after injection of the hormone into young rats (Kumar et al, 1965). This assay had a very poor sensitivity which made it of little use for detecting CT at physiological levels. Recently, an in vitro model, the isolated osteoclast model, has been suggested as a sensitive bioassay system (Chambers and Moore, 1983, Zaidi et al, 1988). This is based on the quantification of osteoclastic activity and can detect femtomolar quantities of the hormone.

I.B.6. Biological activity and physiology.

The most obvious and probably the most important action of CT is to inhibit osteoclastic bone resorption. It has a hypocalcaemic effect when it is administered intravenously to young animals with a high bone turnover (Robinson et al, 1967). This is due to the marked decrease of calcium flow from bone to blood, which occurs after osteoclastic inhibition (Robinson et al, 1967, O'Riordan and Aurbach, 1968). The inhibitory effect of CT on bone resorption has been confirmed by various groups (Milhaud et al, 1965, Friedman and Raisz, 1965, Aliapoulios et al, 1966, Robinson et al, 1967, Raynolds et al, 1968). Besides, Kallio et al (1972) showed that CT produces marked effects on the osteoclast ruffled border 15 minutes after its administration in osteoclast tissue culture. Calcitonin administration also results in reduction of hydroxyproline excretion in urine, which is also consistent with reduced bone resorption (Martin et al, 1966, Aer 1968).

However, apart from its effect on bone, when CT is administered in pharmacological doses, it is also acting directly on kidney to increase calcium and phosphate excretion although this action is not of physiological significance in man (Ardaillou 1975). At high doses it also acts directly on kidney to increase magnesium and sodium excretion and this action is independent of the presence of

PTH (Haas et al, 1971).

Calcitonin is not considered to have a direct influence on bone formation or mineralisation in growing animals (Raisz and Kream, 1983), although there had been great controversy in the literature concerning this issue (Wase et al, 1967, Ziegler and Delling 1972, Boris et al, 1979). The direct effect on the osteoclasts has been very elegantly demonstrated by Chambers and colleagues (Chambers and Moore, 1983), who showed a direct inhibition of osteoclast motility and spreading in their in vitro system. They also showed that CT caused an inhibition of cortical bone resorption by the isolated osteoclast (Chambers et al, 1985, Zaidi et al, 1988).

Because various gastrointestinal hormones have been shown to stimulate CT release, a possible role for the hormone in controlling postprandial hypercalcaemia and conserving ingested calcium has been suggested.

When mild changes in plasma calcium occur, such as those observed after oral administration of calcium salts, an increase in immunoreactive CT can be detected. Cooper et al (1978) have reported that this change is paralleled by an increase in gastrin levels. Austin et al however, failed to detect a correlation between serum gastrin and calcitonin levels after a calcium meal (Austin et al, 1979). Based on these observations they suggested that under physiological conditions calcitonin functions to prevent postprandial hypercalcaemia and that this effect is not mediated by gastrin.

Various actions on CNS have been described for CT, all at pharmacological doses. In experimental animals CT has been found to increase the pain threshold (Pecile et al, 1975, Clementi et al, 1985) and has also been found to induce analgesia in patients with severe pain because of disseminated malignancy. CT also affects PRL and GH secretion probably acting as a neurotransmitter (Looij et al, 1988). The identification of receptors for CT in various areas of the brain and pituitary point to its role as a

neurotransmitter or paracrine regulator.

The levels of CT have been found to be lower in women than in men in most of the studies and some authors have reported that CT levels decline with age. Calcitonin levels have been found to be increased during pregnancy and lactation when the needs for calcium are high. It has been postulated that it probably acts to protect the skeleton at these times (Stevenson et al, 1979). Marked decrease in CT levels such as occurs in totally thyroidectomised patients does not result in any obvious pathological entity which could be attributed to CT deficiency. Likewise, no dramatic effects are seen which can be attributed to CT excess in patients with medullary carcinoma of the thyroid, who sometimes have very high circulating levels, although it is possible that in this case most of the immunoreactive CT is in larger and therefore inactive forms.

The physiological role of CT has not been elucidated in other animals either. It is interesting to note that in cartilagineous animals CT was found to produce a hypercalcaemic effect (Glowacki et al, 1985).

Receptors for CT have been identified on isolated rat osteoclasts (Nicholson et al, 1986), on kidney cells and brain (Marx et al, 1972, Fischer et al, 1981, Tschopp et al, 1985) and in cell lines derived from lymphoid cells and from human lung and breast cancer (Marx et al, 1974, Lamp et al, 1981, Findlay et al, 1981, Moseley et al, 1982). The number of receptors identified on osteoclasts is unusually high ($>10^6$ receptors per cell) thus explaining the sensitivity of osteoclasts to this peptide (Nicholson et al, 1986). There appear to be binding sites for CT in various regions of the brain which are distinct from Calcitonin Gene Related Peptide (CGRP) receptors. The highest concentration in CT binding sites is found in the posterior hypothalamus and median eminence (Fischer et al, 1981). Salmon CT binding to these sites could be inhibited by a 1000-fold higher concentration of CGRP or human CT (Tschopp et al, 1985). Calcitonin and CGRP were found to

interact with each other's receptor and each one inhibited binding at the high affinity receptor of the other (Goltzman and Mitchel, 1985). The presence of CT binding sites in the central nervous system and hypothalamus suggests that CT may have a role as a neurotransmitter under normal circumstances, which remains to be identified.

When CT binds to membrane receptors, it results in an increase in cyclic AMP levels suggesting that cAMP acts as a second messenger for CT (Marx et al, 1972, Heersche et al, 1974, Goldring et al, 1978). CT also results in an increase in cAMP when it is administered to kidney cell lines (Goldring et al, 1978) and to breast cancer cell lines (Lamp et al, 1981).

It has long been observed that when tissues are chronically exposed to CT, they become refractory to the hypocalcaemic action of the hormone despite the continuing presence of biologically active CT (Tashjan et al, 1978). This "escape" phenomenon is characterised by a progressive decrease in the number of its specific binding sites on bone (Tashjan et al, 1978, Findlay et al, 1981). This phenomenon is the result of down regulation of receptors perhaps as a result of continuous occupancy of the receptors by the hormone. Such a desensitisation also occurs with pre-treatment with even non-homologous peptides (cross-desensitisation) (Roos et al, 1986).

To summarise, it is not clear at all whether CT has a physiological role in man, however if it does, then this probably is to protect the skeleton in periods of calcium stress, such as pregnancy, growth and lactation (MacIntyre 1978, MacIntyre et al, 1979).

I.C. OSTEOPOROSIS: THE ROLE OF CALCITONIN AND GENETIC FACTORS

Among the various factors which have been implicated in the aetiology of osteoporosis the most important ones seem to be ageing itself and the lack of oestrogens. The decrease in bone mass is the consequence of an imbalance between bone formation and bone resorption. As calcitonin is a hormone with a strong antiresorptive action on bone, it has been considered as a possible factor playing a role in the conservation of bone mass. On the other hand, it is increasingly recognised that perhaps the most important factor for the degree of osteoporosis at a given instance is the peak bone mass which was achieved at a much earlier age. This is considered to depend on both nutritional, environmental as well as genetic factors. The possible role of genetic factors and of calcitonin adequacy in the development of osteoporosis will be discussed in detail in this section.

I.C.1. The role of calcitonin

Because the levels of circulating CT are lower in women than in men (Hillyard et al, 1978, Deftos et al, 1980) and women are more prone to bone loss than men, it has been suggested that CT deficiency may be causally related to postmenopausal osteoporosis. There have been several reports in the literature supporting this view, although there is no generally accepted view. Decreased basal CT levels were reported by Milhaud et al in one group of osteoporotics (Milhaud et al, 1978), while only stimulated levels were found significantly lower in another group (Taggart et al, 1982). In addition, basal CT levels were

reported to be lower in women after the menopause when compared to premenopausal women (Reginster et al, 1989). Reduced peak CT levels after stimulation were also found in premature menopause (Stevenson et al, 1982a). However, basal and stimulated levels of CT were not found to be affected in postmenopausal women in a different study, where CT was measured by radioimmunoassay in both extracted as well as whole plasma (Tiegs et al, 1985). In the study by Tiegs et al, as well as in a more recent one (Prince et al, 1989), CT levels were even found to be higher in osteoporotics when compared to controls suggesting that excessive calcium release from bone may be even stimulating CT secretion in osteoporosis.

It has been suggested that the well recognised beneficial effect of oestrogens on bone mass after the menopause may be mediated at least in part by CT (Stevenson et al, 1981). The administration of conjugated oestrogens was found to be associated with increased CT levels after calcium stimulation in a study by Morimoto et al (1980). However, oestrogen administration was found to have no effect on CT levels in a recent study where monomeric CT was measured (Body et al, 1989). One of the main reasons why CT has long been suggested to be the mediator of oestrogen action, was the fact that no direct effect on bone cells had been demonstrated, and it was not known whether these cells had oestrogen receptors. However, the issue was resolved recently by Eriksen and colleagues who demonstrated the presence of oestrogen receptors on osteoblast like cells (Eriksen et al, 1988).

A reduced bone mass as assessed by single photon absorptiometry in totally thyroidectomised men, who are therefore CT deficient, has been reported in one study (McDermott et al, 1983). This finding was however not confirmed in a more recent report where both single photon as well as dual photon absorptiometry was used for the assessment of bone mass (Hurley et al, 1987). Evidence for a possible role of CT deficiency in the establishment of os-

teoporosis was provided by the report of an interesting young male patient who was completely CT deficient and developed severe osteoporosis at the age of 19 years. His condition improved progressively with CT treatment (Stevenson et al, 1982b).

From all this data it is obvious that there is no conclusive evidence that CT deficiency itself is one of the factors responsible for the development of osteoporosis, although it is possible that CT deficiency at early ages could impair skeletal development and therefore influence the peak bone mass achieved. In this respect an interesting group to study would be totally thyroidectomised children, who should be followed up with bone mass measurements up to the age of 30-35 years.

Although CT deprivation may not play a major role in the pathogenesis of osteoporosis, it could be one of the many factors involved. For this reason the hormone has been often considered as a therapeutic agent for the treatment of the disease. This was mainly because CT has a proven antiresorptive action on bone, and osteoporosis is a condition where bone resorption occurs in excess of formation. Both the salmon peptide (Gruber et al, 1984, Aloia et al, 1985, Mazzuoli et al, 1986), as well as the human synthetic one (MacIntyre et al, 1988), have been reported to be effective in slowing down bone loss, or even in increasing bone density, on some occasions to the same degree as oestrogen (MacIntyre et al, 1988). In one of these studies (Gruber et al, 1984) the bone mass started to decrease again after twelve months of treatment, while in another it showed a plateau (Aloia et al, 1985), although the treatment was continuing at the same dose. An explanation offered for this reversal of bone gain after a certain period of treatment, is that a low bone turnover state is slowly established, as the reversible remodelling space is filled in (Faturechi and Heath III, 1987).

One of the drawbacks of treatment with CT is the route of administration which is by injection. However, recently intranasal CT preparations have become available; a one year trial has already been published showing that CT administration by this route was effective in preventing early bone loss in perimenopausal women (Reginster et al, 1987).

Overall it appears that it is very unlikely that CT will become a routine treatment for postmenopausal osteoporosis. It probably has a place in the treatment of those cases with a known high bone turnover and the cases where other forms of treatment such as oestrogen are contraindicated (Fatourechi and Heath III, 1987). However, it is likely that only short term benefit should be expected.

I.C.2. Genetic factors

One of the risk factors which is often cited to be predisposing to the development of osteoporosis is a positive family history (Riggs and Melton III, 1986). However, the evidence in the literature is rather weak. There have been studies with bone mass measurements in 1st degree relatives of osteoporotic women which did not find a significant resemblance in bone mass (Sowers et al, 1986). Evans et al (1988) and Seeman et al (1989) have reported a lower bone mass in the spine but not in the forearm (Evans et al, 1988). For this kind of analysis the most interesting studies are those concerning monozygotic twins. In one such study (Pocock et al, 1987), bone mass was more highly correlatd in monozygotic than dizygotic twins and this correlation was more pronounced in the spine, while in another similar study (Dequeker et al, 1987) this correlation was apparent only at a young age. The conclusion from these studies was that there is probably a genetic component affecting the peak bone mass of the axial skeleton and that environmental factors are more important than genetic ones

for the development of bone mass in the hip and wrist.

From all these data it is obvious that familial resemblance in bone mass, when it is found, could result from both shared genetic traits as well as similarities in life style, dietary habits etc. It is also evident that if genetic factors play a role in the development of osteoporosis, this must affect the peak bone mass achieved rather than the rate of bone loss.

I.D. ARE OTHER CALCITONINS PRESENT IN MAN?

Radioimmunological studies in various mammalian and sub-mammalian species have suggested that the calcitonin gene must have duplicated at some ancient time. Perez Cano and colleagues (1982 a, b) detected the presence of both human CT-like as well as salmon CT-like immunoreactive material in eels, salamanders and rats as well as in pigeons and chickens. The salmon CT-like peptide was found to be the major species in eel, chicken and pigeons, while the salamander contained equal amounts of salmon and human CT-like immunoreactive material.

Earlier studies by the same group (Girgis et al, 1980) had shown that the CT-like material present in protochordates is detected with a human, and not with a salmon antibody suggesting that the human type molecule was the ancestor gene, which had subsequently duplicated. Strangely enough, in the frog, only the human CT-like molecule was detected (Perez Cano et al, 1981). In all these studies specific RIAs in combination with high pressure liquid chromatography (HPLC) were used for the characterisation of the peptides.

The process of gene duplication is not unusual in evolution and there are many examples of peptide hormone gene duplications in man. The most characteristic example is perhaps that of growth hormone and prolactin. These peptides share a 34% degree of homology and presumably they have arisen as a result of a duplication of a common ancestral gene. They then diverged in sequence and took on separate functions. The growth hormone gene has duplicated again more recently to give the gene for the closely related peptide somatomammotropin, which is expressed in placenta (Hirt et al, 1987). Similarly, the two hypothalamic hormones oxytocin and arginine vasopressin, which are very closely related in structure, have resulted

from the duplication of an ancestral gene for the peptide vasotocin which can be identified in more primitive organisms (Acher, 1980).

More recently, using specific radioimmunoassays and reverse phase high pressure liquid chromatography Fischer et al and Tobler et al (1983 and 1984) reported the presence of salmon CT like material in medullary carcinoma of the thyroid, periventricular mesencephalon tissue and normal thyroid tissue in man, as well as in the serum of MTC patients (44%) and normal controls (62%). These findings were supported by the identification by the same group of specific binding sites for salmon CT in human brain (Fischer et al, 1981). However, it is known that salmon CT is more potent than the native hormone in humans and that this fact is probably due to the higher affinity of the salmon molecule to receptor. The salmon CT-like material identified by Fischer and colleagues coeluted with sythetic salmon CT in HPLC. These results do not agree with those reported by Perez Cano et al (1982b), who failed to detect salmon CT-like immunoreactivity in human thyroid tissue, either medullary carcinoma or thyrotoxic goitre. It is possible that this discrepancy in the results is due to the different salmon CT antiserum used by the two groups. The possibility therefore remains that one of the antibodies crossreacts with a different so far uncharacterised peptide.

One further study reports the presence of salmon CT immunoreactivity in the serum of 50% of patients with small cell carcinoma of the lung and in the culture medium of lung cancer cell lines (Gropp et al, 1985). In the same material human CT immunoreactivity was detected in only 20% of patients.

One keeps some reservations about this data which does not seem to have been reproduced. No further reports have appeared in the direction of sequence characterisation of these peptides and it is obvious that sequence analysis is needed to provide the final proof that these peptides do

exist.

Some weak and rather inconclusive evidence for the existence of a salmon CT-like peptide in human MTC has been published by Lasmoles et al (1985b) who used molecular biology techniques. They used a chicken (similar to salmon) CT specific probe on mRNA extracted from human MTC and detected cross-hybridisation. Their control was a dot blot of human CT cDNA which did not hybridise to the chicken probe. As DNA-DNA hybrids have different stability from RNA-DNA ones, it is not possible to tell that the mRNA species detected under their conditions was not in fact human CT crosshybridising with the chicken probe. In the same paper, mRNA from MTC directed the in vitro synthesis of a peptide which cross-reacted with salmon CT antiserum. Again, this mRNA and peptide data still awaits confirmation and characterisation.

If all this data is confirmed, and salmon CT is indeed present in man, this will mean that the measurements of circulating CT by RIA may not be reflecting the actual biologically active circulating CT. If this putative salmon CT like peptide could be identified and characterised in man, it could possibly become a useful therapeutic agent.

Footnote to page 41

* These residues, 5 in all, are removed during processing together with the C-terminal proline of the CT peptide.

I.E. MOLECULAR BIOLOGY

I.E.1. Calcitonin mRNA - The calcitonin precursor

Study of the messenger RNA for CT showed that, like many peptide hormones, CT is produced as part of a larger polyprotein, which has a typical structure of a regulatory peptide precursor.

Using cell free translation systems and suitable antibodies various groups were able to isolate the mRNA that directed the synthesis of CT precursor (Jacobs et al, 1979, Alison et al, 1981). This product was found to be larger in vitro than is observed in vivo apparently because it was not cleaved properly in cell free systems. The mRNA for CT was cloned both in rat and man by various groups and analysed in detail (Alison et al, 1981, Jacobs et al, 1981, Craig et al, 1982, Amara et al, 1982a, LeMoullec et al, 1984). It is approximately 1000 nucleotides long and encodes a precursor protein of 141 a/a in man. The 32 a/a peptide is flanked by an 82 a/a N-terminal peptide and a 21 a/a C-terminal peptide. It is separated from its flanking peptides by paired dibasic amino acids, which are recognition sites for proteolytic enzymes.* At the N-terminus this precursor contains a small hydrophobic leader (signal) sequence of the type found in secreted proteins, which is 24 a/a long (Wickener and Lodish, 1985). The CT precursor in the rat is slightly smaller (136 a/a). This difference in size is due to the C-terminal peptide which is 16 a/a in the rat.

The C-terminal peptide was found to be co-secreted with CT in equimolar amounts both in MTC and normal subjects (Amara et al, 1982a, Birnbaum et al, 1983, Roos et al, 1983, Hurley et al, 1988). It is not known whether these N- and C-terminal peptides have any physiological function. The N terminal peptide is apparently cleaved first; inter-

mediates containing the C-terminal (and not the N-terminal) within the precursor have been identified. The N-terminal peptide (57 a/a in the rat) has been recently shown to be co-secreted with CT in equimolar amounts and appears to be coordinately regulated (Burns et al, 1989). More recent studies by the same group suggest that the N-terminal peptide is potent in stimulating the proliferation of osteoblasts in cell cultures (Roos et al, 1989).

As is the case with practically all mammalian genes, the sequence present in the mRNA for CT is represented in the human genome in a discontinuous structure with four exons separated by three intervening sequences (introns). Of the four exons, exon I is non coding, exons II and III contain the information for the signal peptide and the N-terminal peptide of the CT precursor and exon IV contains the information for the last 7 a/a of the N-terminal peptide, CT itself and the 21 a/a C-terminal peptide (fig I.3).

I.D.2. Calcitonin Gene Related Peptide (CGRP)

Shortly after the analysis of the CT mRNA and the characterisation of the precursor polyprotein it was realised that the CT gene is more complex than this. In 1981 while studying CT mRNA in serially transplanted rat MTC cell lines, Rosenfeld et al observed that these lines switched spontaneously from high to low levels of CT production. This change coincided with the appearance of a new, slightly larger, mRNA species (Rosenfeld et al, 1981).

When this new mRNA was cloned and sequenced, it was found that it contained exons I-III of the CT mRNA and that exon III was spliced to two further exons, exons V and VI (fig I.4), (Amara et al, 1982b, Rosenfeld et al, 1982). This new mRNA was found to code for another polyprotein, which did not contain CT sequences. This polyprotein has an N-terminal peptide of 80 a/a, 75 of which are also part of the CT precursor encoded by exons II and III, and then continues with a 37 a/a peptide followed by a C-terminal

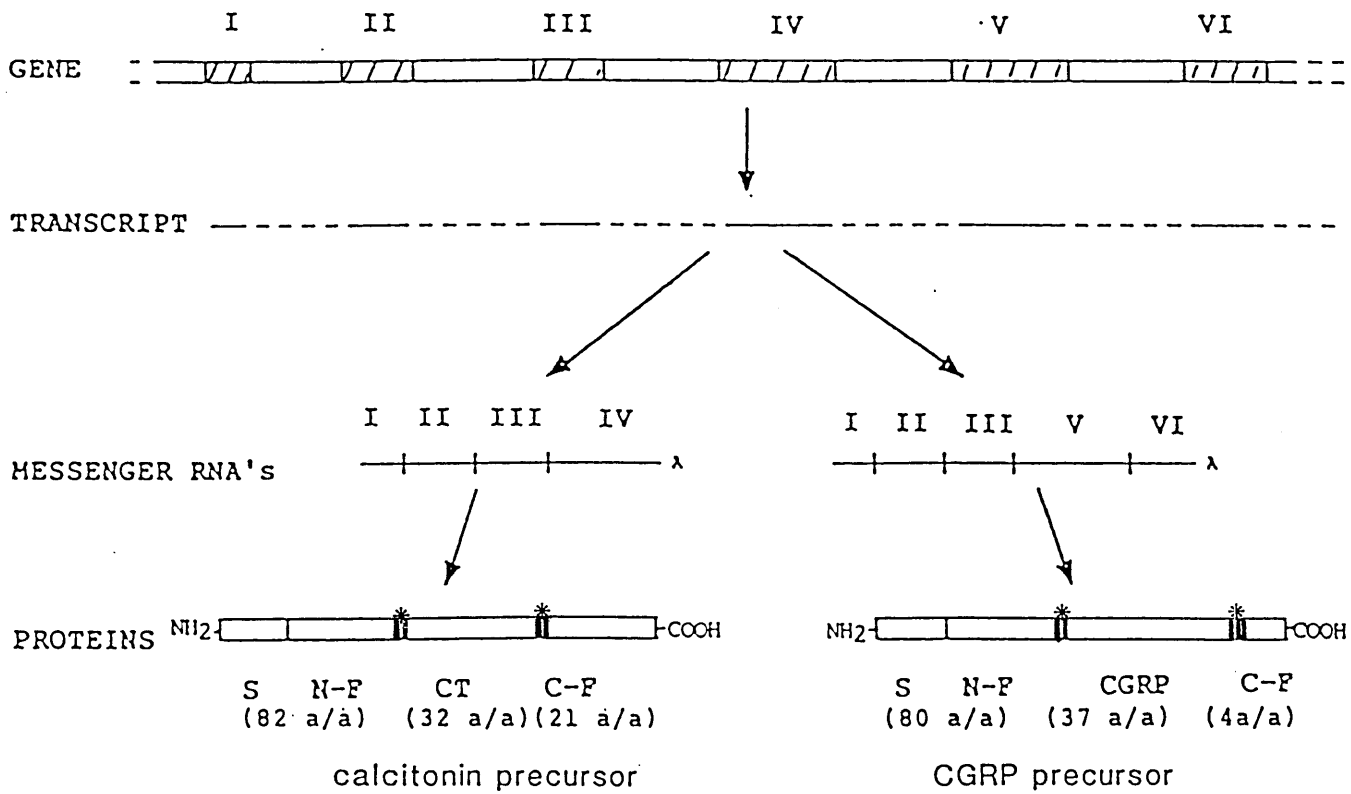

fig 1.3. Structure of the CT and CGRP precursor polyproteins.

The first 75 a/a of the respective amino-terminal flanking peptides (encoded by exons II and III) are identical. 24 a/a at the beginning of this sequence constitute a hydrophobic leader sequence. The dibasic a/a flanking CT (▮*....*▮) are Lys Arg at the N terminus and Lys Lys Arg at the C terminus. In the case of CGRP the respective sequences are Lys Arg and Arg Arg Arg Arg. [S:leader sequence, N-F:N-terminal flanking peptide, C-F:C-terminal flanking peptide].

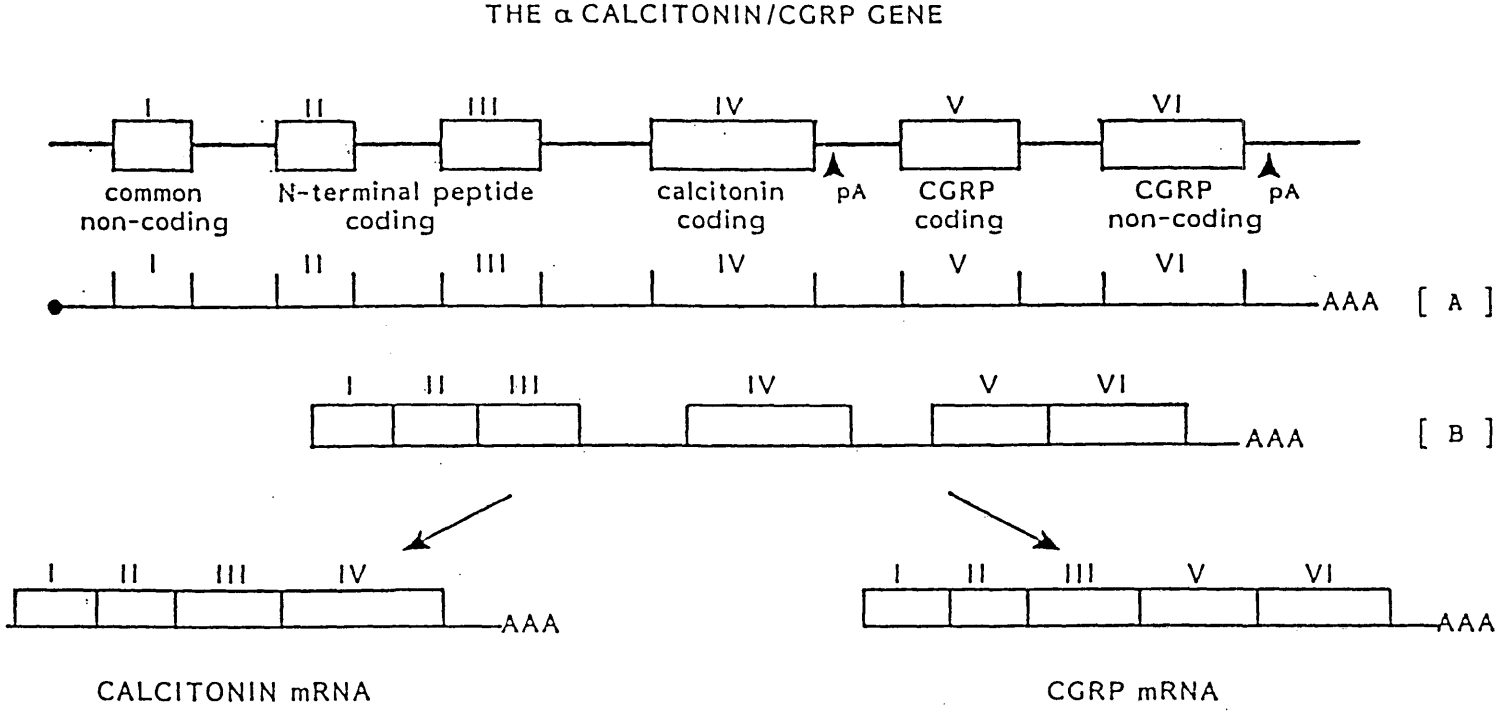

fig 1.4a Structure and differential expression of the CT/CGRP gene.

[A]. Primary transcript of the gene.
[B]. Partially processed intermediate RNA.
(pA: polyadenylation signal).

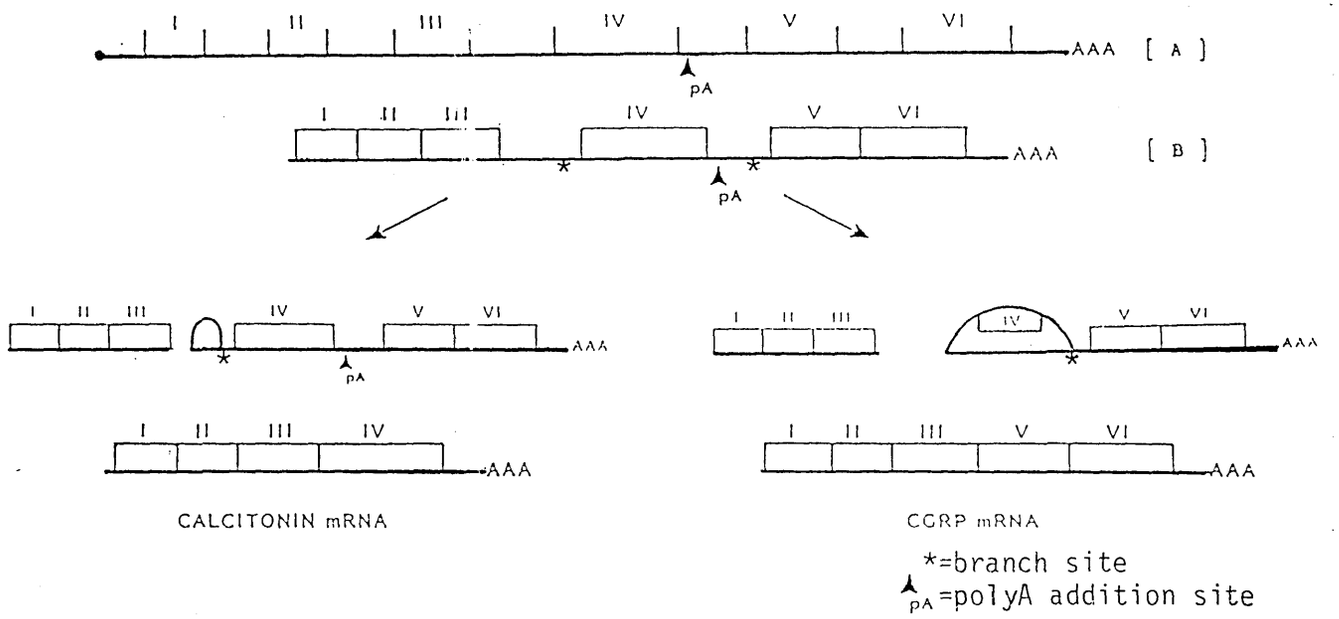

fig 1.4b Alternative splicing of the CT/CGRP gene.

The primary transcript of the gene [A] includes all exon sequences in all tissues. The common partially processed intermediate RNA species [B] will then be cleaved at the 5' of intron III. The alternative splicing will then depend on the 3' splice site and branch point which will be chosen for the lariat formation: the one at the end of intron III will lead to CT mRNA formation (left), while the one at the 3' end of intron IV will lead to CGRP mRNA (right).

tetrapeptide (fig I.3). The last 5 a/a of the N-terminal peptide and the 37 a/a peptide are encoded by exon V, while exon VI is non-coding. Paired dibasic aminoacids are again found between the 37 a/a peptide itself and its flanking N-terminal and C-terminal peptides.

This new predicted peptide was named Calcitonin Gene Related Peptide (CGRP). This peptide has certain structural similarities to CT in that it is similar in size and has a 2-7 disulphide bridge at its amino-terminus. CGRP mRNA was soon identified in man as well (Steenbergh et al, 1984, Nelkin et al, 1984, Edbrooke et al, 1985) and the actual peptide was isolated and sequenced from human MTC tissue confirming the existence of the peptide in man (Morris et al, 1984). CGRP plasma levels were reported to be higher than those of CT (Girgis et al, 1985).

In contrast to CT, CGRP is very well conserved between the various species in which it has been isolated and studied (fig I.5). Human αCGRP differs by four a/a from rat, by four a/a only from chicken, by 5 a/a from bovine (Collyear et al, unpublished) and by six a/a from porcine CGRP (Amara et al, 1983b, Steenbergh et al, 1984, Minvielle et al, 1986, Kimura et al, 1987).

It soon became obvious that CGRP is a neuropeptide as it was found to be widely distributed in the central and peripheral nervous system (Rosenfeld et al, 1983). CGRP has been extracted from rat hypothalami and trigeminal ganglia (Rosenfeld et al, 1983), as well as from spinal cord, pituitary, medulla and pons (MacIntyre 1984). Its mRNA has been localised in various regions of the brain, such as trigeminal ganglia, lateral medulla and mid brain (see section about differential expression below). In trigeminal ganglia 1% of the neurons are CGRP producing and contain 4000-20000 of mRNA copies per cell, a concentration similar to that of prolactin and growth hormone in their respective cells of origin (Rosenfeld et al, 1983).

From the pattern of its distribution in neural tissue

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           *   * * * * * * * * * *     * * * * * *     *   * * * * *   *       * * *

Human A  A C D T A T C V T H R L A G L L S R S G G V V K N N F V P T N V G S K A F G
Human B  A C N T A T C V T H R L A G L L S R S G G M V K S N F V P T N V G S K A F G
Rat   A  S C N T A T C V T H R L A G L L S R S G G V V K D N F V P T N V G S E A F G
Rat   B  S C N T A T C V T H R L A G L L S R S G G V V K D N F V P T N V G S K A F G
Cow      S C N T A T C V T H R L A G L L S R S G G V V K E H F V P T N V R T E A F G
Pig      S C N T A T C V T H R L A G L L S R S G G M V K S N F V P T D V G S E A F G
Chicken  A C N T A T C V T H R L A D F L S R S G G V G K N N F V P T N V G S K A F G
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fig 1.5. Comparison of CGRP peptides from various species.

(reviewed by Zaidi, Breimer and MacIntyre 1987) it has been suggested that CGRP may be acting as a neurotransmitter or a neuromodulator probably having a function in nociception, ingestive behaviour and mineral metabolism (Rosenfeld et al, 1984a). Its physiological function has not been established yet; however, some of its biological properties have been characterised. It is a potent vasodilator (Brain et al, 1985, McCulloch et al 1986) and it has been shown to increase the force and rate of contraction of heart muscle in various species (Tippins et al, 1986, Holman et al, 1986). CGRP has been shown to increase the synthesis of the acetylcholine receptor probably acting as a trophic factor when released from the motorneurons (New and Mudge, 1986). More recently, Denis-Donini has demonstrated a clear neurotropic effect for CGRP: It acts as a differentiation signal for dopaminergic neurons in the olfactory bulb (Denis-Donini, 1989). McCulloch and colleagues have suggested that CGRP probably has a physiological role in regulating cerebrovascular blood flow (McCulloch et al, 1986). It is not known whether CGRP has a true hormonal role. Circulating CGRP probably originates from perivascular nerve endings (Zaidi et al, 1985).

Possibly as a result of its structural similarity to CT, CGRP has been found to interact with CT receptors (Goltzman and Mitchel, 1985). It was thus found to have CT-like effects on bone at a 1000-fold higher concentration than that of CT (Zaidi et al, 1987). It is not unusual for different members of the same gene family occasionally to occupy one another's receptors, thereby simulating each other's action (Fradklin et al, 1989).

I.E.3. Characterisation of the CT/CGRP gene.

Study of the CT gene showed that the six exons span an area of 6.5 kilobases in man. The point of divergence between CT and CGRP mRNAs was found to correspond to a splice junction (Amara et al, 1982b), while nuclear transcripts

were identified which contained both CT and CGRP sequences (Amara et al, 1982b, Nelkin et al, 1984). The human gene was cloned by Jonas et al (1985), as well as by Lips and colleagues (Steenbergh et al, 1985).

Using linkage analysis based on the frequency of recombination Kittur et al (1985) localised the CT/CGRP gene on the short arm of chromosome 11 between the genes for PTH and Catalase. More specifically, another group using the technique of in situ hybridisation localised the gene to 11p13-p15 (Przepiorka et al 1984). In parallel, using human mouse somatic cell hybrids Hoppener et al (1984) placed the gene to roughly the same region (p14-qter) of chromosome 11.

I.E.4. Differential expression of the CT/CGRP gene.

After it became apparent that CT and CGRP were alternative products of the same gene, and that each peptide was encoded by related but different mRNA molecules, a lot of work was done trying to elucidate the mechanism responsible for this differential expression of the CT/CGRP gene.

First of all it was found that the differential expression was tissue specific (Rosenfeld et al, 1983). In thyroid C cells the main product of the gene is CT mRNA with CGRP mRNA representing <10% of the transcripts (Sabate et al, 1985). In neural tissue on the other hand, this gene gives rise almost exclusively to CGRP specific mRNA. An exception to the observations above is medullary carcinoma of the thyroid, in which substantial expression of CGRP mRNA also occurs and is found at varying levels when compared to CT. The relative concentration of CGRP/CT mRNA varies between <2% and >90% (Rosenfeld, Amara and Evans 1984b). In MTC cells CGRP and CT have occasionally been found to be co-localised in the same cell (Sikri et al, 1985, Zajac et al, 1986). Both the alternative mRNAs originating from this gene have also been detected in various lung carcinoma cell lines (Nelkin et al, 1984,

Edbrooke et al, 1985) again at varying levels of expression.

CGRP mRNA has been found in thyroid tissue, MTC and various areas of the brain, such as the hypothalamus, lateral medulla, mid brain and the trigeminal ganglion in the rat (Rosenfeld et al, 1983, Amara et al, 1985, Sabate et al 1985). CGRP mRNA has also been detected in dorsal root ganglia and intestine (Mulderry et al, 1988). In man, expression has been detected in thyroid and MTC tissue and MTC cell lines, as well as in lung carcinoma tissue and derived cell lines (Steenbergh et al, 1984, Nelkin et al, 1984, Edbrooke et al, 1985). It has also been detected in phaeochromocytoma tissue but not in human pituitary tissue (Hoppener et al, 1986). Jonas et al (1985) have however reported identification of very low levels of both mRNAs in human pituitary. A characteristic finding about the expression of this gene in neoplasia is the identification of larger mRNA species of 4.2, 3.3 and 2.3 kb in Northern blots, which apparently represent partially processed immature mRNA molecules (Edbrooke et al, 1985).

I.E.5. Alternative processing of the precursor RNA.

There are various mechanisms whereby multiple mRNAs can originate from a common genomic locus. One possibility would be that alternative sites are used for initiation of transcription or that there is alternative termination of transcription, for example in the end of exon IV in the case of CT mRNA. However, it was shown that in all the cells which express the CT/CGRP gene, and irrespective of the final product, the whole of the gene is always transcribed with transcription starting at the same point and terminating at a distance of around 1 kb after the end of exon VI. Transcription proceeds through CT and CGRP coding exons at the same rate (Amara et al 1984). This observation excludes the possibility that the differential expression could be achieved through the alternative ter-

Footnote to page 50.

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There are three important steps in the processing of a precursor RNA to produce mRNA - capping, polyadenylation and splicing. Capping, the addition of methylated guanine nucleotide to the 5' end of the RNA, is unlikely to be important in the differential regulation of CT/CGRP mRNA production since the two are identical at their 5' ends. Polyadenylation I will take to include the process by which the prospective 3' end of the mRNA is recognised within the precursor and is cleaved and then modified by the addition of 100 - 200 adenosine residues. This is a potential point at which regulation could be achieved as cleavage at the end of the CT exon would clearly preclude CGRP mRNA production (see figure 1.4a). The other likely point of differential control is splicing - the process by which intervening sequences (introns) are removed from the precursor RNA and exon sequences become joined together to produce the mature mRNA. This process is illustrated in figure 1.4b. The first step in splicing is cleavage of the RNA at the 5' end of the intron. The intron sequence then forms a looped structure (a lariat) in which its 5' end is covalently joined to a base located near its 3' end, usually at a distance of 15 to 40 bases from the following exon. The lariat is then removed by cleavage at its 3' end and the two exons are spliced together. Splicing therefore requires the presence and correct recognition of a pair of 5' and 3' splice sites and a suitable branch point sequence for lariat formation. The CT and CGRP specific splices both involve the same 'splice donor' sequence (exon III). If there is differential splicing then attention must be focussed on the sequences at the 3' ends of introns III and IV and/or the factors recognising those sequences.

mination of transcription. The differential regulation of expression must therefore be done at the post-transcriptional level and must affect either RNA processing or RNA stability (Leff, Rosenfeld and Evans, 1986). *

There is no doubt that the polyA addition site used in the two alternative products of the gene is different: it is located at the end of exon IV in the case of CT and at the end of exon VI in the case of CGRP. The polyadenylation signal at the end of exon IV has the sequence AATAAA, while the one at the end of exon VI has the alternative sequence ATTAAA. The molecular basis for the alternative RNA processing therefore involves either alternative polyadenylation or alternative splicing patterns or a combination of the two. The question then was whether the selection of the polyA site was determined first and splicing followed, or whether it was the splicing choice which was the regulated event and this then directed the polyadenylation selection.

A detailed analysis of the various intermediate RNA forms (products of partially processed RNA) detected in an MTC cell line led Bovenberg et al (1986) to the conclusion that polyadenylation at the end of exon IV generally occurs after some introns have been removed. They suggested that the final common intermediate RNA form (a 3.3 kb species) still contains introns III and IV as is schematically presented in fig I.4 and that this is the decisive step in commiting the cell to produce one or the other mRNA product. At this point therefore cleavage and polyadenylation at the end of exon IV leads to CT mRNA after splicing of intron III, or alternatively, splicing of exon III to V gives rise to CGRP mRNA. Detailed study of all the possible partially processed RNA intermediates has not been possible, apparently because they are short lived (Bovenberg et al, 1986).

The mechanism whereby the differential expression of this gene is achieved, was somewhat clarified recently after the elegant work of Leff et al, who introduced constructs of

the gene to cell lines which were known beforehand to have a characteristic pattern of expression (Leff et al, 1987). For example, a lymphoid cell line was used for their study, which expressed almost exclusively CT mRNA. Another cell line used was derived from a carcinoma of the adrenal medulla and expressed predominantly CGRP mRNA. The CT gene was appropriately modified and re-introduced to the lymphoid cell line to see if the mode of expression could be altered. It was found that when the CT polyadenylation site was mutated, long transcripts were accumulating and the expression of the gene was not converted to the alternative product, as if the necessary machinery for CGRP production was not present in these cells.

When CGRP producing cells were transfected with a mutated gene lacking exon III, they were able to recognise the exon IV poly-A signal, which means that CGRP is normally produced because exons III and V are spliced together and not because the exon IV poly-A site cannot be recognised. They also found that when most of intron III had been deleted, the lymphoid cell line was then able to splice exon III to V and produce CGRP.

From these experiments Leff et al conclude that cleavage and polyadenylation at the end of exon IV could occur in all types of cells; cells producing CGRP appear to have a necessary factor or factors, which, perhaps by affecting the secondary structure of the precursor RNA , allows the splicing of exon III to V to take place and somehow "hides" the exon IV poly-A site thus commiting the cell to CGRP production (see fig I.4).

Data in support of a "splice commitment factor" exclusive to neuronal tissue was also presented by Crenshaw et al (1987) from the same group, who introduced a metallothionein calcitonin fusion gene to transgenic mice. They found that the gene produced almost exclusively CT mRNA in every tissue of the animal except in neural tissue, where it produced mostly CGRP mRNA. From this work it appeared therefore that CT mRNA production is a "null-choice" and

does not require the presence of a specific factor.

In a more recent paper by the same group (Emeson et al, 1989) regions of the gene were clarified which are important for the regulation of processing of the CT/CGRP gene transcript. The authors used again two cell lines expressing predominantly one or the other product. When the cell lines were transfected with a variety of mutant genes, it was shown that the selection of the alternative splice site is regulated by active elements near the CT specific 3' splice junction. It was suggested that there must be sequences near the CT splice acceptor site which inhibit the formation of CT in CGRP producing cells. Deletion of approximately 40 bases in this region (-58 -17) resulted in increased production of CT in cells which were previously unable to make CT. When this region was intact, and the CGRP splice acceptor mutated, there was no switching to CT production. This is consistent with the view that CGRP producing cells have a cell specific inhibition of the CT splice acceptor, which requires that the CT acceptor sequences are intact. Moreover, cells which normally produce CT, were able to produce CGRP when the CT splice junction was removed. This last observation suggests that for CGRP production there is no absolute requirement for a neural cell specific factor.

I.E.6. Regulation of transcription.

The factors that regulate transcription of this gene are not known. Vitamin D has been suggested as an important regulator of CT gene transcription in a recent study (Naveh Many and Silver, 1988). In this study $1,25(OH)_2$ D3 administration at low doses was shown to markedly inhibit CT gene trancription to <10% of controls at 48 hrs in rat thyroids; this vitamin D dose had not caused a rise in calcium levels. This effect was even seen with doses as low as 12.5 pmol of $1,25(OH)_2$ D3. The mRNA detected was of

normal size suggesting that the effect was not in RNA processing. Vitamin D administration in very high doses (1μg/kg body weight) had been shown to cause a transient 2-fold increase in CT mRNA in a previous study (Segond et al 1985). On the contrary, there was no effect of high calcium (up to 5mM) on CT mRNA production from thyroid slices in vitro (Jacobs et al 1983). Acute hypercalcaemia induced in vivo resulted in a 4-fold increase in immunoreactive CT, which occurred as early as 2 min after calcium administration, but had no effect on CT mRNA level (Segond et al, 1984).

Glucocorticoids and in particular dexamethasone has been found to increase transcription of the gene, resulting in an increase in cellular levels of immunoreactive CT in an MTC cell line, which occured after 48 hrs and reached its maximum after 7 days (Muszinsky et al, 1983). Administration of other steroids such as oestradiol, methyltestosterone or vitamin D to the same MTC cell line had no effect on cellular CT levels. In a different study, dexamethasone was again found to increase CT mRNA levels, while at the same time decreasing CGRP mRNA levels (Cote and Gagel, 1986), thus suggesting that this was perhaps an effect on the splicing mechanism, which favoured the production of CT over that of CGRP mRNA in the MTC cell line studied. In a more recent work (Russo et al 1988), where a different MTC cell line was used, it was shown that dexamethasone mainly increased the transcription rate of the gene and perhaps also slightly favoured CT over CGRP mRNA production.

Treatment with phorbol esters (which are considered to be activators of protein kinase C) and dibutyryl cAMP (activator of protein kinase A) induces a differentiated phenotype in MTC cells in culture and resulted in an increase in CT and CGRP expression (deBustros et al, 1985 and 1986). The increase was 2-fold after treatment with phorbol esters and affected equally CT and CGRP. Introduction of the viral oncogene Harvey ras into MTC cells which had lost their differentiated characteristics (low CT/high CGRP

producers), caused a switch of the splicing pattern from CGRP to CT production (Nakagawa et al, 1987).

The degree of methylation of the 5' region of the gene in various cancer cell lines has been studied extensively by one group. This region was found to be hypermethylated in lung cancer and lymphoma cells, while it was hypomethylated in MTC cells (Baylin et al, 1986). In the lung cancer cells there was a striking correlation between the distribution of neuroendocrine features (such as L Dopa-Decarboxylase activity) and the methylation status of the CT gene. In another study, where cells from several different sorts of leukemias were studied, it was found that the degree of methylation of this region of the CT gene was closely linked to the malignant phenotype of the cell and was found to vary with the remission state of the patient (Baylin et al, 1987). Hypermethylation of this particular gene is not considered to have any functional implication for neoplasia, but probably reflects the methylation pattern in neoplastic cells. However, it was suggested that the study of the methylation of the 5' region of the CT/CGRP gene could be used as a molecular marker to monitor the clinical status of the patient in malignancies.

I.E.7. The beta CGRP gene.

A lot of the work on the molecular biology of the CT genes had already been published when it was realised that there were further sequences in the human genome which were related to this gene. The first indication came from the study of genomic DNA by restriction analysis, where it was realised that extra bands, not corresponding to the known gene, could be seen in whole genome Southern blots when CT cDNA (Hoppener et al 1984) or CGRP specific (Edbrooke et al, 1985) probes were used. Similar findings suggesting the possible existence of a CGRP related gene for the rat had already been reported (Rosenfeld, Amara and Evans, 1984a).

cDNA copies of messenger RNAs originating from this related gene were soon isolated and characterised both in man (Steenbergh et al 1985) and rat (Amara et al 1985) in both cases originating from MTC cells. This new mRNA was found to encode a new CGRP molecule, very similar to the known one, which was named beta CGRP and its gene, the beta gene. The well characterised CT/CGRP gene then became known as the alpha gene.

Beta CGRP differs from alpha CGRP by three amino acids in man and by one amino acid in rat. The mRNA for beta CGRP has a similar structure to that found in alpha and in the predicted precursor, beta CGRP should be cleaved in the same way as is predicted for alpha. The exon V CGRP-coding regions as well as the exon III N-terminal peptide coding regions were found to be highly homologous between the two genes (>90% homology in nucleotide sequence). On the contrary, the 3' non coding region of the mRNA (corresponding to exon VI) was found not to be so well conserved, with 65% structural homology. Specific probes for alpha and beta CGRP respectively were made from this region, which could be used to detect expression of one or other gene.

Beta CGRP was found to be expressed in various regions of the brain in rat, showing roughly the same distribution as alpha CGRP, although it was usually, but not always, expressed at lower levels (less than 20% those of alpha) (Amara et al 1985). For example predominantly beta CGRP was found to be expressed in oculomotor nucleus, while alpha CGRP was mainly expressed in the trigeminal ganglion. Beta CGRP, as well as alpha CGRP, has been extracted and partially sequenced from normal human tissue (spinal cord) (Petermann et al, 1987). These authors report that more beta than alpha CGRP can be identified in pituitary, thalamus and cerebellar cortex. In a recent paper by Mulderry et al (1988) it was found that only beta CGRP mRNA could be detected in enteric autonomic neurons, while equal amounts of alpha and beta CGRP mRNA could be detected in dorsal root ganglia. Peptide data from the same group

showed that beta CGRP was predominantly found in colon and ileum, while in all the other tissues studied such as heart, lung, bladder and dorsal spinal cord there was mainly alpha CGRP immunoreactivity present.

As the various CGRP molecules are very closely related in structure (fig I.5), it would be unlikely that they have radically different functions. Nevertheless, the two peptides have been reported to have small differences in biological potency. They were found to be equipotent in their vasodilator activity in rat skin (Brain et al, 1986) and in increasing the force of contraction in the isolated rat atrium bioassay (Tippins et al, 1986). However, beta CGRP was found to be three times more potent than alpha in increasing the rate of contraction in the same assay (Tippins et al, 1986). Holman et al compared their vasodilator activity with that of sodium nitroprusside and found that beta CGRP was 240 times as potent, while alpha CGRP was 50 times as potent in rat coronary vasculature (Holman et al, 1986). Finally, Mulderry et al found that the two peptides have equal potency in increasing the rate and force of atrial contraction, but that alpha CGRP was 2.7 times more potent in relaxing colonic smooth muscle (1988). It is not currently known whether this difference in potency reflects differences in interaction to receptor or perhaps difference in stability. It also remains unknown if the two molecules are ever co-produced in the same cell under physiological conditions. It remains to be seen what advantage is offered to some cells by the selective expression of beta rather than alpha CGRP.

As the two mRNAs are expressed in the same tissue, but at variable levels, they must be regulated independently. In a recent report (Russo et al 1988), it was shown that in a rat MTC cell line, dexamethasone treatment caused an increase in αCGRP mRNA without affecting βCGRP mRNA. It was suggested that perhaps the two genes are differentially regulated by cell specific factors or hormonal stimuli. If the two genes are indeed regulated independently, then this

mechanism must provide some functional advantage by increasing the potential flexibility of CGRP expression.

An interesting observation was that beta CGRP was found to be exclusively expressed in cell lines established from Ewing sarcomas (Hoppener et al, 1987). Beta CGRP could not be detected in a series of other neoplasias nor in a range of normal tissues from man, such as lung, liver, pituitary, thyroid, (embryonal, neonatal or adult), intestine or hypothalamus, thus suggesting that beta CGRP is not an abundant transcript in human tissue (Hoppener et al, 1987).

The beta CGRP gene was localised to chromosome 11 (q12-pter) using human rodent-somatic cell hybrids by Hoppener et al (1985). This gene was reported not to contain any CT related sequences in man (Hoppener et al, 1985, Steenbergh et al, 1985), or rat (Amara et al, 1985), while Jonas et al (1985) reported that they could detect CT hybridising regions in a human genomic clone which represented the beta gene. The possibility that a second CT might be encoded by the beta CGRP gene was very interesting particularly in view of the reports about CT related peptides in man which were mentioned in the previous section. If a second calcitonin is present in the beta gene, then its sequence must have diverged significantly, as it could not be detected under standard hybridisation conditions.

ADDENDUM ONE.

I.F. AIMS OF STUDY

Two important questions are raised by this review of the physiology and molecular biology of calcitonin. What if any role does calcitonin play in the maintenance of bone mass and is there a second, possibly more potent, calcitonin sequence encoded by the human genome?

As has already been discussed, there is no clear evidence that calcitonin plays an important role in the development of osteoporosis and several studies point to the lack of any such role (for example, Hurley et al 1987, Prince et al 1989). However it should be noted that none of these studies concerned patients with a life-long calcitonin deficiency and in particular none of them cover the period of adolescence when bone mass is increasing most rapidly. However, I have already mentioned the case of a young male patient with severe osteoporosis who was calcitonin deficient (Stevenson et al 1982b). If a defect in his calcitonin gene could be demonstrated it would provide an explanation of his calcitonin deficiency and would therefore support the contention that calcitonin plays an important role in the preservation of skeletal integrity. I therefore decided to study the calcitonin gene from this patient and the results of this study are presented in chapter III.

The second question concerns the possibility of other calcitonin sequences in the human genome. As has already been mentioned, there is no convincing evidence to support this contention but a number of findings indicate that there might be a second human calcitonin having features in common with the type of calcitonin found in fishes and birds (Fisher et al 1983, Tobler et al 1984, Gropp et al 1985, Lasmoles et al 1985b). As calcitonin is encoded by the same gene as CGRP (see figure 1.3) and a second (beta) CGRP gene had been described, it seemed possible that there might be a

second calcitonin similarly encoded in this gene. I therefore decided to isolate the beta CGRP gene and to try to identify any calcitonin related sequences within it. The 'beta calcitonin' sequence which I characterised is presented in chapter IV. I then used the techniques of low stringency hybridisation developed in cloning the beta sequence to look for any further calcitonin-like sequences which might be present in the human genome. The characterisation of the 'gamma sequence' and several other sequences which proved to have only a chance relationship with the calcitonin gene is presented in chapter V.

CHAPTER II

MATERIALS AND METHODS

A. METHODS

II.1. DNA PREPARATION METHODS.

1.1.Making genomic DNA from blood.

Two different methods were followed to make genomic DNA. One was to lyse the cells and prepare nuclei using the detergent NP40. With this method whole blood DNA is prepared. The other was to separate the white cells from the red cells before lysis. Both methods were used on different occasions.

1.1.1.Preparing DNA from white cells.

1) 20 ml of blood into heparin tubes were taken.

2) These were mixed with 20 ml of Phosphate Buffer Saline (PBS) to give 40 ml. After putting 20 ml of Ficoll/Hypaque into each of two 50 ml centrifuge tubes, 20 ml of the diluted blood were layered carefully over the Ficoll/Hypaque.

3) After making sure that they are balanced, the tubes were centrifuged at room temperature for 30 min at 400g.

4) The white cell layers were removed from the interface.

5) They were then transferred to two more centrifuge tubes and filled and balanced with PBS.

6) The two tubes were centrifuged at 400 g for 15 min.

7) The supernatant was taken off and the white cells resuspended in 5 ml of PBS, without vortexing. The two cell

suspensions were combined in a single tube, filled with PBS and centrifuged again. Then the cell pellet was left for 1-2 hours on ice.

8) One ml of urea lysis buffer was added to the cell pellet and they were resuspended using the end of a Pasteur. A second 1 ml of lysis buffer was added and the lot transferred to a disposable 10 ml propylene tube.

9) 400 µl of 20 % SDS were added and mixed with the Pasteur.

10) The mixture was icubated at 37 °C.

11) 1 ml of chloroform was added and mixed vigorously.

12) 2 ml of phenol were added and mixed vigorously.

13) The tube was centrifuged at 4000 rpm for 15 minutes.

14) The upper (aqueous) phase was carefully collected, making sure that the interface is avoided.

15) This was transferred to another tube and steps 11 to 14 were repeated.

16) The aqueous phase was extracted with 1 ml of chloroform in order to remove phenol from the DNA.

17) The final aqueous phase was transferred to another polypropylene tube and 2-3 volumes of ethanol were added and mixed by inversion. The precipitate of DNA was transferred with a "hooked" Pasteur to 1 ml of 70 % ethanol in a microfuge tube.

18) The precipitate was spun down at maximum speed for 5 min.

19) All ethanol was removed and the pellet dried.

20) The pellet was resuspended in 100 µl of TE buffer.

21) Finally, this was left overnight at room temperature to dissolve properly.

1.1.1. Whole blood method (Old 1986).

1) 20 mls of blood were taken in a heparinised "Universal" vial.

2) 30 mls of distilled water (GDW) were added and mixed by inversion.

3) The sample was centrifuged at 3,000 g for 10 min and

the supernatant removed.

4) To the nuclei pellet were added 25 mls of 0.1% NP40 and mixed.

5) The sample was centrifuged again and the supernatant removed.

The urea lysis method was then followed (Foroni et al, 1987) as described in the previous protocol from step 8 onwards.

1.2. MiniPreps of Supercoiled DNA from Bacteria.

The method used is a variation on the method of Birnboim and Doly (as described in Maniatis et al, 1982), which is based on the lysis of bacteria by SDS at high pH, followed by neutralisation.

Culture.

1) A single colony was used to innoculate 2ml of L broth containing appropriate antibiotics and grown overnight in a 25 ml Sterilin vial on an orbital incubator rotating at about 120 rpm.

2) For large-scale preps of plasmid DNA a single colony was used to innoculate 10 ml of L broth/antibiotic, grown for about 4-5 hours, then used to innoculate 400 ml of medium.

3) This was grown to an OD_{600} of 1-1.5 and chloramphenicol added to 1.5 mg/ml and then grown overnight. Large cultures were occasionally grown overnight without chloramphenicol. Use of chloramphenicol increased the yield of plasmid by a factor of 2.

The volumes described here are those for the small-scale plasmid preparation, which were scaled up for large culture volumes.

Preparation of DNA.

1) 1.5 ml of the culture was chilled on ice in a microfuge tube, then centrifuged 5 min, 6 k rpm, 4 °C.

2) The cell pellet was resuspended in 0.5 ml TSE, then centrifuged 5 min, 6 k rpm, 4 °C.

3) Cells were resuspended in 90 µl GTE, then 10 µl Lysozyme solution was added and left 10 min at room temperature.

4) Alkaline SDS (200 µl) was added to the cell suspension, mixed gently by inversion and left 5 min on ice.

5) Cold 5 M potassium acetate (150 µl) was added, mixed by inversion and left at least 15 min on ice, then centrifuged 10 min at 12000 rpm, 4 °C.

6) The supernatant was transferred into a clean microfuge tube, taking care not to carry over the precipitated cell debris. Nucleic acid was precipitated by the addition of 0.6 volume of isopropanol and centrifuged for 10 min at 12000 rpm at room temperature.

7) The pellet was drained, dissolved in 100 µl TE, RNase A added to give a final concentration of 10 µg/ml and incubated at 37 °C for 20 mins.

8) The crude plasmid DNA was deproteinised by extraction with 100 µl of phenol:chloroform by vortexing for 30 seconds. Traces of phenol were removed by ether extraction.

9) Finally, the plasmid was ethanol precipitated, the pellet rinsed with 70% ethanol, air-dried and then dissolved in 20 µl GDW. This DNA was usually clean enough to cut with an excess of restriction enzyme; however, if problems were experienced, the DNA was purified further by PEG precipitation.

1.3. Purification of plasmid DNA on CsCl-EthBr gradients.

1) The plasmid miniprep protocol scaled-up for the 400 ml culture was followed as far as step 6, then the precipitate was resuspended in 12.75 ml of TE.

2) The crude plasmid was digested with RNase at 100 µg/ml for 15 min at 37 °C, followed by proteinase K (PK) digestion at about 10 µg/ml for a further 15 min.

3) Thirteen grams of CsCl was dissolved and 0.25 ml of 10 mg/ml ethidium bromide added before transferring into a vertical rotor (TV865B) tube. The tube was sealed and centrifuged overnight at 25 °C and 45,000 rpm.

4) The plasmid band was visualised by UV and was removed from the gradient using a 200 µl capillary linked to a peristaltic pump.

5) The ethidium bromide was removed by 3 or 4 extractions with CsCl saturated isopropanol until all traces of pink colouring disappeared, then the plasmid was dialysed against TSE for 1-2 hours at room temperature.

6) To remove any PK contaminating the sample, it was extracted with phenol/chloroform and ethanol precipitated.

The plasmid was finally purified by PEG precipitation to remove oligoribonucleotides.

1.4. M13 replicative form preparation.

Small-scale preparation of replicative form M13 DNA was done essentially by combining the culture conditions of the ss M13 preparation and the plasmid DNA miniprep. Thus a 1.5 ml culture of male E.coli cells were infected with a single M13 plaque and grown as described in section II.1.7, the cells were centrifuged and the supernatant saved to make single-standed DNA or to infect further cells.

Replicative form (double stranded) DNA was prepared from the cell pellet as described in section II.1.2.

1.5. Lambda phage stocks and DNA preparation.

1.5.1. Making plating cells for lambda phage.

1) 10 ml L-broth / 10 mM $MgSO_4$ ("LBM") supplemented with 0.2% maltose was inoculated with Q358 or Q359 cells picked from a single, well-defined colony and the culture grown overnight.

2) Cells were pelleted at 6K rpm, for 10 mins, room

temperature. The supernatant was discarded and the pellet resuspended in sterile 10mM $MgSO_4$ (0.5 x original culture volume). The cells were stored at 4 °C and used for up to 3 weeks. For best results, freshly prepared cells were used.

1.5.2. Streaking out phage on lawn of cells and picking plaques.

1) A small (90mm) LBM/1.5% agar plate was dried and kept warm in the 37 °C incubator before pouring the soft agar. 0.1 ml of plating cells was mixed with 3 ml molten 0.7% agar/LBM (kept at 45 °C) and poured quickly onto the plate, tilting and swirling to distribute evenly. This was allowed to set for 10-15 minutes, without its lid in the laminar flow cabinet so that moisture could escape.

2) A loop of phage stock was gently streaked across the soft agar and then incubated upside down at 37 °C. The lawn of cells grew in 3-4 hours and plaques were visible in 7-8 hours.

3) Plaques were picked when small and well separated, using a sterile pasteur with a bulb. The pasteur was stabbed through the chosen plaque into the hard agar beneath and the agar plug gently sucked into the pasteur and ejected into 0.5 ml SM phage buffer. A drop of chloroform was added and the phage stored at 4 °C. Phage particles are supposed to remain viable indefinitely if stored at 4 °C in SM containing 0.3% chloroform.

1.5.3. Small plate lysate stocks.

1) 50,000-100,000 plaque forming units (pfu) were used per plate to prepare the plate lysates. The phage were added to 0.1 ml plating cells and pre-adsorbed at 37 °C for 20 min. This was mixed with 3 ml molten agar LBM and poured onto a wet, pre-warmed (37 °C) 90 mm plate and incubated, right-side up, at 37 °C for 7-8 hours.

2) Confluent lysis had usually occurred by this time - i.e. the plaques had expanded until they just touched, giving a mottled looking lawn. The plates were then put into the cold room (or fridge) to chill for at least 1 hour. Each plate was overlaid with 5 ml cold SM and a few drops of $CHCl_3$ added, then left O/N (or for a minimum of 3 hours) to harvest the phage.

3) As much as possible of the SM was removed with a pasteur and 0.1 ml $CHCl_3$ added, vortexed briefly and centrifuged at 4000g for 10 minutes at 4 °C. The supernatant was removed and $CHCl_3$ added to 0.3%, then stored at 4 °C.

1.5.4. Liquid culture method for preparing lambda DNA.

The method followed here is suggested by Maniatis et al (1982).

1) On day 1 a 10 ml O/N culture of Q358 was set up.

2) On day 2, one fresh plaque of lambda was picked into a 100 ml flask and 0.25 ml of O/N Q358 was added. This was left for 20 min at 37 °C. Then 25 mls of LBM was added and left shaking O/N at 37 °C. A fresh O/N culture of Q358 was set up.

3) On day 3, 500 mls of LBM were inoculated with 2.5 mls of the O/N Q358 culture and was incubated shaking at 37 °C for 2.5 hours (until OD_{600} was 0.3). Meanwhile 0.25 mls of chloroform was added to the lambda culture and shaken. The debris was spun out and the supernatant was stored at 4 °C. After the 2.5 hours 2.5 mls of this phage suspension was added to the Q358 culture and incubation was continued (shaking) for a further 3-4 hours until lysis.

After lysis, 2 mls of chloroform was added amd shaken for 5 min. The debris was spun out and the supernatant was incubated with 100µg/ml DNase and RNase at room temperature for 1 hour. NaCl was added to 2% and dissolved. PEG was then added to 8% and dissolved. The phage were precipitated O/N at 4 °C.

4) On day 4 the phage precipitate was centrifuged for 10 minutes at 8k. The supernatant was discarded and the walls dried to remove PEG. The pellet was resuspended in 8 mls SM phage buffer and CsCl was added to a concentration of 0.75g/ml. This was ultracentrifuged for 24 hrs at 50k at 20 °C in the SW55 rotor.

5) On day 5 the phage band was collected by puncturing the side of the tube with a wide gauze needle. It was dialysed against 10mM NaCl, 50mM Tris/HCl pH 8.0, 10mM $MgCl_4$. EDTA was added to 20mM, proteinase K to 50µg/ml and SDS to 0.5%. This was incubated at 65 °C for 1 hour. The lambda DNA was then extracted with phenol and finally ethanol precipitated (see steps 9 and 10, section 1.5.b. below).

1.5.5. Plate lysates and minipreps of lambda DNA clones.

The plate lysate method of preparing phage and the DNA miniprep was modified from the method described by Maniatis et al (1982).

1) The phage were plated in the morning so that their growth could be monitored, using about 10^4 pfu to infect 100 µl of plating cells. A range of volumes of phage stock were used to infect the plating bacteria to get optimal density of plaques. Typically, 5-10 µl of phage eluate gave the correct number of plaques. These were incubated for 20 min at 37 °C, mixed with 3 ml of soft LBM agarose and poured onto a 90mm LBM agarose plate. Freshly poured plates were used. The plates were incubated at 37 °C, right side up so that the surface stayed moist.

2) After 7-8 hours the plaques were usually not quite confluent, at which stage the plate was chilled for an hour at 4 °C. The plate was overlaid with 5 mls of SM and left overnight at 4 °C.

3) The lysate was removed, centrifuged for 5 min at 5k rpm to pellet any cells and the supernatant transferred into a clean tube.

4) DNase I was added to the supernatant to give a

final concentration of 2 µg/ml and incubated for 30 min at 37 °C.

5) 5ml of 2 M NaCl/20% PEG in SM was added and the lysate put on ice-water for 1 hr.

6) The phage precipitate was centrifuged for 10 min, 12k rpm at 4 °C and all the supernatant carefully removed and discarded.

7) The phage pellet was resuspended in 500 µl of SM, transferred to a microfuge tube and made 40 µg/ml RNase A, then incubated at 37 °C for 1 hr.

8) EDTA was then added to a concentration of 10mM, a few grains of PK and SDS to 0.2% and this was heated to 65 °C for 20 min.

9) The DNA was extracted once with an equal volume of phenol, mixing by inversion, not vortexing and then extracted with phenol/chloroform and finally with chloroform.

10) The DNA was precipitated at room temperature by addition of 75 µl of 2 M Na acetate and 1 ml of ethanol. There was usually a white "veil" of precipitate immediately visible. This was pelleted by centrifugation for 5 min at 6k rpm, room temperature. The pellet was carefully rinsed with room temperature 70% ethanol, air-dried and resuspended in 100 µl of TE. The yield of DNA was usually 20-30 µg and was usually cut by restriction enzymes at a 5-10 fold excess. If there were problems with cutting, this could sometimes be overcome by PEG-precipitating the lambda DNA.

1.6. PEG precipitation of DNA.

This method precipitates high molecular weight nucleic acids (greater than about 500bp) and is a good way of removing RNA fragments from plasmid or other DNA preparations (Lis 1980). It also seems to remove other impurities such that the DNA is easier to cut with restriction enzymes.

1) DNA had to be at an estimated concentration of > 100 µg/ml in TE or GDW, to ensure quantitative recovery after precipitation.

2) The sample was adjusted to final concentrations of 0.5 M NaCl and 6.5% PEG, incubated on ice for 60 min, then centrifuged at 10 k rpm for 20 min at 4 °C.

3) The supernatant was removed carefully and discarded, the remaining soft pellet washed with cold 70% EtOH. The DNA was dissolved in an appropriate volume of GDW or TE. If the solution appeared sticky, the sample was re-precipitated with ethanol to remove residual PEG.

1.7. Preparation of M13 single-stranded DNA.

1) An overnight culture of male cells (JM 101 or JM109) was diluted 1:50 with 2x YT or L broth and was left to incubate shaking) at 37 °C for 1-2 hours. The culture was then distributed in 1.5ml aliquots into 25 ml plastic 'universals' (Sterilin).

2) An individual plaque was touched with a sterile inoculating loop which was then dipped into an aliquot of cells.

3) The culture was grown for 5-6 hours, then transferred to a microfuge tube.

4) The cells were pelleted by centrifugation at 12k rpm, 5min.

5) The supernatant was transferred into a clean tube taking care not to transfer any cells.

6) Next 200 µl of 20% PEG/2.5 M NaCl solution was added to the supernatant, mixed well and left at room temperature for 5 min.

7) The phage precipitate was pelleted by centrifugation at 6000 rpm for 5 min. If the phage growth had been successful, a pellet was visible at this stage. The supernatant was removed and disposed, then the pellet re-centrifuged briefly and any remaining liquid carefully removed.

8) The pellet was dissolved in 100 µl of TE by pipetting vigorously. An equal volume of buffered phenol was added, the mixture was vortexed for 30 seconds and then centrifuged at 12k rpm for 5 mins. About 80% of the aqueous layer was carefully removed, avoiding the white interface material.

9) Residual phenol was removed from the aqueous material by extraction with 0.5 ml of ether and traces of ether evaporated by warming to 37 °C for 10 minutes.

10) An equal volume of 5 M NH_4 acetate was added, followed by 2.5 volumes of ethanol, mixing well. The DNA was pelleted by centrifugation at 12k rpm for 10 min, the ethanol allowed to drain out of the inverted tubes. The pellet was not visible at this stage. One ml of cold 70% ethanol was added to the tube, re-centrifuged for one minute and the ethanol drained away, any remaining droplets being carefully removed with a Pasteur attached to a water-pump vacuum. Pellets were air-dried and resuspended in 20 µl TE. The yield was usually at 1-5 µg of single-stranded DNA from a 1.5 ml culture.

1.8. Polymerase Chain Reaction (PCR).

1) Genomic DNA was diluted to 25 ng/µl and checked by spotting on an ethidium bromide plate.

2) The reaction was assembled as follows:

41 µl water
15 µl 10X Taq polymerase buffer
24 µl dNTP mix (1.25mM each all 4 dNTPs)
5 µl 5'primer
5 µl 3'primer
60 µl DNA (1.5 µg)

3) Proteases were inactivated by heating to 70 °C for 10 minutes.

4) The condensation was spun down and 50 µl of the reaction was taken out as an unreacted control. The rest was used for the PCR. 1 µl of enzyme was added to the 100

µl, was mixed and was then overlaid with 100 µl of paraffin oil. It was then transferred back to the 70 °C waterbath.

5) The reaction was run for 30 cycles using 2 min at 95 °C, 2 min at 48 °C and 3 min at 70 °C.

6) 30 µl of the reaction and 30 µl of the unreacted control was analysed on an ethidium bromide gel with appropriate markers.

II.2. SUBCLONING METHODS.

The main purposes of subcloning were to enable sequencing of a clone and/or to provide convenient quantities of restriction fragments as probes. Two approaches were taken to obtain particular subclones:

a. "Shotgun" subcloning in which a restricted clone consisting of a large number of fragments was ligated into a vector. Plaque/colony lifts were used on this occasion and individual transformants were characterised.

b. Individual restriction fragments were isolated on an agarose gel and were subsequently used to ligate into a vector. Plasmid or M13 vectors were used for subcloning and these were subsequently used to transform or infect appropriate host cells. Digestion with restriction enzymes, electrophoresis, DNA fragment purification, ligation and transformation will be described in this section.

2.1. Restriction enzyme digests.

Restriction digests of 1 µg of plasmid or double stranded M13 DNA were usually performed in 20 µl reaction volumes set up in a 0.5 ml microfuge tube. Reactions were usually scaled up for digestion of larger amounts of DNA.

x µl of DNA in water
2.0 µl of 10 x reaction buffer
1.0 µl of 2 mg/ml BSA
15.5 - x µl of GDW

The components were mixed together on ice then 1-3 µl of enzyme was added and mixed thoroughly by flicking the tube or 'pumping' with a micropipette. Droplets were collected at the bottom of the tube by centrifuging briefly in a microfuge.

Reactions were usually incubated in a water bath for at least 1 hour at 37 °C. Digestions with Taq I, an enzyme extracted from a thermophilic bacterium, were performed at 65 °C.

For digestions of genomic DNA a 30-50 µl reaction was usually set up containing 5-10 µg of DNA. After original incubation with a 3fold excess of enzyme, a further "booster" dose of 10 units of enzyme was added and the reaction usually continued O/N.

Enzyme activity.

The definition of enzyme activity is that 1 unit should cut 1 µg of DNA in an hour under optimal conditions. However, an excess of enzyme was often used to ensure complete digestion. Typically, this varied from 2-fold for a DNA preparation which was known to be quite pure, to about 10-fold for preparations which often contained impurities, such as genomic DNA or lambda minipreps. If difficulties were experienced with digestion, the DNA was purified further by a variety of methods, such as phenol extraction, ether extraction, ethanol precipitation, and/or PEG precipitation. (See sections 1.2 and 1.6). Care was taken to not use greater than 0.1 volume of enzyme in a reaction, as glycerol could inhibit the activity or alter the specificity of the enzyme.

2.2. Non-denaturing agarose gels.

These gels were used for most analyses of DNA molecules larger than about 50 bp. The standard buffer was Tris-Borate-EDTA (TBE).

1) Between 0.7 and 2.0 gm of agarose was melted in 100 ml of 1 x TBE in a 500 ml conical flask in a domestic microwave oven. This typically required 3-4 minutes at low power.

2) The agarose was allowed to cool to about 60 °C before adding 5 µl of 10 mg/ml ethidium bromide. Waterproof tape was used to form a mould around the edge of a clean glass plate, (measuring 11 by 12 cm) and a comb was positioned about one cm from the end using Bulldog clips.

3) The molten agarose was poured into the mould and allowed to set for at least 20-30 min. The comb was carefully removed without damaging the slots, the waterproof tape peeled off and the gel, supported by the plate, put into the gel tank. The tank was filled with 1 x TBE to just above the surface of the gel.

4) 0.1 volume of loading dye was added to the sample, mixed, loaded and run. Gels were electrophoresed at between 20 and 150 Volts.

For genomic Southern blots ethidium bromide was omitted from the gel which was briefly stained after electrophoresis to visualise the marker DNA. On this occasion electrophoresis was always run O/N at 20-30 Volts as this gave sharper bands in the autoradiography.

2.3. Purifying restriction fragments from agarose gels.

Restricted DNA was electrophoresed as described above and specific fragments isolated by electroelution.

1) The restriction fragments were visualised by staining briefly with ethidium bromide and the required band excised with the minimum amount of extraneous agarose using a sterile scalpel blade.

2) The gel band was transferred into a piece of dialysis tubing. The tubing was sealed at one end with a plastic clip and 200 µl of TBE buffer was added. The other end was sealed after all air bubbles had been carefully

removed.

3) The tubing was immersed in 1XTBE buffer in a tank and electrophoresed at 150 Volts for 15 minutes. In this time the majority of the DNA had left the gel fragment and migrated against the tubing. This was monitored using a portable long-wavelength UV lamp. Longer time was needed for fragments which were >2kb.

4) The polarity of the current was reversed for 10 seconds to elute the DNA from the walls of the tubing. The buffer containing the DNA was removed from the tubing and tansferred into a microfuge tube.

This DNA was sufficiently pure to be used directly in random -primer labelling reactions. However, if the DNA was to be religated or restricted, further purification was needed with phenol/chloroform extraction and ethanol precipitation.

2.4. Ligation reactions.

The conditions described here were used for ligating DNA fragments into plasmid and M13 vectors. The reaction conditions were altered for ligating into lambda vectors.

x µl, 20 ng of linearised vector
y µl, insert DNA (3-fold molar excess)
1 µl 10 mM rATP
1 µl 100 mM DTT
1 µl 10 x ligation buffer
6 - (x+y) µl GDW

These components were mixed on ice and 1 unit of T4 DNA ligase in 1 µl was added, mixing gently by pipetting. The reaction was centrifuged briefly and incubated overnight at 15 °C. The reaction worked with 4 hours of incubation but gave better results O/N.

2.5. Cloning in M13.

All the DNA fragments to be sequenced were subcloned in

M13. Recombinants were introduced into E coli (JM101) and plated in soft agar.

The methodology usually followed for subcloning in M13 was to set up three different ligation reactions in which usually an excess of inserts as compared to vector was used. It was found that using these conditions the desired recombinant phage were usually obtained with the firt attempt. The amount of vector was kept constant in all three ligations (25ng) and the amount of inserts was usually 5, 50 and 100 ng. A mock ligation reaction was always incubated in parallel containing the cut vector but no inserts or ligase.

The M13 cloning system includes a colour selection to aid in the identification of recombinants. The cloning site of the M13 cloning vector is located in the coding sequence of the amino terminal region of the beta galactosidase gene. The host cells carry a mutant form of beta galactosidase and the bacterial and phage genes complement each other to produce beta galactosidase. In recombinant phage the beta galactosidase gene function is inactivated. A chromogenic substance (BCIG) is included in the soft agar as well as an inducer of the enzyme (IPTG). Wild type phage produce beta galactosidase activity and convert BCIG to a blue substance. Recombinants (usually) lack this activity and give rise to clear plaques. The mock ligation was used as a control to check the background contributed by uncut vector.

2.5.1. Preparation of competent cells.

This is based on the original Mandel and Higa method of preparing competent E. coli (as described in Maniatis et al, 1982). M13 RF DNA could be introduced with efficiencies of between 5 x 10^5 and 2 x 10^6 pfu per µg, but this could be improved to about 10^7 pfu/µg by using the alternative high efficiency method described below.

An overnight 10 ml culture of cells, usually JM101, was

grown from a colony picked from a minimal medium plate. This was subcultured (1 to 50) and grown in 2x YT until the OD_{550} reached about 0.5. This was subcultured again and grown to an OD of about 0.3. The cells were gently pelleted for 5 min at 5K rpm, 4 °C and resuspended in 10 ml of ice-cold 50mM $CaCl_2$. The remains of the original culture was diluted and grown for later use as "lawn" cells. After 20 min on ice, the cells were pelleted at 5K rpm, 4 °C and resuspended in 2 ml of 50 mM $CaCl_2$. The cells were then left at least 30 minutes on ice before use.

2.5.2. Transformation.

The competent cells were dispensed in aliquots of 200 µl into Eppendorf tubes. Up to 50 µl of a ligation reaction was added to an aliquot of cells, mixed gently and left on ice for 30-60 min. This was subjected to heat shock for 90 sec at 42 °C, then returned briefly to ice. Just before the end of the incubation on ice the following was added to 3 ml of molten top agar at 45 °C in a Sterilin vial, mixing well:

20 µl of BCIG solution.
20 µl of IPTG solution.
200 µl of lawn (growing) cells.

Finally, the transformation was added to the soft agar and mixed, then poured onto a pre-warmed agar plate. This was allowed to set, then inverted and grown overnight.

2.5.3. Screening M13 clones.

When one type of insert DNA fragment (such as gel eluted fragments) was present in the reaction, any clear plaques were considered as certain recombinants, DNA was prepared directly from them and the screening step of taking lifts and hybridising was omitted. When many molecules were present in the reaction, proper screening was done by taking plaque lifts as described for lambda (section 4.6.1.) and the positive clones were identified after hybridisation.

Finally, on certain occasions, when no probe was available for the region which was being isolated, random clones were grown from clear plaques and the size of the insert was characterised, or the sequence determined when necessary.

In those cases where the insert was cloned in one orientation only, the other orientation clone was obtained by using the M13 vector which carried the opposite orientation of polylinker: after the insert was cut out with two enzymes, it was religated to the new vector and was thus turned "around".

When single stranded M13 DNA was prepared, the sequence of the T track only was often determined as a first step in these experiments, in order both to identify identical clones and save unnecessary sequencing, as well as to check for the quality of the DNA, which was very important for a successful sequencing reaction afterwards.

2.6. High efficiency transformation protocol.

This method is based on that of Hanahan (1983) and gave efficiencies of up to 2 x 10^7 colonies / µg of supercoiled plasmid DNA, when using the DH1 strain of E. coli. Although this protocol gave better transformation efficiencies with most strains than the protocol described for M13 in section 2.5.1., it was most often used for DH1. The transformation buffers contain 15% glycerol and so the competent cells were routinely made in batches and frozen.

2.6.1. Preparation of competent cells.

1) These were streaked from frozen stock of DH1 onto psi agar plate and incubated at 37 °C overnight.

2) A single fresh colony was picked and used to innoculate a 5 ml culture in psi broth (filtered prior to autoclaving). This was allowed to grow at 37 °C for 2 hours (to OD_{550} = 0.3).

3) Subcultures 1:20 were done into 100 ml psi broth

(prewarmed to 37 °C) and allowed to grow for about 2 hours (optimal ODD_{550} = 0.48).

4) These were chilled for 5 min on ice and spun in the Sorvall in 30 ml Corex tubes, at 6000 rpm for 5 min at 4 °C.

5) The cells were resuspended in 2/5 volume of Tfb1.

6) Then they were left on ice for 5 min, then spun at 6000 rpm for 5 min at 4 °C.

7) The cells were resuspended in 1/25 volume of TfbII and left on ice for 15 min.

8) Aliquots of suitable volumes (usually 200 µl) were taken into microfuge tubes (using prechilled pipette and tubes) and stored at -70°C.

2.6.2. Transformation.

1) An aliquot of cells was thawed with hand heat until a small lump of ice remained then left on ice for 10 min. DNA was added (up to 2/5 volume of cells and no more than 100ng per 200 cells) and left on ice for 15-45 min.

2) Cells were subjected to heat shock at 42 °C, 90sec, then returned to ice for 1-2 min.

3) Four volumes of L-broth (at room temperature) was added and then the cells were incubated at 37 °C for 45 min.

4) A suitable fraction of the transformation was plated on L broth + antibiotic.

II. 3. LABELLING METHODS.

3.1. Nick Translation.

This method of labelling double-stranded DNA (Rigby et al, 1977) was used for a few experiments. It is particularly sensitive to impurities in DNA eluted from gels.

The enzyme mixture (see Materials) was checked whenever a new batch of either enzyme was used as the method depended

on the optimal activity of both enzymes.
Reaction:

x µl of DNA containing 10 - 20 ng
2 µl of nucleotide solution
1 µl of 3000 Ci/mmol dCTP
6 - x µl of dist. water

The above were mixed on ice in a 0.5 ml microfuge tube and 1 µl of enzyme solution added, pumping gently with the micropipette to mix, then centrifuged briefly. The reaction was incubated for 60 min at 15 °C and stopped by the addition of 90 µl of TE/SDS. Labelled DNA was separated from unincorporated nucleotides by gel filtration on a Sephadex G-50 minicolumn (see section 3.5) Incorporation was usually 40-60%.

3.2. Random Primer Labelling.

This method was reported by Feinberg and Vogelstein (1983) and was the main method used to label single-stranded DNA as it was more reliable than nick-translation.

1) 10-20 ng of insert DNA were taken in a volume of 13 µl with GDW, were mixed and heated at 100 °C for 3 min, then chilled rapidly on ice.

2) Then the components below were added:

5 µl of ABC buffer (materials section)
5 µl of 2 mg/ml BRL BSA
1 µl of 3000 Ci/mmol alpha-^{32}P-dCTP (10 µCi)

3) Finally, 1 µl of diluted Klenow enzyme (1unit/µl) was added and the reaction incubated for 60 min at 37 °C or overnight at room temp. The efficiency of incorporation was usually 50-80% and the probe was purified on a Sephadex G-50 minicolumn before use in hybridisations.

3.3. Primed synthesis of probes on M13 templates.

This method was convenient for preparing a probe from an M13 clone, based on synthesis of a labelled complementary

strand using Klenow enzyme.

1) The following were mixed in a 0.5ml microfuge tube:

1 µl 10 x Klenow buffer

1-5 µl M13 clone (ssDNA)

1 µl universal primer

The volume was adjusted to 10 µl with GDW.

This was left to anneal as described in section II.8 for sequencing.

2) Then the following were added:

1 µl dATP, dGTP, dTTP, 1 mM each.

2 µl 0.1 M DTT

1 µl alpha-^{32}P-dCTP (10 µCi)

1 µl Klenow enzyme (1 unit)

and the reaction was incubated at 50 °C for 20 min.

3) 1 µl 0.5 mM dCTP was then added and the reaction incubated for a further 15 min to ensure synthesis through the clone insert and into the M13 polylinker. The polymerase was inactivated by heating to 70 °C for 10 min and then the DNA was restricted by either one or two enzymes, depending on whether a single or double-stranded probe was required.

4) The restricted probe was electrophoresed on a 6% acrylamide/8M Urea gel. Electrophoresis was stopped when the bromophenol blue dye had reached the bottom of the gel.

5) The gel plates were separated and the wet gel wrapped in cling film. It was briefly exposed (for 30-60 seconds) to x-ray film. The hot band was then excised from the gel using a sterile scalpel blade and the probe was eluted O/N in TE/SDS. This probe could be used directly in hybridisation experiments as it was single stranded.

3.4. Tailing of oligonucleotides for use as probes.

Oligonucleotides were labelled by tailing with alpha-^{32}P-dCTP using the enzyme terminal transferase. The standard 20 µl reaction was as follows:

4 µl 5xTailing buffer (BRL)

2 µl oligonucleotide (74 ng - about 4 pmol)
2 µl isotope (6.6 pmol)
10 µl GDW
2 µl terminal transferase (30 units)

The reaction was incubated for 1 hour at 37 °C. The incorporation was usually 25-40%, assayed by running the reaction on a G50 Sephadex minicolumn.

3.5. Sephadex G-50 minicolumns.

Sephadex G-50 was swollen in TE containing 0.1% SDS and autoclaved. A column was made by drawing out a 2 ml glass Pasteur to give a capillary end, which was then blocked with sterile, siliconised glass wool. The G50 mix was pipetted into the column and allowed to settle, with the gel bed surface about 5 mm from the top of the Pasteur. The sample was loaded in a volume of 200 µl and then the column was run using TE/SDS as eluent. Fifteen to sixteen 200 µl fractions were collected. The DNA usually eluted in fractions 4-7.

The fractions were counted on the 10 sec setting in the mini-counter. The % incorporation was calculated and hence the specific activity of the DNA.

3.6. Geiger counting of samples to estimate incorporation of ^{32}P.

A Geiger tube connected to a scaler was used to estimate incorporation of labelled nucleotide into DNA probes. Microfuge tubes containing the labelled samples were held in a fixed geometry relative to the window of the tube, allowing consistent measurements. The detection efficiency of this system was uncertain and probably quite low, however, it was sufficient for the purpose.

II 4. NUCLEIC ACID BLOTTING AND HYBRIDISATION METHODS.

4.1. Formaldehyde denaturing gels for RNA blots.

Formaldehyde gels (Lehrach et al 1977) were used for all Northern blots.

1) 1-1.5 g of agarose was melted in 87.5 ml of water, then 5 ml of 20 X MOPS buffer and 7.5 ml of formaldehyde were added, mixed well and the gel poured.

2) For a sample of 10 µl containing up to 50 µg of RNA, 20 µl of sample buffer were added and the RNA denatured at 60 °C for 15 min.

3) Samples were allowed to cool, 4 µl of orange G dye marker added to each and then loaded onto the gel.

4) Gels were run at about 75-100 Volts (constant) in 1X MOPS buffer containing 75 ml formaldehyde per litre and were transferred onto nitrocellulose or nylon membranes without any pre-treatment.

Normally, no more than 10 µg of RNA was loaded per track.

5) To visualise the RNA by UV illumination, 1 µl of 5 mg/ml ethidium bromide was added to the sample before loading the gel, allowing about 2 µg of total RNA to be detected.

4.2. Southern Transfer to Nylon filter.

This was based on the original method described by Southern (1975).

1) After electrophoresis, an oblique cut was made in the gel, in the right hand corner furthest away from the origin. This allowed the correct orientation of the gel. The region behind the origin was discarded.

2) The gel was soaked in 1.5 M NaCl/ 0.5 M NaOH for 60 minutes, changing the buffer once.

3) The gel was washed twice (5 minutes each) with a large volume of GDW.

4) The gel was then transferred into 1.5 M NaCl/ 0.5 M

Tris/HCl pH 7.4 for two hours. The buffer was replaced with fresh every half hour.

5) The filter was cut accurately to match the size of the gel, with one corner trimmed off. Once cut to size, the filter was marked in pencil across the bottom to allow identification later. It was floated on GDW for a few minutes before transfer.

6) 500 ml of 20 x SSC was put into a cut-out lunch-box, which acted as buffer reservoir and support for the blot. A gel plate was put onto the lid and a wick of two pieces of 3MM paper wetted with buffer laid over this. Two rectangles of wetted 3MM were put over this, a little bigger than the gel. These sheets were soaking wet to prevent buffer (and DNA) being sucked out of the gel when it was put on top.

7) The neutralised gel was laid on the apparatus with the lower surface of the gel uppermost. The nitrocellulose or nylon filter was carefully put on top of the gel with the cut corner matching the cut on the gel. Air bubbles were expelled by rolling a pipette gently over the surface. Saran-Wrap was placed around the gel to prevent any contact between the wicks and the stack of towels which might bypass the gel. A piece of dampened 3MM the same size as the gel, was placed over the filter and rolled with a pipette to ensure good contact and expel any bubbles. Another damp sheet and a couple of dry ones were placed on top, making sure there was good contact and no air space. On top of this were placed about 1.5 inches of cut hand towels. Two gel plates were placed on top of the stack to compress the stack and ensure good contact and uniform blotting.

The transfer was performed overnight.

8) The filter was allowed to air-dry on a sheet of 3MM, then baked for 2 hours at 80 °C .

4.3. Filter Hybridisations.

Stringent hybridisations were performed at 42 °C in the presence of 50% formamide.

For reduced stringency hybridisations, the temperature was lowered to 34 °C and the formamide concentration unchanged. For experiments in which the signal was expected to be quite weak (eg whole-genome Southerns, most Northerns and screening genomic DNA libraries) 10% dextran sulphate was included in the hybridisation buffer to improve the signal. Hybridisations were done in plastic heat-sealable bags.

1) Prehybridisation.

At least 100 µl of prehybridisation mix was used for each cm^2 of filter (ie about 10 ml for a standard Northern or Southern blot).

5 x SSPE
0.1% dried milk powder
0.15% SDS
50% formamide

Prehybridisation was performed in this buffer for two hours. If using dextran sulphate in the subsequent hybridisation, the prehybridisation buffer was discarded and replaced by buffer (2-3 ml for a standard blot) containing dextran sulphate and prehybridised for an additional 15 minutes. In the original experiments, the dried milk powder was replaced by 1 X Denhardt's reagent and 100 µg/ml sonicated, denatured herring-sperm DNA. This made very little difference to the result of the hybridisation.

2) Hybridisation.

Hybridisation was performed in a 5ml volume (for Southern or Northern blots) of the buffer described above, containing dextran sulphate if necessary. If the labelled probe was double-stranded, it was boiled for 5 minutes and then left on ice for a few minutes. It was then mixed with the hybridisation buffer. The hybridisation mixture was added to the filter and the plastic bag heat-sealed, excluding as

much air as possible. The bag was then put into a water bath and left shaking gently overnight at 42 °C.

3) Washing.

The washing buffer for a standard hybridisation was 0.2 X SSPE/0.1% SDS. The following washes maintained the same stringency as the hybridisation, except for the final wash, which was equivalent to about 15 °C below Tm for a typical DNA-DNA hybrid. For low-stringency hybridisations the wash buffer was 2 X SSPE/0.1% SDS.

1) The bag was opened and the probe poured out, saving if desired.

2) The filter(s) were transferred into 100-200 ml of wash buffer in a sandwich box.

3) They were washed with vigorous shaking for 5 min at room temperature. This was repeated with fresh buffer.

4) Filters were washed twice for 30 min at 42 °C.

5) The final wash was for 30 min at 60 °C or 50 °C for a low-stringency hybridisation.

These conditions were changed if the probe was very short, such as a labelled oligonucleotide. Occasionally further washing was necessary to reduce the background.

After washing, filters were usually either exposed whilst sealed into a bag or wrapped in Saranwrap, to keep them moist and so make probe removal easier.

4.4. Probe removal and reuse of filters.

Southern blots and lifts: Probes were removed by putting nylon filters into 0.4 M NaOH at 42 °C for 30 min, followed by neutralisation in 0.2 M Tris HCl pH 7.5/0.1XSSC/0.1%SDS at 42 °C for 30 min.

When a nitrocellulose filter was to be de-probed, boiling distilled water was poured onto the filter and allowed to cool to room temperature.

For RNA blots (either nylon or nitrocellulose), probes were removed by incubation at 85 °C in TE / 0.1%SDS for 15-

30 minutes, depending on the strength of the signal. Probe removal was difficult if the filter had been allowed to dry out and sometimes required extended treatment. Probe removal was checked by exposure to film.

4.5. Making colony replicas.

Transformed cells were spread on a nylon filter placed on an L agar plate supplemented with appropriate antibiotics. This was the master filter and was marked clearly with a soft pencil to allow identification. The cells were grown until colonies about 0.5-2mm in diameter appeared and replicas made. This protocol is essentially as described by Hanahan and Meselson (as in Maniatis et al, 1982).

1) The master filter was removed from the plate and placed on 3-4 dry Whatman 3MM filters.

2) A new nylon filter was marked and wetted by placing on a fresh L plate. Filter forceps were used to transfer the filter from the plate and to lay it on top of the master filter, 'wet' side down.

3) 3-4 Whatman 3MM filters were laid on top of the colony filter sandwich and gentle downward pressure exerted by rocking a sealed plastic bag half filled with water over them. This ensured efficient replica formation.

4) Holes were made through both colony filters using a needle in a pattern of one, two and three holes around the edge, thus ensuring correct orientation of master and replica. The filters were then peeled apart and returned to their respective plates, colony side up.

5) The master plate was then stored at 4 °C, until ready to pick positive colonies.

6) The replicas were grown on L plates until colonies were easily visible (0.5-1 mm diameter), which usually took 3 to 5 hours.

7) The filters were denatured and neutralised as described below for lambda (section 4.6.1), and the DNA bound to the nylon filters by baking.

8) Debris was washed off before prehybridisation by washing for a few hours at 65 °C in 2XSSC/0.5%SDS.

4.6. Screening lambda plaques.

4.6.1. Plaque lifts.

1. The phage were grown until almost touching. Before lifts were taken the plates were always allowed to cool for at least 1 hour at 4 °C.

2. A nylon filter was marked with a soft pencil and placed carefully on the surface of a phage-infected lawn using gloves and left for 1-2 minutes. The orientation of the filter was marked by piercing the filter and the agar below a symetrically with a needle in three positions, then the filter was peeled off using flat filter forceps.

3. A piece of 3MM paper was soaked with Southern denaturing solution, but not made too wet. Similarly, a second piece of 3MM paper was soaked with Southern neutralising solution. Filters were then transferred to the denaturing pad for 5 minutes to disrupt the phage particles and denature the DNA. The filters were then transferred to the neutralising pad and left again for 5 minutes.

4. The filters were baked at 80 °C for 2 hours to fix the DNA.

5. The filters were prehybridised and hybridised in the same way as Southern blots (section 4.3).

4.6.2. Plaque amplification.

Two methods for plaque amplification were tested:

a. Method of Woo et al, 1978. Filters were soaked in 50 mls of a 10% dilution of O/N Q358 cell culture. They were left to dry on sterile 3MM paper. Lifts were taken with these dry filters and were transferred -plaque side up- to fresh LBM plates. They were left to incubate at 37 °C for 3 hours.

It was found that this method resulted in an unequal amplification of the different size plaques (a much bigger

amplification of the big plaques compared to that of the smaller plaques). For this reason this method was not used, as the signal coming from smaller plaques could be very easily lost during screening.

b. The "sandwich" amplification method is recommended by Maniatis et al, 1982. With this method a clean filter is placed on an empty LBM plate. After the plaque lift, the filter is transferred and placed on top of the clean one on the empty LBM plate, plaque side down, and is left to incubate for 4-12 hours. A lawn of cells with phage plaques grows between the two filters. The filters are subsequently processed as usually.

Although some amplification of signal was achieved with this method, there was also a lot of smearing of the signal, apparently caused during the separation of the two "sandwich" filters; this method was therefore not subsequently used either.

At a later stage, and when the plaque purification step was reached, a different method was used to amplify the signal coming from the plaques; this was based on using a "poorer" culture medium for the plates (removing yeast extract from LBM), so that the bacteria would be starved. The phage plaques became bigger using this medium and shorter exposures were needed to get a signal (Tom Williamy, personal communication).

4.7. Plaque dots ("macroplaques").

This is a method of producing an amplified signal from a phage plaque, by elution of the phage particles and then spotting the eluate onto a lawn of host cells. An area of confluent lysis results, which is transferred onto a filter and the filter is probed in the normal manner.

1) 200 µl of lawn cells were mixed with 3 ml 0.7% agarose/LBM, poured onto a small agar/LBM plate and allowed to set.

2) 0.5-5 µl lambda phage eluate was spotted onto the

surface and allowed to soak in. The plate was then inverted and incubated overnight. Filter lifts were taken in the normal manner, after chilling the plate.

II 5. RNA PREPARATION.

5.1. Extracting RNA with guanidinium isothiocyanate.

Guanidinium thiocyanate is very effective at denaturing proteins as it is the most strongly chaotropic of salts. So, it is used in many methods for extracting RNA. These methods also often use beta-mercaptoethanol, which helps to inactivate ribonucleases in the tissue by reducing disulphide bridges.

1) Tissues were collected at surgery, then immersed into liquid nitrogen and stored at -70 °C.

2) Frozen tissue was ground under liquid nitrogen using a ceramic pestle and mortar then dispersed in guanidinium mix and homogenised, using 5 to 10 ml of mix for every gm of tissue. Homogenisation was performed with a motor driven glass and Teflon homogeniser. The viscosity of the homogenate was checked with a Pasteur and, if needed, it was squirted through an 18 gauge needle repeatedly until the viscosity was reduced. It also helped to use a larger volume of guanidinium mix.

3) After homogenisation, insoluble material was pelleted by centrifuging at 10k rpm , 5 minutes, room temperature and the supernatant transferred to a clean tube.

5.2. CsCl Method.

This method is based on a protocol describede by Maniatis et al (1982) and depends on the different buoyant densities of RNA and DNA in CsCl. RNA was pelleted through a cushion of caesium chloride while the DNA formed a band, using the SW 55 or AH627 ultracentrifuge rotors.

1) 1 gm of CsCl was added for each 2.5 ml of the supernatant homogenate.

2) This was then layered over a cushion of 5.7 M CsCl in 0.1 M EDTA. The cushion volume was 1.2 ml for an SW 55 tube and 8ml for an AH627 tube.

3) It was centrifuged overnight at 20o at 35K rpm for the SW 55, or at 24K rpm for the AH627.

4) The supernatant was discarded and the walls of the tube dried carefully, without touching the pellet.

5) The glassy-looking RNA pellet was dissolved in TE/0.1% SDS/proteinase K (a microspatula end) by pipetting and incubated for 30 min at 37 °C.

6) The RNA was extracted with an equal volume of phenol/chloroform and the organic layer back-extracted with a half-volume of TE/SDS.

7) The aqueous layers were pooled and then extracted with chloroform.

8) The RNA was ethanol precipitated using 2.5 volumes Ethanol and Na acetate (250 mM final concentration).

5.3. Poly U Sepharose purification of mRNA.

1) Total RNA was dissolved in 8.6 ml of loading buffer without NaCl - care was taken to ensure it was thoroughly dissolved and the volume was doubled if necessary. The RNA was heated to 60 °C for 2 min, chilled briefly on ice, then adjusted to 0.7 M NaCl.

2) A 1 ml bed volume column of poly-U Sepharose was pre-run with 5ml of elution buffer and then 5ml of load buffer.

3) The sample was loaded then the column rinsed with a further 5ml of load buffer and finally 10 ml of wash buffer. The wash was monitored by collecting 1 ml fractions and spotting 1 µl of each fraction onto an ethidium plate (simply 1% agarose/TBE containing 5µg/ml ethidium bromide poured in a 90 mm petri dish).

Footnote to page 89

* The markers used were a Bam H1 digest of EMBL4 (19,400, 13,700, 9,242 bp) and a HinD III digest of phage lambda DNA.

4) When there was no detectable fluorescence in the wash fractions, elution buffer was applied to the column and 0.2 ml fractions taken.

The fractions containing A+ RNA were identified again by spotting 1 µl onto an ethidium plate, they were then pooled, made 2 M in ammonium acetate and precipitated with 2.5 vol. of EtOH, O/N at -20 °C.

The yield of RNA was estimated by absorbance at 260 nm. RNA was then re-precipitated and dissolved in sterile water at 1 mg/ml and stored at -70o. The poly-U Sepharose column was stored at 4 °C in TE/SDS containing 10 µg/ml of proteinase K to prevent degradation by ribonucleases.

II.6. GENOMIC LIBRARY.

6.1. Partial digestion of genomic DNA.

6.1.1. Control digestion (Maniatis et al, 1982).

1) A 10 µg quantity of the patient's DNA was taken in a volume of 150µl with the appropriate salts.

2) This quantity of DNA was distributed in several aliquots such that the first one contained 30 µl (i.e. 2µg of DNA) and all the others contained 1 µg of DNA. Four units of the enzyme (Sau 3A) were added to the first tube.

3) A series of twofold enzyme dilutions were then done from tube 1 through to tube 8 by taking 15 µl from the first tube, mixing well with the second, then taking 15 µl from the second , mixing well with the third etc. No enzyme was added to the ninth tube.

4) The samples were incubated for 1 hour at 37 °C and were subsequently electrophoresed in a 0.5% agarose gel with appropriate size markers in the range of 10-30 kb*. The lanes were compared in the gel and the concentration of the enzyme that produced the maximum amount of fragments corresponding to the desired size was identified.

5) 250 µg of genomic DNA were subsequently digested

using this concentration of enzyme and identical conditions as those of the control. The digested DNA was run on a gel to check for distribution of fragments in the desired range and the DNA was phenol extracted, ethanol precipitated and resuspended in 500 µl TE. It was then fractionated through a 10-40% sucrose density gradient.

6.1.2. Sucrose density gradient centrifugation.

1) The gradient was prepared in a 14 ml SW40 Ti rotor tube by overlaying 3.25 ml each of 10, 20, 30 and 40% sucrose solutions dissolved in 1M NaCl/TE. The layers were allowed to diffuse into a concentration gradient by standing O/N at 4 °C.

2) The partially digested DNA was overlaid on the gradient and centrifuged at 26000 rpm, 25 °C, for 20 hrs.

3) 0.5 ml fractions starting from the bottom of the tubes were then collected by use of a peristaltic pump. A small amount (10µl) of every third fraction was run in a 0.5% agarose gel with appropriate markers. The samples containing the markers had previously been adjusted for sucrose and salt concentration. Those fractions containing DNA fragments in the desired size were thus pooled. The sucrose concentration of the fractions was diluted to <10% with TE and the DNA was subsequently ethanol precipitated. It was finally resuspended in TE at a concentration of 0.1 µg/µl.

6.2. Preparation of vector "arms".

100 µg of lambda EMBL4 DNA was cut with the enzyme BAM HI and was checked for successful digestion in a 0.5% agarose gel. The expected fragments are 19400, 13700 and 9242 bp. Before electrophoresis the DNA was always heated at 68 °C for 10 minutes to disrupt the cohesive ends of lambda. After it was identified that digestion was complete, the DNA was also digested with the enzyme Sal I, which has recognition sites in the "stuffer" fragment only. The

resulting digested DNA was phenol extracted, phenol traces were removed with ether and it was finally ethanol precipitated. Finally, it was resuspended in TE at a concentration of 1 µg/µl.

6.3. Ligation of vector "arms" to genomic DNA.

The control ratios of arms to inserts were 6:1, 5:1, 3:1, 2:1, and 0.7:1. The ligations were performed as described in section II.2.4. in 20 µl reactions containing a maximum of 4µg per reaction and incubated for 15-18 hours at 15 °C. 1/4 of the ligation reaction was packaged in vitro (see section 7.3) using reliable packaging extracts. From these pilot ligations the ratio of vector to insert was found which would give the highest phage titres. Various packaging reactions were carried out using these optimal conditions until all the ligated DNA was packaged. This was titrated and the total number of recombinant phage produced was calculated. Each packaging reaction containing $4x10^4$ - $1.5x10^5$ pfu/µg was kept in a total volume of 100 µl after dilution with SM. A library of $1X\ 10^6$ clones was thus constructed.

6.4. Plating out and amplification of the library.

The aim was to get a total of 10000-20000 plaques per 135mm plate.

1) 1/5 of a standard packaging reaction was used each time to transfect 200 µl of indicator plating cells (see section 1.5) (strain Q359). The cells were incubated for 20 minutes at 37 °C to adsorb.

2) This was mixed with 7 mls of prewarmed (45 °C) molten agar and poured into a prewarmed LBM 135 mm plate. The plates were incubated for 8-10 hours. The incubation was stopped when the plaques were starting to touch one another to avoid "scrambling" of the library.

3) 12mls of SM were placed on each plate which was left

O/N at 4 °C on a level surface. The 12 mls were collected the following day and the plate was washed with a further 3 mls of SM. All the phage suspensions were brought together in a pot and chloroform was added to a concentration of 5%. It was incubated for 15 minutes at room temperature with occasional shaking. The library stock was then centrifuged for 5 minutes at 4000g at 4 °C, to remove the bacterial debris, agar and chloroform. The supernatant was transferred to a sterile glass container. Chloroform was added to a concentration of 0.3% and the library was kept at 4 °C shielded from light.

II.7. PREPARATION OF LAMBDA PACKAGING EXTRACTS.

This protocol is based on that described by Maniatis, Fritsch and Sambrook (1982), with minor modifications.

Cells of each packaging strain (BHB2690 and BHB2688) were streaked out on two LBM plates from frozen stocks. One plate was incubated at 32 °C, the other at 42 °C. Both strains should grow only at 32 °C. If there were signs of growth at 42 °C, this meant that the cells had become contaminated with revertents that had lost one of the mutations necessary to make efficient packaging extracts. Sonicated extracts and freeze-thaw lysates were prepared on separate days.

7.1.Sonicated extract (SE).

1) A single BHB2690 ("prehead donor") colony from the 32 °C plate was used to inoculate 50 mls of LBM in a 1 litre flask and incubated overnight at 30-32 °C.

2) 8 ml of the overnight culture was used to innoculate 500 ml of aerated, prewarmed LBM in a 2 litre flask and incubated at 32 °C, shaking vigorously to ensure good aeration until the OD_{600} reached 0.3 - 0.35 (2-3 hours).

3) The flask was then placed in a 65 °C water-bath,

swirling continuously until the temperature of the culture reached 45 °C, then the flask was transferred to a 45 °C water-bath and incubated for 15 minutes. This treatment induces expression of the lambda genes, causing large amounts of phage proteins to be made without host lysis.

4) After induction the culture was incubated at 38-39 °C for 2-3 hours, shaking vigorously. Induction was checked by adding a few drops of chloroform to a 3-4 ml sample of the culture. If the cells had been successfully induced, the sample cleared within a few minutes and became a little sticky, with small amounts of visible debris.

5) The cells were chilled rapidly by swirling the flask in an ice-water bath until the temperature fell below 5 °C. From this stage onwards, the temperature was kept below 5 °C. The cells were centrifuged at 4.5k rpm for 10 minutes at 4 °C, then drained well on ice, also drying the interior of the centrifuge tube.

6) The pellet was resuspended thoroughly in 3.6 mls of freshly prepared, ice-cold sonication buffer first by using a glass rod and then pipetting gently with a wide-bore Pasteur. The cells were then transferred into a clear plastic 25 ml 'universal' tube in ice-water.

7) Sonication was performed using an MSE sonicator. The cells were sonicated using short bursts of 5-10 seconds at maximum power, with "tune" set at 14, so that the amplitude was about 14. The temperature was not allowed to exceed 4 °C and this was monitored carefully, the sample being left to cool for 20-30 seconds between bursts. Sonication was continued until the sample became translucent and the viscosity reduced. When sonication was complete, the sample was transferred to a centrifuge tube and centrifuged for 10 minutes at 10k rpm at 4 °C.

8) The supernatant was transferred into a clean tube containing an equal volume of ice-cold sonication buffer and 1/6 volume of fresh packaging buffer and mixed gently. This extract was dispensed in 50 µl aliquots into precooled microfuge tubes on ice, which were capped and snap-frozen

in liquid nitrogen. The sonicated extract (SE) was stored at -70 °C.

7.2. Freeze-thaw lysate (FTL)

1) A single BHB2688 colony from the 32o plate was used to inoculate 50 mls of LBM in a 1 litre flask and incubated overnight at 30-32oC.

2) Three x 2 litre flasks each containing 500 ml of aerated, prewarmed LBM were each innoculated with 8 ml of the overnight culture. The three cultures were incubated at 30-32 °C with vigorous shaking until their OD600 reached 0.3-0.35 (2-3 hours). They were then treated exactly as described above in steps 3 to 5.

3) The cells from each culture were resuspended in 1 ml of ice cold sucrose solution, using a glass rod and then pipetting gently with a wide-necked Pasteur.

4) The suspension was distributed evenly between six precooled eppendorfs on ice, adding 25 µl of fresh ice cold lysozyme solution to each, mixing gently. The tubes were quickly capped and plunged into liquid nitrogen, then allowed to thaw on ice, which took about an hour.

5) 25 µl of freshly prepared packaging buffer was added to each tube and mixed, then the thawed extracts were combined in a centrifuge tube and centrifuged at 20k rpm for one hour at 4 °C. The freeze-thaw lysate (FTL) was dispensed in 10 µl aliquots into precooled microfuge tubes on ice, which were then snap-frozen in liquid nitrogen and stored at -70 °C.

7.3. Packaging reaction.

To check the efficiency of the packaging extracts, wild lambda DNA was used as control. One microgram of DNA was packaged each time according to the following method.

1) One tube of "freeze-thaw" lysate (10 µl) and one tube of sonicated extracts (15 µl) were taken out of the

freezer and placed on ice.

2) When it could be seen that the freeze-thaw one was starting to thaw, 1 μg of control DNA in a volume of 5 μl was added to the tube followed immediately by the contents of one tube of sonicated extracts.

3) These were mixed gently and left to incubate at 30 °C for 1-2 hours.

4) 100-500 μl of SM phage buffer was then added to the tube followed by 2 drops of chloroform. The packaged DNA was briefly centrifuged to remove debris and afterwards transferred to a clean tube and kept over chloroform at 4 °C until further use, usually not longer than one week.

The packaging efficiency was calculated in the following way using freshly prepared plating bacteria.

1) 10 μl quantity was taken from the packaged DNA and was brought to a tube containing 90 μl of SM.

2) Six 10fold serial dilutions were then done by taking 10 μl of this stock originally and adding to 90 μl of SM after thorough mixing. 10 μl from the second tube were then mixed with 90 μl of SM in the third tube etc.

3) 50 μl of these dilutions were immediately used to transfect plating bacteria and the following day the number of plaques were counted on each plate and the packaging efficiency was calculated.

Several batches of packaging extracts were prepared to improve the efficiency using minor modifications, usually of the degree of sonication.

II.8. DIDEOXY CHAIN-TERMINATION SEQUENCING OF DNA.

This method, originated by Sanger and co-workers (1977) depends on the enzymatic synthesis of a nested set of DNA molecules which have one end (the primer) in common and their other ends terminated by the incorporation of a specific dideoxynucleotide. These DNA molecules are

resolved by a denaturing polyacrylamide gel able to fractionate fragments differing in size by a single residue. The M13 system provided a convenient single-stranded template and several 'universal' primers which were used for sequencing.

1) Template/primer annealing

5-8 µl M13 clone (about 0.5-1.0 µg)
1 µl 10 x Klenow buffer
1 µl sequencing primer
Adjusted to a final volume of 10 µl with GDW

The above were mixed in a 0.5 ml microfuge tube (with a hole in the lid), heated at 100 oC for 3 minutes and annealed at 37 °C for 1 hour, allowed to cool to room temperature and then centrifuged briefly to collect condensation.

2) Whilst the annealing reaction was proceeding the dNTP' mix/ddNTP solutions were prepared from the stocks for each of T,C,G and A. Different stock solutions were used, depending on the choice of labelled nucleotide. The amount of each of the stocks which was required to give the correct degree of termination was determined by trial and error and for ^{32}P-dCTP was:

T: 5µl dTTP' mix, 5µl ddTTP
C: 5µl dCTP' mix, 1µl ddCTP, 4ul GDW
G: 5µl dGTP' mix, 5µl ddGTP
A: 5µl dATP' mix, 3µl ddATP, 2ul GDW

For ^{35}S-dATP, the mixtures were:

T: 5µl dTTP' mix , 3µl ddTTP , 2ul GDW
C: 5µl dCTP' mix , 4µl ddCTP , 1ul GDW
G: 5µl dGTP' mix , 4µl ddGTP , 1ul GDW
A: 5µl dATP' mix , 5µl ddATP

At this stage, the Klenow polymerase/isotope mix was

prepared, adding the enzyme last of all, immediately before aliquotting. For four clones, using ^{32}P-dCTP as label this consisted of:

4 µl 0.1M DTT
1 µl alpha ^{32}P dCTP
4 µl 10 µM dCTP
22 µl 10 mM Tris-HCl, pH 8.0
1 µl (4 units) Klenow polymerase

When using ^{35}S-dATP as label the mixture was:

4 µl 0.1M DTT
2 µl ^{35}S-dATP
25 µl 10 mM Tris-HCl, pH 8.0
1 µl (4 units) Klenow polymerase

3) Reaction. When the primer had annealed, 2µl aliquots were added to each of 4 microfuge tubes (appropriately marked T,C,G,A) followed by 2µl of the appropriate dNTP'/ddNTP mix. Then 2 µl of enzyme/isotope was added, mixing thoroughly by 'pumping' the solution with the micropipette, trying to avoid air bubbles. The reactions were incubated at 50 °C for 15 minutes (20 minutes for the less efficiently incorporated ^{35}S-dATP), then 2µl of 0.5mM dCTP was added to each reaction (0.5 mM dATP for reactions using this labelled nucleotide), mixed well and incubated for a further 15 min at 50 °C.

The reactions were stopped by the addition of 4 µl of formamide/dye. The reactions were then either stored frozen (for no more than 1-2 days for ^{32}P-label and 1-2 weeks for ^{35}S-label) or boiled for three minutes and immediately loaded onto a gel.

4) Running the gel (See also section II.9). For best resolution the gel should be thin (0.4 mm). The wells were given a final rinse and then 3-5 µl of each reaction was loaded in the order T,C,G,A. The wells near the edge of the gel were avoided . The gel was usually run at about

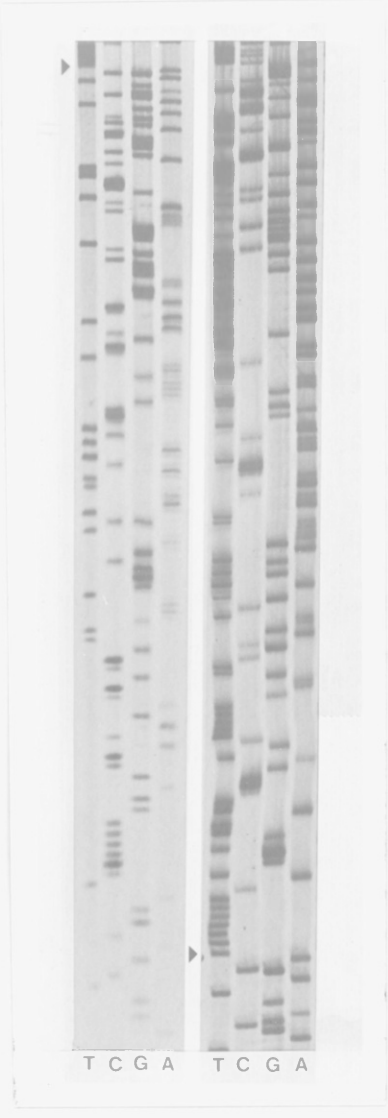

fig 2.1. Demonstration sequencing gel.
A short run [approx 2 hrs at 1200 Volts] is shown on the left and a longer run [6-7 hrs] is shown on the right. The arrowheads point to the same sequence in the two gels.

1000-1100 V and the voltage was adjusted to try to achieve a gel temperature of approximately 50-60 °C, which helps to keep the DNA fully denatured. Electrophoresis was usually stopped when the bromophenol blue was about an inch from the bottom of the gel. To determine longer sequences, the gel was run for longer times, sometimes for 8-10 hours.

5) Fixation, washing and drying of the gel. The buffer was discarded and the gel sandwich removed from the tank and cold water run over the surfaces to cool them down. The gel plates were carefully prised apart with a scalpel blade and at this point the gel was usually stuck to only one of the plates. The DNA was fixed and urea washed away by immersion of the gel, still stuck to the plate, in 2 litres of 10% acetic acid, 10% methanol for 20 min and then drained briefly. To allow drying of the gel it was transferred onto a sheet of Whatman 3 MM paper, carefully but firmly pressing down from the centre outwards and then peeling off the filter paper with the gel stuck to it. The gel was protected by a layer of Saranwrap and dried under vacuum at 80oC for 30-40 min.

6) Exposure to film, development and analysis. Saranwrap was removed and the dried gel exposed to X-ray film, at room temperature without a screen. The signal from either ^{32}P or ^{35}S usually gave a readable sequence after an overnight exposure to Kodak XAR-5, but occasionally longer exposures were necessary, especially with ^{35}S label. The use of intensifying screens and exposure at -70oC with ^{32}P could give quicker results, but also reduced resolution.

Klenow polymerase produces several characteristic artefacts in sequences, which are outlined below:
In a doublet, the upper C is always more intense than the lower C. Similarly, the upper G is often more intense than the lower G, particularly when the double G is preceded by

a T. Finally, an upper A is often less intense than the lower A.

II.9. BUFFER GRADIENT SEQUENCING GELS.

Buffer gradient gels allowed the determination of longer sequences than those with the standard, uniform buffer concentration.

1) Before pouring gels, the glass plates, spacers and comb were washed thoroughly with 1% SDS, followed by water then ethanol. The first time plates were used, they were both siliconised and rewashed with water followed by ethanol. Thereafter, only the smaller of the two was siliconised, to encourage the gel to stick to the other plate when separating them.

2) The plates were formed into a sandwich with the spacers and taped together with waterproof tape, leaving the gel unclamped as this made it easier to pour the gradient. The amounts of solutions required for each size of gel plates is in the table below:

	Top gel	Bottom gel
Large gel plates	45ml	10ml
Small gel plates	25ml	6ml

3) The top and bottom gel solutions were kept at 4 °C and not allowed to warm before adding the 25% AMPS and TEMED, thus slowing the rate of polymerisation of the acrylamide and so making it easier to pour the gel. 33ml (18ml) of top gel was taken up into a 60ml (20ml) syringe and 9ml (5ml) of top gel into a second, 20ml syringe. 9ml (5ml) of bottom gel was then carefully drawn into this second syringe, and a few bubbles allowed to pass up the syringe to mix the layers, forming a crude gradient.

4) This mixture was squirted between the plates, down the middle, holding the sandwich almost vertically. The gel plates were gradually lowered to a shallower angle, and

the remaining top gel was then used to fill the sandwich. The comb was inserted and the plates clamped together with bulldog clips. The gel was allowed to polymerise for at least 30 minutes whilst doing the sequencing reactions. When polymerised, the comb was removed, and the wells immediately washed out with TBE to remove any unpolymerised acrylamide. The gel sandwich was clamped into the gel apparatus and about 400ml TBE buffer added to each reservoir. The wells were given a final rinse prior to loading, to remove the urea. Gels were electrophoresed at 40-50 (30-40) watts for 2-3 hours.

II.10. IN SITU HYBRIDISATION ON HUMAN CHROMOSOMAL METAPHASES.

For in situ hybridisation a region of the beta gene was chosen for use as a probe, which lacked strong homology to the alpha gene so that it would not cross hybridise to it. Because it was a genomic fragment, before use it was checked for the presence of repeats in the following way. It was used as a probe on a genomic Southern blot and was considered suitable for in situ only when it was found to hybridise exclusively to the band it was derived from. In addition, total genomic DNA was nick translated and used as a probe on a Southern blot of the genomic clone DNA. The probe was suitable when no hybridisation could be detected.

In situ hybridisation was done with the help of Miss Feyruz Rassool as described by Rassool et al (1988).

1) Human female metaphase chromosomes were prepared from peripheral lymphocytes.

2) The chromosomal DNA was denatured in 70% formamide / 0.1 mM EDTA / 2 X SSC (pH 7.0) at 65 ^{o}C for 4 minutes.

3) The probe was labelled with ^{125}I by nick translation (Boyd et al 1984) to a specific activity of 5X10^{8} dpm/μg. It was then lyophilised and resuspended in hybridisation buffer.

4) The hybridisation buffer contained 50% formamide, 5 X Denhard's solution, 5 X SSPE, 10% dextran sulphate and 20µg/ml salmon sperm DNA.

5) The probe was boiled for 10 minutes to denature and 30 µl of probe mixture at a concentration of 0.004 ng/ml was hybridised with the denatured chromosomal DNA O/N at 42 $_{o}$C.

6) The slides were then rinsed in 5X SSC, washed in 2 X SSC (2 hours at room temperature and 1 hour at 65 °C). The final wash was in 0.2 X SSC for 30 minutes at 60 °C.

7) The slides were then dipped in Ilford L4 nuclear emulsion and were stored in the dark for 7-14 days at 4 °C. They were then developed in D19 for 8 minutes at 15 °C and banded with the Wright's stain according to Harper and Saunders (1981). Hybridisation of DNA sequences in situ was seen as one or more black grains over a particular chromosome. Metaphases were then photographed and analysed. The slides were exposed for an average of one week. The number of grains was counted on 50 metaphases and a histogram was designed showing the distribution of grains on the various chromosomes.

II.11. BIOASSAY OF SYNTHETIC BETA GENE PEPTIDE AND CLOSE ANALOGUES.

The predicted CT like peptide and two close analogues were synthesised. They were all three tested for biological activity using a recently developed bioassay which is based on calcitonin induced inhibition of motility of freshly disaggregared rat osteoclasts (Chambers and Moore, 1983). These experiments were performed by Dr Mone Zaidi.

With this method, long bones are obtained from neonatal rats, they are cut accross their epiphyses and the shafts are curetted with a scalpel blade in a petri dish in the presence of culture medium containing 20% FCS. The bone slices are allowed to incubate for a short time so that the

cells can be released into the medium. The debris are removed and the cells are incubated for 30 minutes at 37 °C. They are then transferred to coverslips and their motility studied under the microscope. A microtitre plate containing 24 wells is then used and at least 10 isolated osteoclasts are placed into the wells. CT is then added to the culture medium, incubated for 1 hour and its effect on the motility of the osteoclasts is recorded. Immotile cells are counted and expressed as a percent of total.

B. MATERIALS.

1. DNA PREPARATIONS

Ficoll/Hypaque — Pharmacia Lt

Lysis Buffer:
300 mM NaCl
10 mM EDTA
10 mM Tris/HCl pH 7.5
7 M urea
(make up with solid urea on the day)

Phenol:
99.9 % phenol
0.1 % 8 hydroxyquinoline
(water saturated & neutralised)

Chloroform:
98 % chloroform
2 % isoamylalcohol

TSE washing solution:
100 mM NaCl
10 mM Tris/HCl pH 7.5
100 µM EDTA, neutralised.

Glucose solution GTE:
50 mM glucose (18% = 1 M)
25 mM Tris pH 8.0

10 mM EDTA

Alkaline SDS - make fresh ! 200 mM NaOH
1% SDS
(add the water first and leave at room temperature)

5 M Potassium acetate pH 4.8: 3.0 M K Acetate
2.0 M Acetic Acid
This is not really pH 4.8 - probably around 5.0. The solution is 3 M with respect to potassium anions and 5 M in acetate. For 1 litre you make 600 ml of 5M K Acetate and add 115 ml of glacial acetic acid and 285 ml of water.

Boiled RNase A:
Dissolve BDH RNase at 10 mg/ml
in 10 mM Tris/HCl pH 7.5 / 15 mM NaCl
and boil for 10 min. Cool to room temp and store in frozen aliquots (-20°C).

Lysozyme: (5 mg/ml in GTE - make fresh!)

TE: 10mM Tris
0.1mM EDTA

SM phage buffer:

for 1 litre:	NaCl	5.8 gr
	MgSO4.7H2O	2.0 gr
	Tris7.5 (1M)	50 mls
	Gelatin (2%)	5 mls

PEG 6000 was bought from BDH.
(For preparing phage and DNA).

Polymerase Chain Reaction:
10 X Taq buffer: 100 mM Tris / HCl pH 8.3
500 mM KCl
15 mM MgCl2

0.1% gelatin

All 4 dNTPs: 1.25mM each, mixed

Primers: 100 µg/ml in water

2. SUBCLONING.

Enzymes were obtained mainly from Amersham, but some were also bought from BRL, New England Biolabs and Boehringer.

Storage was at -20o C. Enzymes were usually supplied in a buffer containing 50% glycerol and sometimes were diluted in this buffer to a convenient working concentration.

Restriction enzyme buffers:

For most restriction enzyme digests, the manufacturers' recommended 10 x reaction buffer was used.

Ligations:

Ten times ligation buffer: 660 mM Tris/HCl pH 7.6
66 mM MgCl2

Ten times DTT: 100 mM DTT

Ten times rATP: 10 mM rATP

Cloning in M13:

BCIG 20 mg/ml in dimethyl formamide.

IPTG 24 mg/ml in water.

L agar plates (1.5% agar/L Broth).

Top agar - L broth with 0.7 agar.

2 x YT: 16 gm/l Bacto Tryptone
10 gm/l Yeast extract
5 gm/l NaCl

L Broth: 5 gm/l NaCl
10 gm/l Bacto Tryptone
5 gm/l Yeast Extract Adjust pH 7.5

[for LBM, add 2.5 gr MgSO4 per litre].

Competent DH1 cells:

Psi Agar Medium: 5 g per litre Bacto Yeast Extract
20 g per litre Bacto Tryptone
5 g per litre $MgSO_4$ with KOH
14 g per litre Bacto Agar

Psi Broth: as above, ommitting the agar.

Tfb1:	MW	100ml
30 mM potassium acetate	98	294mg
100 mM $RbCl_2$	121	1210mg
10 mM $CaCl_2$	147	147mg
50 mM $MnCl_2$	198	990mg
Add glycerol to 15% (v/v)		15ml

The pH was adjusted to 5.8 with 0.2M acetic acid (1:85 dilution) and the solution was sterilised by filtration.

TfbII:	MW	100ml
10 mM MOPS	209	209mg
75 mM $CaCl_2$	147	1103mg
10 mM $RbCl_2$	121	121mg
15% (v/v) glycerol		15ml

The pH is adjusted to 6.8 with KOH and the solution sterilised by filtration.

3.LABELLING METHODS.

Nick translation:

5x buffer/nucleotide solution:
100 µM dATP
100 µM dGTP
100 µM dTTP
250 mM Tris/HCl pH 7.8
25 mM MgCl2
50 mM 2-mercaptoethanol

Nick translation enzyme solution:

500 Units/ml DNA polymerase I
5 ng/ml DNase
100 ug/ml BSA
[Make up in Klenow dilution buffer]

Solutions for random primer labelling:

Buffer O: 125 mM $MgCl_2$
1.25 M Tris pH 8.0

Buffer A: 1 ml of buffer O
+ 18 µl beta mercaptoethanol
5 µl 100 mM dATP
5 µl 100 mM dTTP
5 µl 100 mM dGTP

Buffer B: 2 M HEPES pH 6.6
(titrate with 4 M NaOH)

Buffer C: random oligonucleotides. 112 ug/ml.

Mix A, B & C in the ratio 100 : 250 : 150 and store at -20o. This is referred to as "ABC" buffer.

Final concentration in ABC buffer:

$MgCl_2$	25 mM
Tris pH 8.0	250 mM
Beta mercaptoethanol	50 mM
Each dNTP	100 µM
HEPES pH 6.6	1 M
Oligos	33.6 µg/ml

4. DNA ANALYSIS.

Agarose: for standard gels low gelling agarose is to be avoided.

Ten times Tris/Borate: 108 gm/l Tris base
55 gm/l Boric acid
9.3 gm/l EDTA, pH 8.3

Loading dye mix: 0.25 % Bromophenol blue
50 % Glycerol
10 mM EDTA

Nitrocellulose sheets: Schleicher & Schuell BA 85

Denaturing buffer: 0.5M NaOH / 1.5M NaCl.

Neutralising buffer: 0.5M Tris7.5 / 1.5M NaCl.

20 x SSPE: 3.6 M NaCl
200 mM Sodium phosphate pH 6.8
1 mM EDTA

20x SSC: 3 M NaCl
300 mM Na_3citrate

Denhardt's reagent, one times = 0.02% each: Ficoll, Polyvinylpyrolidone, B.S.A.

5. RNA ANALYSIS.

20 times buffer:		for 500 mls
(for Northerns)	MOPS 400 mM	41.8 gm
	NaAc 100 mM	4.05 gm
	EDTA 20 mM	3.72 gm

This was titrated to pH 7.0 with 1 M NaOH (about 100 ml)
Then it was made 0.1 % in formaldehyde for storage.

Sample buffer: 100 µl formamide
30 µl formaldehyde

10 µl 20 times buffer.

A 40 % solution of ordinary grade formaldehyde was used.

6. RNA PREPARATION.

4 M Guanidinium mix:

10 gm guanidinium isothiocyanate (Fluka)
100 ml dist water
10 ml 1 M Tris/HCl pH 7.5
4 ml 500 mM EDTA

Warm to dissolve, then add (in the fume hood)

12 ml 35% Sarkosyl (sodium laurylsarkosinate)
2 ml beta mercaptoethanol

Make volume up to 200 ml and sterile filter.
Store at -20 °C in 20 ml aliquots.

Poly U Sepharose solutions:
Loading buffer:

25% formamide
10mM PIPES pH 7.0
1mM EDTA pH 8.0
0.1% SDS

Wash buffer:

Same as loading buffer, but 50% formamide.

Elution buffer:

Same as loading buffer, but 96.5% formamide.

7.PACKAGING EXTRACTS.

Sonication buffer: 20 mM TrisCl(pH 8.0)
1 mM EDTA
5 mM beta mercaptoethanol

Packaging buffer: 6 mM TrisCl(pH 8.0)
50 mM spermidine
50 mM putrescine
20 mM MgCl2
30 mM ATP
30 mM beta mercaptoethanol

Sucrose solution: 10% sucrose
50 mM TrisCl(pH 8.0)

Lysozyme solution: 2 mg/ml lysozyme in
250 mM TrisCl(pH 8.0)

E.coli strains:
BHB2690 lambda packaging strain (prehead donor)
BHB2688 lambda packaging strain (packaging protein donor)

8. SEQUENCING.

10 x Klenow buffer: 50 mM MgCl2
100 mM Tris/HCl pH 8.0
300 mM NaCl

Materials for sequencing with alpha ^{32}PdCTP:

Dideoxytriphosphate (ddNTP) solutions:
Working solutions are 0.5 mM ddTTP
0.3 mM ddATP
0.3 mM ddGTP
0.1 mM ddCTP
These solutions, suitable for use with labelled dCTP, are altered accordingly for different labelled nucleotides.

Deoxytriphosphate (dNTP') mixes:

	dTTP'	dATP'	dGTP'	dCTP'
0.5 mM dTTP	1 µl	20 µl	20 µl	20 µl
0.5 mM dATP	20 µl	1 µl	20 µl	20 µl
0.5 mM dGTP	20 µl	20 µl	1 µl	20 µl
50 mM Tris pH 8.0} /0.1 mM EDTA}	5 µl	5 µl	5 µl	5 µl

Materials for sequencing with alpha ^{35}S dATP:

Dideoxytriphosphate (ddNTP) solutions:

Working solutions are 0.5 mM ddTTP
25 µM ddATP
0.1 mM ddGTP
0.1 mM ddCTP

Deoxytriphosphate (dNTP') mixes:

	dTTP'	dCTP'	dGTP'	dATP'
0.5 mM dTTP	5 µl	100 µl	100 µl	100 µl
0.5 mM dCTP	100 µl	5 µl	100 µl	100 µl
0.5 mM dGTP	100 µl	100 µl	5 µl	100 µl
50 mM Tris pH 8.0/} 0.1 mM EDTA}	40 µl	40 µl	40 µl	40 µl
Sterile water	155 µl	155 µl	155 µl	155 µl

Alpha ^{35}S deoxyadenosine thiotriphosphate : 400Ci/mmol from Amersham. All other solutions were the same as for sequencing with ^{32}P-dCTP.

Formamide dye:

200 mM EDTA, 0.03g Xylene cyanol, 0.03g Bromophenol blue in 100 ml de-ionised formamide.

9. ACRYLAMIDE SEQUENCING GELS.

The urea mix described below was used at thickness of 0.4

mm. For a 35 x 42 cm gel, 45 ml are required.

For 60 ml of gel solution the recipe requires:

29.9 gm urea
22.9 ml distilled water
6.0 ml 10 x tris/borate buffer
9.0 ml acrylamide solution- 38 % acrylamide
2 % bisacrylamide

Before pouring the gel are added:

120 µl of 25 % ammonium persulphate
120 µl of TEMED

10 x TBE:
108.0g Tris
55.0g Boric acid
9.3g $Na_2EDTA.2H_2O$
(Made up to 1 litre)

40% acrylamide:
38.0g acrylamide
2.0g bis-acrylamide

Made up to 100ml with GDW, then de-ionised by stirring gently with 5g Amberlite MB-1 for 30 min. Filter, and store at 4oC in the dark.

25% Ammonium persulphate (AMPS): Dissolve 250mg in 1ml GDW, store at 4oC. Prepare fresh AMPS solution every 2-3 days.

TEMED (NNN'N'-tetramethylenediamine): Sigma Chemicals. Store at 4oC.

Gradient gels:

Top gel:
230.4g urea
27.4g acrylamide }
1.44g Bis acrylamide }
(OR 72mls 40% acrylamide stock)
24mls of 10 x TBE

Made up to 480mls with water and filtered. Store at 4°C.

Bottom gel: 43.2g urea

9.0g sucrose

5.13g acrylamide }

0.27g Bis acrylamide }

(OR 13.5 mls 40 % acrylamide stock)

0.01g bromophenol blue

22.5mls of 10 x TBE

Made up to 90mls with water and filtered. Store at 4 °C.

CHAPTER III

MOLECULAR ANALYSIS OF THE CALCITONIN GENE OF AN OSTEOPOROTIC PATIENT WITH CALCITONIN DEFICIENCY

CONTENTS:

Abstract. A young male osteoporotic patient presented at the age of 19 with a history and clinical findings suggestive of severe osteoporosis, which had started 3 years earlier. After detailed laboratory examinations he was found to be completely calcitonin deficient; this was considered as a probable cause for his condition. He subsequently received CT treatment, to which he responded well. The possibility of a defect in his calcitonin gene was considered and a detailed structural study of the gene was undertaken. Genomic Southern blots were done with various restriction enzymes, which did not reveal any gross abnormalities in the gene. A genomic library was then constructed using the EMBL 4 lambda cloning vector, into which 17-20 kb genomic fragments of partially digested DNA were introduced. Two independent clones containing most of the alpha CT/CGRP gene were isolated and one of them was analysed in detail. The nucleotide sequence of the coding regions of the gene was analysed and found to be normal, ie coding for normal precursor polypeptides. The region around the polyadenylation site, and the splice junction sequences were also normal. The only abnormality identified was a single base insertion in the intron separating exons IV and V. The affected sequence is homologous with an intron sequence from the globin gene which is implicated in splicing, and lies next to a CTGAC sequence, the consensus sequence for lariat formation during RNA processing. It was therefore originally speculated that this change might disrupt normal RNA maturation and be responsible for the patient's condition. However, application of the PCR method allowed sequencing of normal non osteoporotic individuals with normal CT levels and showed that this sequence is also present in the population suggesting that it is a neutral polymorphism. No explanation can therefore be offered for this patient's lack of CT.

III A. INTRODUCTION

As has already been discussed there is no clear consensus as to whether CT deficiency is important in the development of postmenopausal osteoporosis, the most common form of this disease. Although there are claims that CT deficiency in thyroidectomised patients is associated with a reduction in bone mass, this has not been confirmed by other studies (McDermott et al, 1983, Hurley et al, 1987). However, in none of these studies had the CT deficiency started to affect the patients at an early age, when the skeleton was still developing. It is at this time that the hormone is considered to exert its physiological function.

In 1982, a young male patient was referred to the Endocrine Unit at the Hammersmith Hospital at the age of 19 with severe osteoporosis (Stevenson et al, 1982b). This patient has subsequently been under the care of Dr John Stevenson who performed all the clinical investigations. His CT levels were undetectable under standard conditions as well as after stimulation with the three standard provocative tests for CT. CT deficiency was therefore considered as a possible cause for his condition. Katacalcin (the C-terminal peptide co-secreted with CT) was also undetectable. This patient has responded well to CT treatment over the past seven years. His CGRP levels were found to be normal, although it is not known whether this was alpha or beta CGRP as the radioimmunoassay does not distinguish between the two.

The CT assays have been repeated several times on plasma from this patient over the course of the last 7 years. 'Normal' basal CT levels are close to the threshold for detection (80pg/ml). This means that it would be possible that this patient is 'normal' for CT and has levels just below the limit of detectability under basal conditions. However, it has been the lab's experience that normal male

footnote to page 116
* unless this patient represents a sporadic case (new mutation).

volunteers have detectable CT levels after stimulation. Nevertheless there is no proof that this patient's condition is caused by a lack of CT.

If lack of CT is responsible for the osteoporosis, then we have to consider what the primary defect might be. One possibility might be that CT production was normal but neutralising antibodies were present - no anti CT antibodies could be detected however, and the satisfactory response to CT therapy would also indicate that CT is not being neutralised in this patient. Another possibility might be that there are no C-cells in his thyroid due to agenesis or auto-immunity. These conditions have never been described and would be difficult to test.

The other possibilities concern the CT gene itself. A defective CT gene would be expected to have a recessive phenotype and the absence of any family history would rule out the inheritance of a dominant disorder in this case,* It is unlikely that the CT/CGRP gene is completely absent or is otherwise incapable of expression as CGRP levels are normal. Although the assay does not distinguish between alpha and beta CGRP, one would expect that there would be some clinical signs if the alpha species were absent, as this is the major species present in most tissues. The most likely possibilities would be those affecting CT expression only. Mutations specifically affecting the processing of CT mRNA would be expected to lie in introns III or IV or in exon IV whilst those affecting the translation or maturation of the protein would be found in exon IV.

Although there could be no proof that this patient's CT gene was defective, it was nonetheless decided to pursue this further. If a defect could be demonstrated, it would provide an explanation of his CT deficiency and by inference would support the importance of CT in the maintenance of skeletal integrity. Such a finding would have obvious implications for the use of CT in the treatment or

even prevention of osteoporosis.

The ideal way to study the function of the gene would be to study its expression in the cells where CT is normally produced, the C-cells of the thyroid. However, the C-cells are not accessible for direct analysis of the gene's expression. Therefore, a structural study of the gene was undertaken to look for any changes which might explain the failure to produce CT.

As a first step, Southern blot analysis was performed to look for any deletions or insertions in the region of exon IV. As this technique was not then being used for whole genome analysis in the lab, it was first necessary to improve the methods to achieve the required sensitivity. Because no gross alterations to the gene were apparent from this study, it was then decided to clone this patient's CT/CGRP gene and subclone appropriate regions for DNA sequence analysis.

III B. RESULTS AND DISCUSSION

III.1. Southern Blot Analysis.

This technique was not being used for whole genome analysis in our unit and it was necessary to improve the sensitivity of hybridisation before it could be used for this purpose. The problem appeared to lie with the labelling of the DNA probes. These were restriction fragments eluted from agarose gels and then labelled by nick translation (Materials & Methods, section II.3.1.) but incorporation was often low because of inhibition by residual agarose. There were reports that single stranded RNA probes gave the best results so it was decided to try these (Melton et al, 1984). To make an RNA probe one has to transfer the cloned sequence into a special plasmid vector containing the promoter sequence for an RNA polymerase adjacent to the cloning site. At the time, vectors containing an SP6 promoter were the only ones available, SP6-4 and SP6-5 (Melton et al, 1984). The DNA probes in these experiments were derived from the plasmids phTB3 (CT cDNA probe (Craig et al, 1982)) and phTB58 (CGRP specific probe (Edbrooke et al, 1985)), which were kindly provided by Dr Roger Craig. Of these, plasmid phTB3 contains the whole of exon IV and most of exon III of the human alpha CT/CGRP gene. Plasmid phTB58 contains the cDNA of a partially processed transcript of the gene which contains exons V and VI, intron IV and a portion of exon IV but the plasmid we received lacked exon IV and all but the last 24 bases of intron IV.

The scheme for transferring the phTB3 insert into the SP6 vectors is shown in Fig 3.1. The CT insert was excised from the plasmid pBR322 by digestion with Pst 1 and ligated to Pst 1 cleaved SP6-4 (Materials and Methods, section 2.6.). Recombinants were introduced into competent DH1

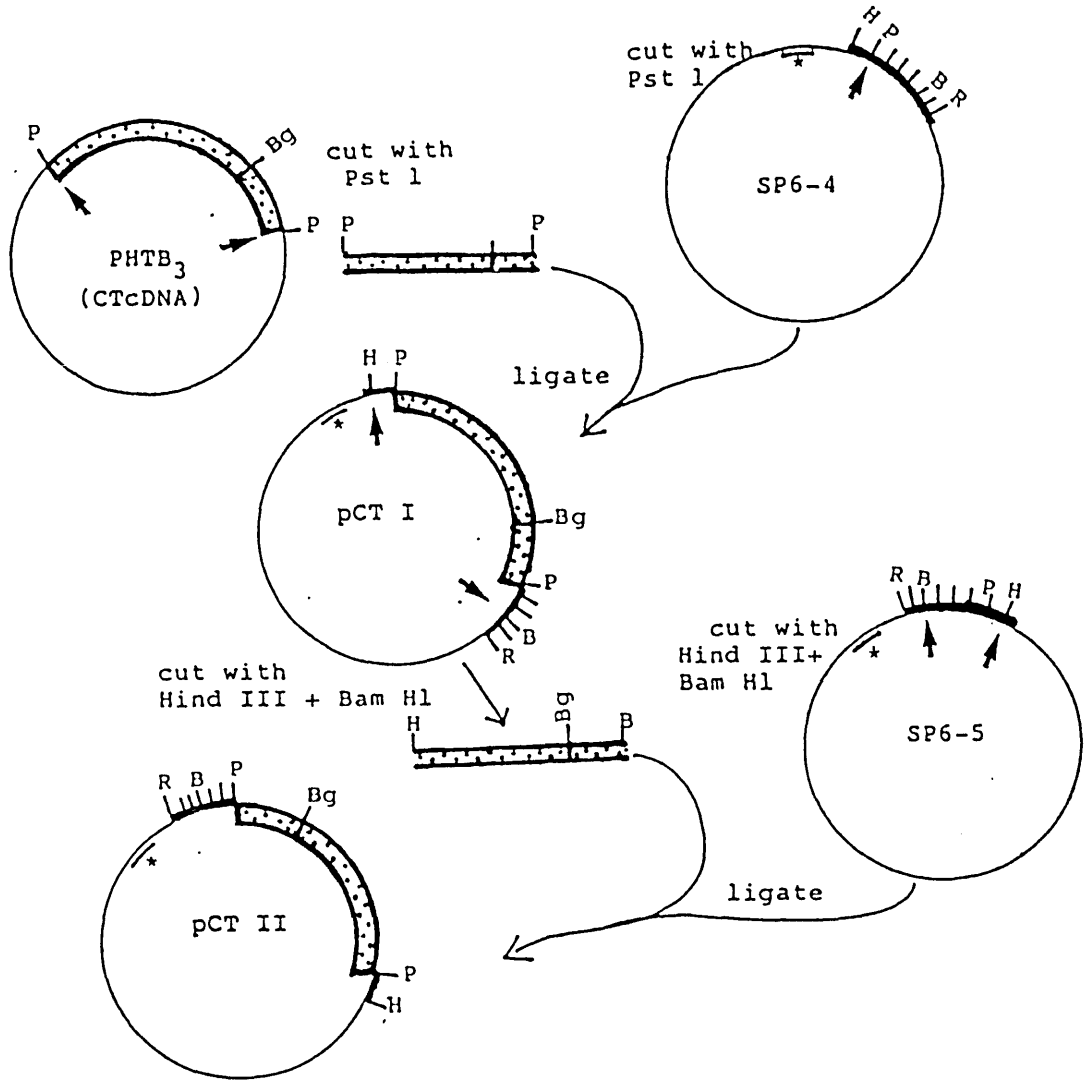

fig 3.1. Scheme for transferring CT cDNA insert to SP6 vector.

PhTB3 was digested with Pst I and the CT cDNA fragment was subsequently cloned into the Pst I site of the SP6 4 vector (recombinant plasmid pCTI). To obtain both orientations relative to the SP6 promoter [*] the vector SP6 5 was used. Plasmid pCTI was digested with the enzymes Hind III and Bam HI and the resulting fragment was cloned into the Hind III/Bam HI sites of the vector SP6 5 (recombinant pCTII). The Bgl II site present in CT cDNA close to the exon III/IV junction is also indicated. [P:Pst I, R:Eco RI, B:Bam HI, H:Hind III, Bg:Bgl II].

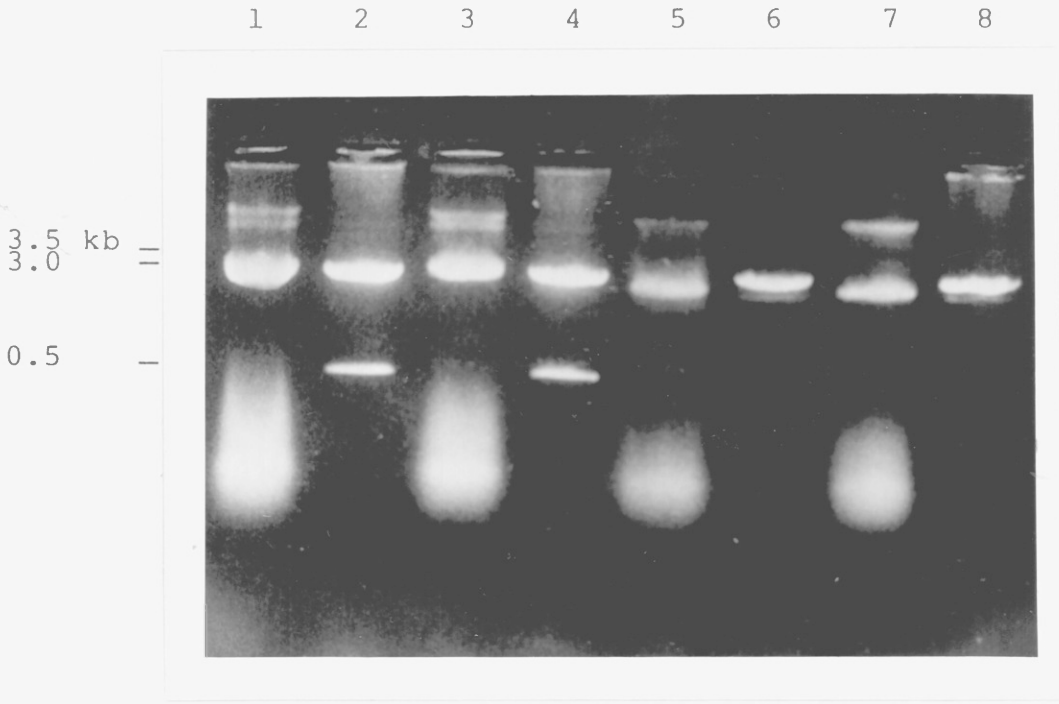

fig 3.2. Restriction digests of pCT recombinants.

4 colonies positive by hybridisation were picked and grown. DNA was prepared (see materials and methods section 1.2), digested with the enzyme Pst I and analysed by electrophoresis on an ethidium bromide stained agarose gel. Lanes 1,3,5,7: uncut plasmid. Lanes 2,4,6,8: Pst I digests. Lanes 1-4: 1 µg of DNA, lanes 5-8: 0.5 µg of DNA.
The cloned CTcDNA insert is of the expected size.

cells (Materials and Methods, section 2.6.) and spread on ampicillin containing nutrient plates. Filter replicas of the colonies were then screened with the CT specific probe and 12 positive colonies were analysed (Materials and Methods, section 4.5.). All had inserts of the expected size (fig 3.2). The cloning procedure should allow the insert to be cloned in both orientations allowing one to make both sense as well as antisense probes, that is having the same or the complementary sequence to the mRNA. Since it was possible that these probes would be used to analyse mRNA at some stage, it was important to get both. The two orientations could be distinguished by digestion with either Hinf 1 or Hae III. However, all 12 recombinants gave identical restriction patterns with both of these enzymes. One of these was selected for further use and was named pCTI.

To force the insert to clone in the opposite orientation with respect to the SP6 promoter, the other SP6 vector, SP6-5, was used. SP6-5 has its cloning site in the opposite orientation to SP6-4. The insert was excised from pCTI with Bam H1 and Hind III and the SP6-4 sequence was inactivated by further digestion with Eco R1. This digest was then ligated to Bam H1 / Hind III digested SP6-5 (fig 3.1). Only one positive colony (pCTII) was obtained after hybridisation screening. When this was grown it was noted that it grew more slowly than expected. Its growth rate was only half that of pCTI (Fig 3.3). It would appear that, in this orientation, the CT sequence was somehow harmful to its host probably explaining why it was so difficult to clone. It is not clear whether or not the CT itself might be responsible for this effect.

Although much effort was expended in obtaining these clones, RNA probes actually proved to be somewhat disappointing in my hands and these clones were simply used as a source of CT insert for the method which was subsequently used, random primer labelling (Materials and Methods, section 3.2.). This method gives high specific activities

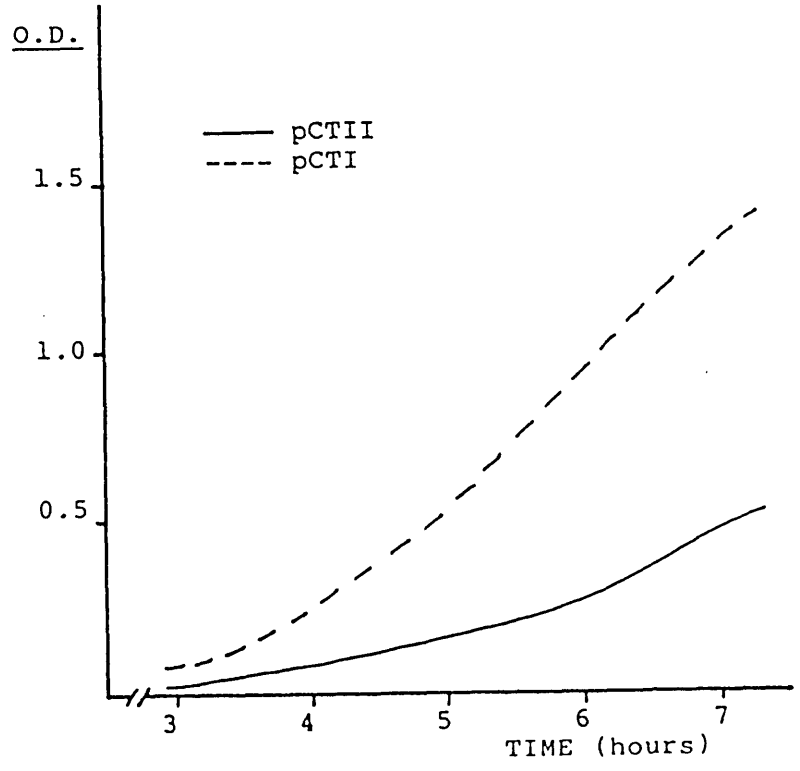

fig 3.3. Growth curves of plasmids pCTI and pCTII. OD: Optical Density at 550nm.

The unusually slow growth of pCTII may indicate that the cloned sequences are somehow harmful to its host in this orientation.

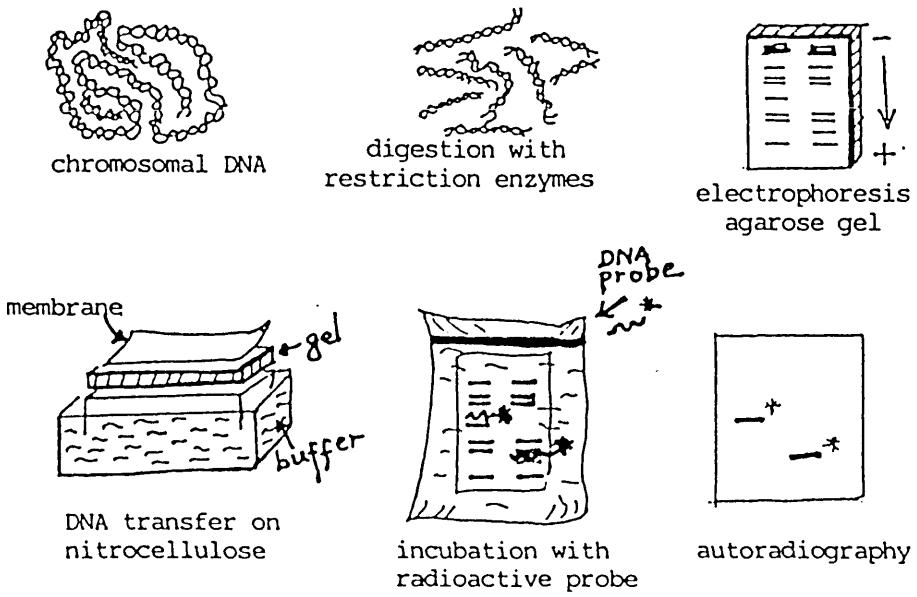

fig 3.4. Southern blot analysis in a schematic representation.

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* This 4.2 Kb polymorphic Taq fragment is detected with a CGRP probe and has not been reported by others. However its frequency has not been assessed.

(c 10^9 dpm / μg) and is not affected by the presence of contaminating agarose in the DNA.

Once the problem of obtaining suitable probes had been resolved it was possible to proceed with the Southern blot analysis. In this procedure, DNA is digested with a restriction enzyme and the resulting fragments are separated by size by electrophoresis on an agarose gel. The fragments are rendered single-stranded by treatment with alkali and transferred to a nitrocellulose membrane by capillary blotting (Materials and Methods, sections 2.2. and 4.2.). The membrane is baked to stabilise the DNA and is then hybridised with a specific probe. Finally, the filter is washed to remove unhybridised probe and used to expose a sheet of X-ray film (fig 3.4).

DNA was prepared from peripheral lymphocytes (Materials and methods, section 1.) and digested with the enzymes Eco R1, Pvu II, Pst 1, Bam H1 and Taq 1. Parallel digestions were performed on control DNA obtained from volunteer members of the laboratory who were apparently normal. These digests were analysed by Southern blotting using both CT and CGRP specific probes. Examples of autoradiographs of these blots are shown in fig 3.5. In all cases the hybridisation pattern obtained from our patient's DNA was the same as that obtained from the controls except in one instance, Taq 1 digestion, in which an extra band is visible in one of the controls. This represents a previously unreported polymorphism in the gene, which might be useful if ever it becomes necessary to follow the inheritance of this gene.*

From the Southern blot analysis it became obvious that no gross alterations were present in the CT gene of our patient. This type of analysis does not reveal changes which are less than about 100 bases, unless they happen to affect one of the restriction sites. Most gene defects are

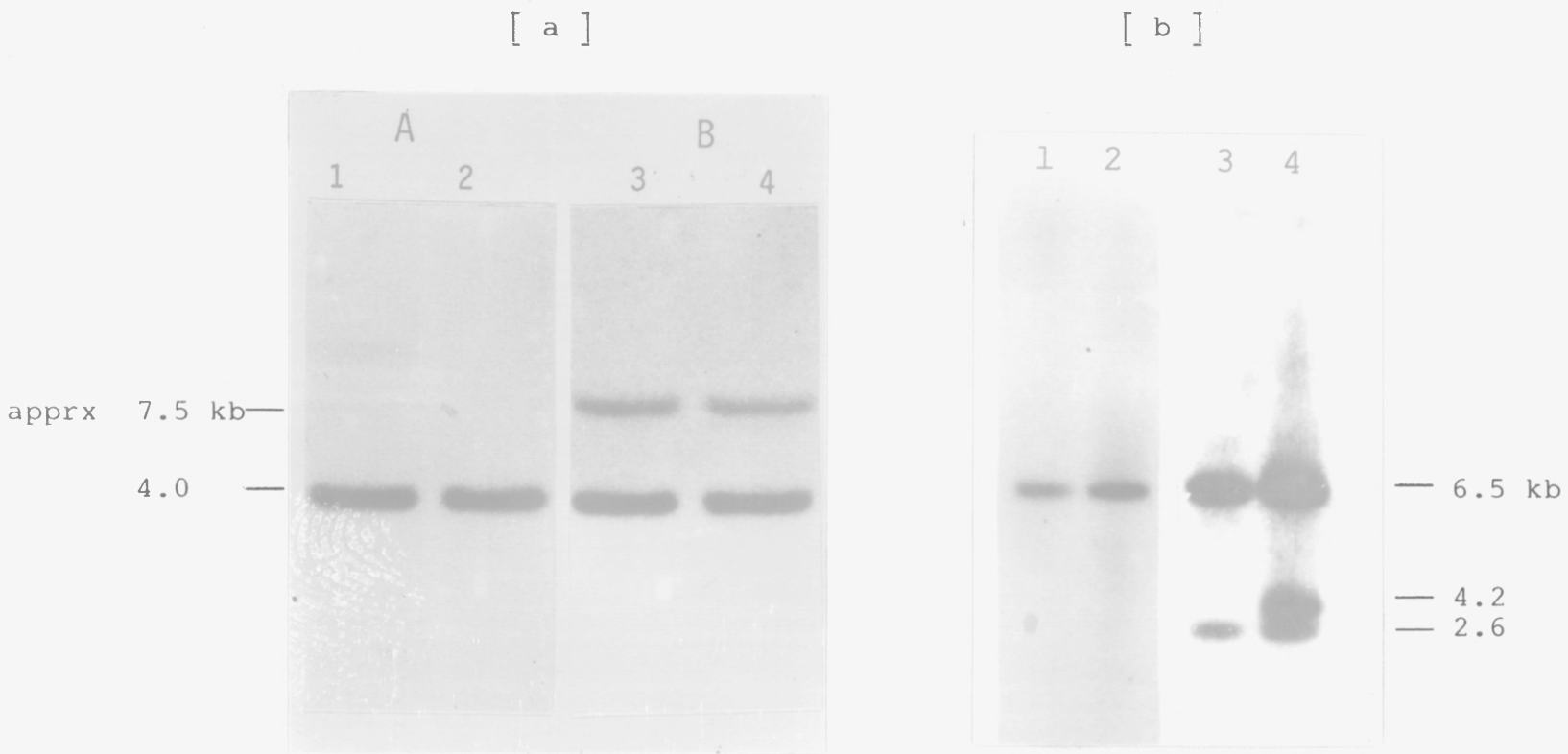

fig 3.5. Genomic Southern blots of patient DNA.

[a]. 10 micrograms of patient DNA (lanes 1+3) along with 10 micrograms of control DNA(lanes 2+4)were digested with the enzyme Pst I. Panel A: Southern blot probed with pCTI. Panel B: The same Southern blot reprobed with phTB58.
[b]. Genomic Southern blots of patient (lanes 1 and 3) and control (lanes 2 and 4) DNA using the enzyme Taq I. Lanes 1 and 2 are hybridised with pCTI and lanes 3 and 4 are hybridised with phTB58.

caused by point mutations which may affect either the coding sequences or other sequences important for the correct processing of the mRNA (Kazazian and Boehm, 1988). Mutations of this kind can only be recognised by sequence analysis. To pursue these studies further it was therefore necessary to make a genomic library from the patient's DNA and clone the gene for sequence analysis.

III.2. Construction of genomic library.

A genomic library is a population of recombinant clones, in which in theory all the DNA present in the organism should be represented. In order to construct a representative library, which has a good chance of containing all the genes of the organism, and thus the gene under study, one has to calculate the number of clones which should be present in the library. The formula used to calculate this number (N) is: $N=\ln(1-P)/\ln(1-\alpha/\beta)$, where P is the probability that a gene is represented in the library, α is the average size of genomic DNA fragments contained in the library and β is the size of the genome. If for example one uses 17kb fragments, for a P=95% one has to construct a library with 535,000 clones (Maniatis et al, 1982).
The steps involved in constructing a library are (fig 3.6):

1. Vector choice and preparation.
2. Generation of random genomic fragments to be cloned.
3. Preparation of packaging extracts so that the recombinant DNA can be introduced into E Coli (for lambda and cosmid vectors).
4. Ligation reactions to find the optimum insert to vector ratio, followed by large scale ligation and plating out of the library.

2.a. Vector.
For the construction of the genomic DNA library the vector

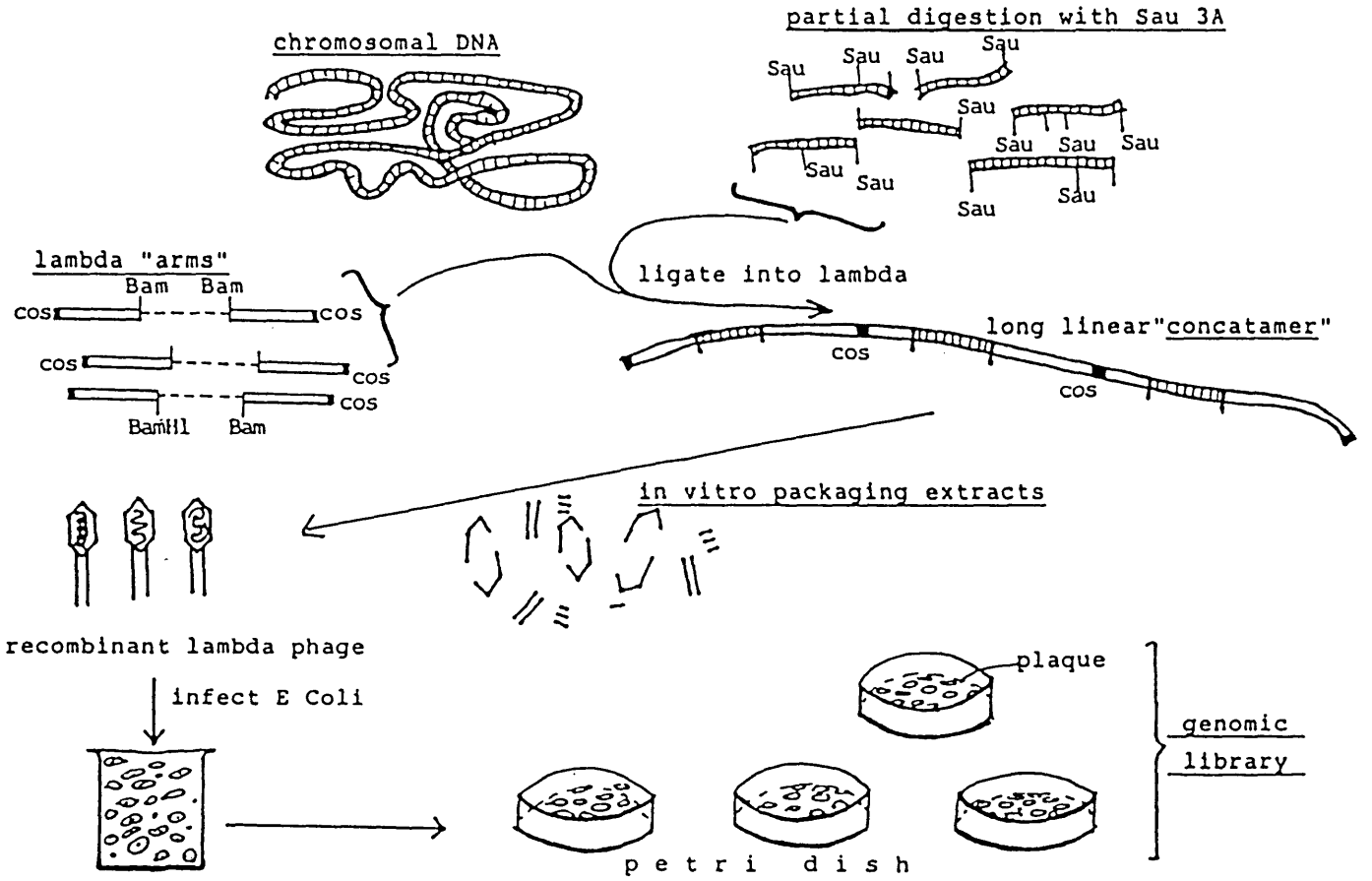

fig 3.6. Construction of genomic library in lambda phage.

lambda phage EMBL4 was chosen. It is a substitution vector and is suitable for cloning 15-20 kb fragments after removal of the central "stuffer" fragment, which is not essential for lytic growth. The wild type phage has the "spi" phenotype (short for: "sensitive to P2 interference"), which means that it cannot infect cells that carry P2 prophages. The genes which confer this phenotype reside in the central part of its genome which is replaced by the insert. For this reason, when a foreign DNA sequence is cloned into this vector, the resulting recombinant phage are spi^-. This provides a selection means; indicator bacteria carrying P2 prophage are used, which are only infected by phage which are spi^-, i.e. recombinant ones.

The EMBL4 cloning vector has a Bam HI cloning site on each side of the "stuffer" fragment. Furthermore, one can use the enzyme Sal I, which only cuts the DNA in the "stuffer" fragment, to reduce the chance that this will religate to itself during the cloning experiment. This then means that one can obviate the need for preparation of vector "arms" by centrifugation through a sucrose gradient (Materials and Methods, section 6.2.). EMBL4 lambda phage DNA was therefore prepared using the liquid culture method (Materials and Methods, section 1.5.4.) and this was digested with Bam HI and Sal I. The indicator bacteria used were the strain Q359, which is a $recA^+P2$ strain and the strain used for growing both recombinants as well as non recombinants was Q358, which is $recA^-P2$.

2.b. Genomic fragments.

The lambda vector which was used for the construction of this library accepts substitution fragments of 15-20 kb between its Eco R1 or Bam H1 sites. One has to ensure that all sequences have a chance of being cloned and that there will not be a systematic exclusion of particular sequences. If genomic DNA were digested with Eco R1 or Bam H1, many sequences would be excluded because they were not found in fragments of the appropriate size. To overcome this

problem, partial digestion with enzymes that recognise frequently occurring tetranucleotide sequences within eucaryotic DNA, yields a population of fragments which is close to random. The enzyme which would be suitable for the vector EMBL4 would be Sau 3A 1 for the Bam H1 site and the "star" activity of Eco R1 for the Eco R1 site. The latter would prevent the Eco R1 site being used to excise the insert and is notoriously difficult to work with. The enzyme Sau 3A 1 was therefore used which creates "sticky" ends compatible with Bam H1.

A test digestion was first performed to define the concentration of the enzyme, which should be used to generate partially digested fragments of the size of 17-20 kb. The optimum conditions were found to be incubation at 37 °C for 1 hour with 0.25 units of Sau 3A 1 per microgram of genomic DNA (Materials and Methods, section 6.1.1.). A total of 250 µg of genomic DNA was then digested and size fractionated through a 10-40% sucrose gradient and the appropriate fractions were pooled and ethanol precipitated (Materials and Methods, section 6.1.2.). The yield of the appropriate fractions was 80 µg.

2.c. Packaging extracts.

The recombinant lambda DNA molecules have to be packaged in vitro into viable bacteriophage particles, so that they can infect appropriate host cells. The packaging extracts were prepared from two different strains of E Coli, which carry two mutants of bacteriophage lambda in which the genes responsible for the assembly of the particles are affected. Of these proteins, E protein is required for prehead formation. E mutants assemble all the components of the particles and serve as packaging protein donor (strain BHB 2688). D protein is required for insertion of lambda DNA in the capsid. D mutants accumulate preheads and serve as prehead donor (strain BHB 2690). The extracts were prepared separately from each strain and were then mixed when the DNA was added. The method followed is

described in Materials and Methods, section 7.. The extracts which were derived from BHB 2688 are referred to as "freeze-thaw" lysates and the ones which are derived from BHB 2690 are referred to as "sonicated extracts". When the two extracts are combined on ice with lambda DNA, infectious phage particles form, which are then used to infect E coli. After a number of trials, packaging extracts with an efficiency of 10^7 pfu/µg of wild type lambda DNA were prepared.

2.d. Ligations.

The aim of the ligation between the lambda arms and the genomic fragments is to create recombinant linear molecules, which can most easily be packaged into infectious particles. In vivo, the substrate for packaging is a linear concatamer of lambda DNAs. The aim of the ligation is therefore to produce linear molecules in which the fragments have been joined together in the order ...left arm : insert : right arm : left arm : etc. From this concatamer, "lambda" monomers between 78% and 105% the length of wild type lambda can be excised and packaged into infectious particles. Successful packaging therefore depends on the formation of the right molecular arrangements with correctly sized inserts during the ligation reaction.

The number of compatible cohesive ends in the reaction should be equally distributed between inserts and each of the two vector fragments. Because vector arms have one such site each, at one end only, and inserts have two, one on each end, the molar ratio of arms to inserts should be 2:1. One can theoretically calculate the ratio as well as the absolute concentration of DNA fragments to be ligated, which should be present in the ligation reaction, so that the optimal yield of long concatamers with correct size recombinants can be achieved. If one assumes that all the fragments in the reaction have perfect ends, then the calculated insert concentration in the reaction should be 43 µg/ml and the arm concentration should be 135 µg/ml

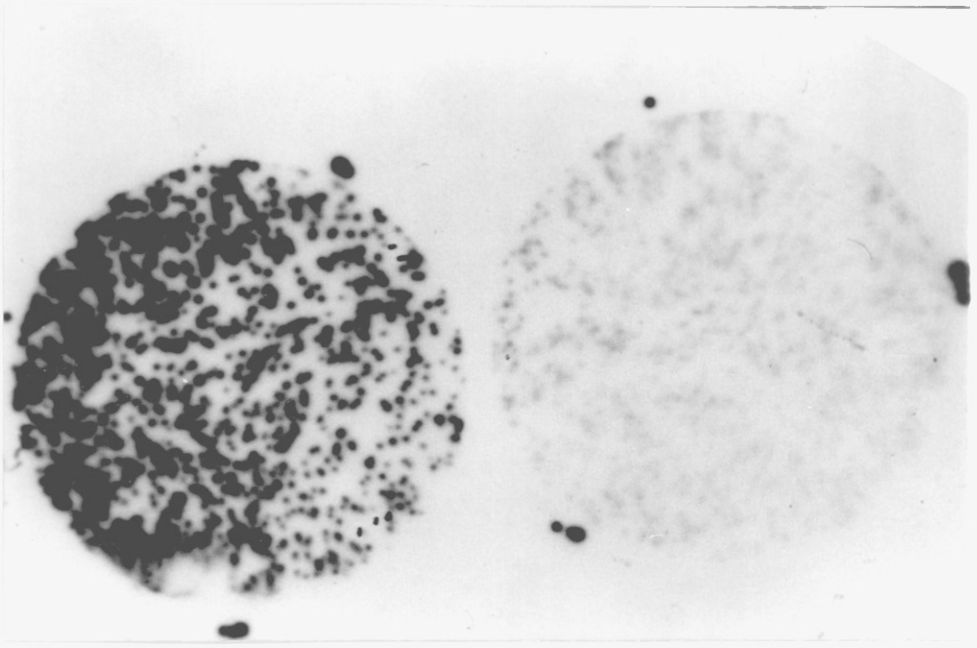

fig 3.7. Comparison of nitrocellulose with PALL nylon membrane for plaque lifts.

Lifts were taken from lambda EMBL4 plaques and were subsequently probed with nick-translated lambda EMBL4 DNA. Left: nitrocellulose. Right: PALL nylon membrane.

The signal obtained with nitrocellulose is at least three times stronger; this was therefore used in my experiments.

(Maniatis, Fritsch and Sambrook, 1982). One has, however, to adjust the conditions empirically doing control experiments with the preparations which are available until the optimal results can be obtained.

Based on these recommendations various ligation reactions were set up (Materials and Methods, section 6.3.) to find the optimal ratio of arms to insert. Using a weight ratio of 5 : 1, an efficiency of 4.5 x 10^4 pfu/μg of DNA was achieved. A total of 24 packaging reactions were then performed under these conditions giving a titre of 400,000 pfu/ml and a total of 980,000 recombinants.

III.3. Screening the library.

Before the actual plating and screening of the library was done, control experiments were carried out in the hope of improving hybridisation signals. Two methods have been described in which phage plaques are amplified before hybridisation screening (Woo et al, 1978, Maniatis, Fritsch and Sambrook, 1982). Both of these involve growing plaques on the replica filter (Materials and Methods. section 4.6.2.) and both were effective in amplifying the signals. However, the method of Maniatis et al resulted in a considerable loss of signal definition and the method of Woo et al gave a very unequal degree of amplification between plaques. Neither method was therefore adopted. Two types of transfer membranes were tested - nitorcellulose was found to give a signal which was about 3-fold stronger than ^{R}PALL membranes (fig 3.7). The library was therefore screened without amplification using nitrocellulose membranes.

A total of 600,000 plaques were screened on 12 135mm Petri dishes. The primary screening was done with a CT cDNA probe excised from pCT 1 as described in Materials and Methods, section 4.6.1.. Four weakly positive clones were identified. These were eluted from the plates and a por-

tion of the eluate was spotted onto a lawn of indicator bacteria (Materials and Methods, section 4.7.). After overnight incubation "macroplaques" were visible on the plates and when these were screened, strong signals were obtained in all 4 cases. Phage were then plated out at low dilutions and re-screened to obtain pure preparations. DNA was prepared from these (Materials and Methods, section 1.5.5.) and subjected to restriction enzyme analysis. DNA was digested with Eco R1 which has a recognition site on either side of the Bam H1 site and the restriction patterns of the phage were compared. Two different groups of clones were identified. One of these, lambda 2, was analysed in detail.

The filters were subsequently reprobed with a CGRP specific probe. The CT positive clones were also positive with CGRP and there were 15 further clones which were positive for CGRP but not for CT. These could represent alpha gene clones containing exon V but not exon IV sequences, or they could be beta gene clones since the exon V sequence is highly conserved and would cross-hybridise efficiently even under the stringent hybridisation conditions which were used. These clones were analysed at a later stage (see chapter IV).

III.4. Analysis of genomic clone lambda 2.

4.a. Exon IV.

The region of the gene of greatest interest was the CT coding part of the gene, exon IV. The first step in isolating this region was to generate an exon IV specific probe. As the insert from pCT 1 also contains part of exon III it is therefore not suitable to use as an exon IV specific probe. At the 5' end of exon IV in the cDNA clone there is a Bgl II recognition site (fig 3.1). This was used to separate exon IV from exon III sequences. PCT 1 was digested with Bgl II and Hind III, which has a recognition site in the polylinker. An exon IV specific fragment was

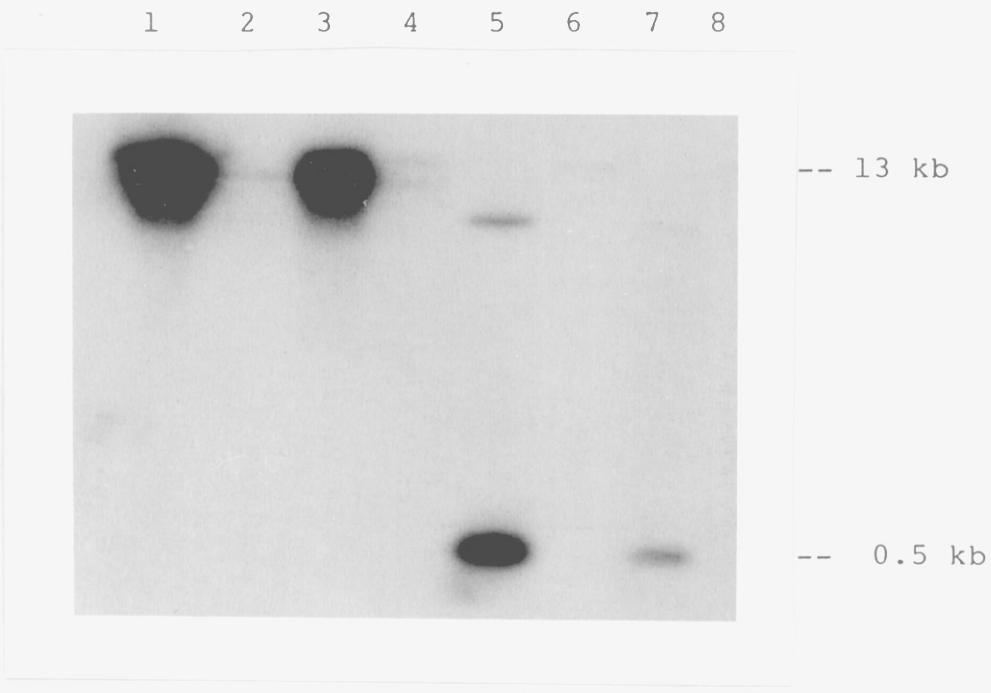

fig 3.8. Southern blot analysis of genomic lambda clones.

Recombinant phage lambda DNA was digested with Eco RI (lanes 1-4) and Bgl II (lanes 5-8). Clones are λ2(lanes 1,5), λ5(lanes 2,6), λ8(lanes 3,7), λ23(lanes 4,8). The blot was probed under stringent conditions with a CT specific probe. The results show that CT homologous regions are present in the DNA of clones 2 and 8 (lanes 1,3,5 and 7) indicating that these represent the alpha gene. The non-hybridising clones 5 and 23 in lanes 2, 4, 6 and 8 (track 8 only partially shown), were later shown to represent the beta gene. [Other very faint bands which may be visible represent non specific hybridisation].

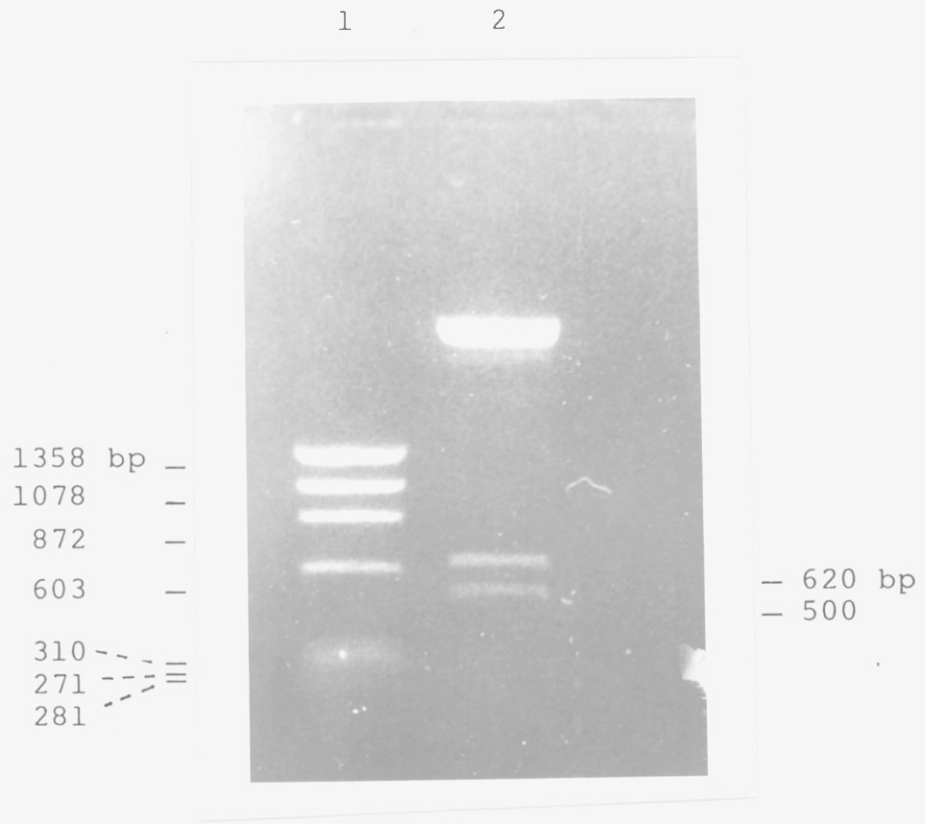

fig 3.9. Bgl II digest of plasmid p3.

Lane 1 is a marker (Hae III of phi X153). From the two smaller Bgl II fragments the 500 bp one represents exon IV and the 620 bp one represents intron IV.

Plasmid p3 is a subclone of the alpha gene lambda clone. This was obtained by cloning a 3.3kb BamH1/PvuII fragment starting from intron III which includes the whole of the exon IV - exon V region. Lane 1 is a marker (Hae III of phi X153). From the two smaller BglII fragments the 500bp one represents exon IV while the 620 bp one represents intron IV.

thus isolated. This was subsequently cloned between the Bam H1 (which has compatible ends with Bgl II) and Hind III sites of M13 phage mp8 (Materials and Methods, section 2.5.) and an exon IV specific probe was made. A single stranded DNA radioactive probe was made from this clone using the Klenow fragment of DNA Polymerase I (Materials and Methods, section 3.3.).

This probe was used on a Southern blot of the genomic clone, lambda 2, and regions hybridising to the exon IV of the patient's gene were identified (fig 3.8). Exon IV sequences were found in a Bgl II fragment of approximately 500 bp. Because plasmid clones are easier to grow (Materials and Methods, section 1.2.) and handle than lambda clones, before undertaking the subcloning of exon IV, a region of the gene containing exons IV and V was subcloned into a plasmid. The region subcloned was a 3.3 kb Bam H1 - Pvu II fragment starting from intron III and it was cloned in the Bam H1 - Sma I site of the plasmid SP6-5 (Sma I leaves blunt ends which are compatible with those obtained with Pvu II). This recombinant was named p3. Fragments derived from p3 were then cloned into M13 for sequencing. After digestion of P3 with Bgl II two small fragments were identified, one of 500 bp and one of 650 bp (fig 3.9).

The 500 bp Bgl II fragment was subcloned in both orientations in the Bam H1 site of mp9. Both strands of the DNA were sequenced in multiple determinations using the dideoxy- chain termination method (Materials and Methods, sections 8. and 9.). This Bgl II fragment contains all but 12 bases of exon IV from the 5' end of the exon. The missing sequence was obtained from one end of a 950 bp Bam H1-Bgl II fragment isolated from the genomic clone. This fragment was predicted from the published restriction map of the gene (Steenbergh et al, 1985) and was isolated as a band from an agarose gel (Materials and Methods, section 2.3.). These two fragments gave the entire sequence of exon IV. Both the coding region as well as the poly-A ad-

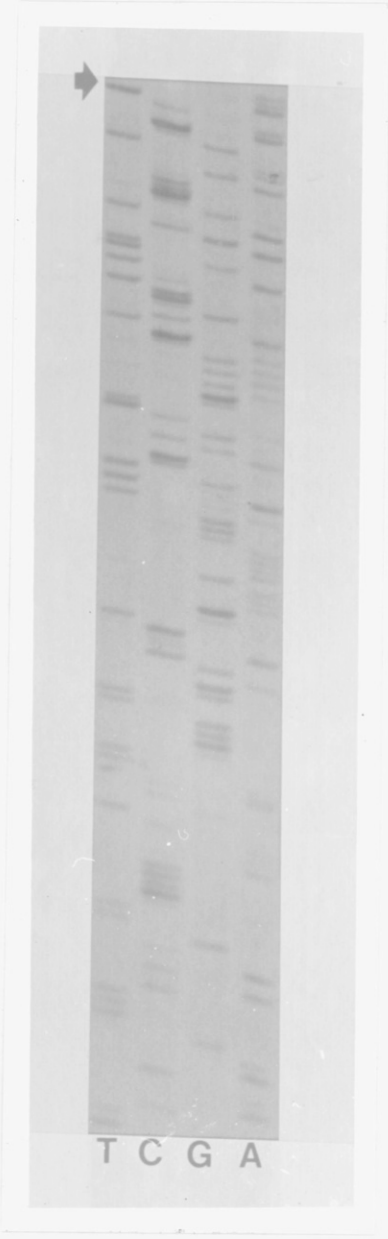

fig 3.10a. Sequencing gel of exon IV region of the alpha gene (part of the 500bp BglII fragment).

The arrow points to the termination codon TAA (underlined in fig 3.10). The sequence shown here reads TTCAA... starting from the nucleotide at position 33 on the second line in fig 3.10.

gtatgtgttttccctgcagCCTGGAGAGCCCCAGATCTAAGCGTGCGGTAATCTGA
GTACTTGCATGCTGGGCACATACACGCAGGACTTCAACAAGTTTCACACGTCCCAA
ACTGCAATTGGGGTTGGAGCACCTGGAAAGAAAAGGGATATGTCCAGCGACTTGGA
GAGAGACCATCGCCCTCATGTTAGCATGCCCCAGAATGCCAACTAAACTCCTCCCT
TTCCTTCCTAATTTCCCTTCTTGCATCCTTCCTATAAGTTGATGCATGTGGTTTGG
TTCCTCTCTGGTGGCTCTTTGGGCTGGTATTGGTGGCTTTCCTTGTGGCAGAGGAT
GTCTCAACTTCAGATGGGAGGAAAGAGAGCAGGGACTCACAGGTTGGAAGAGAATC
ACCTGGGAAAATACCAGAAAATGAGGGCCGCTTTGAGTCCCCCAGAGATGTCATCA
GAGCTCCTCTGTCCTGCTTCTGAATGTGCTGATCATTTGAGGAATAAAATTATTTT
TCCCCAaagatctgagctggtggtc...

---

fig 3.10.
Nucleotide sequence of exon IV of the alpha CT/CGRP gene.

---

dition site were normal, as well as the rest of the exon. The full sequence of exon IV in our patient is presented in fig 3.10.

4.b. Exon III.

The possibility was then examined that the CT precursor could not be formed. The rest of the exons participating in the formation of the CT precursor polyprotein were subcloned and studied by sequence analysis. As no exon III specific probe was available, a suitable probe containing most of exon III was subcloned from pCT1 as a 100 bp Bgl II/Bam H1 fragment in M13. Using this probe exon III was then subcloned from the genomic clone, lambda 2, as a 500 bp Sau 3A fragment. Both orientations were cloned and the nucleotide sequence was determined on both strands of the DNA and found to correspond to the published sequence (fig 3.11).

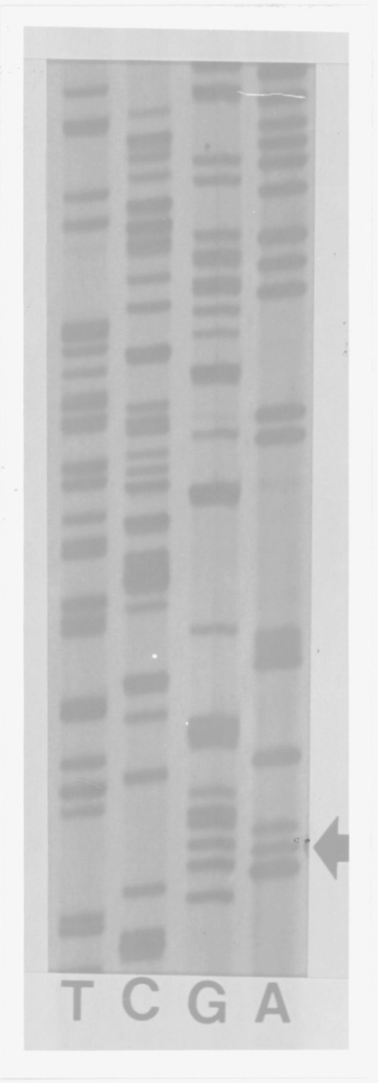

fig 3.12a. Sequencing gel of the exon II region of the alpha gene.

The arrow points to the first A of exon II and the sequence reads AGAGGTG....

agtttgcttcccctccacagGTCTGCCCTGGAGAGCAGCCCAGCAGACCCGGCCAC
GCTCAGTGAGGACGAAGCGCGCCTCCTGCTGGCTGCACTGGTGCAGGACTATGTGC
AGATGAAGGCCAGTGAGCTGGAGCAGGAGCAAGAGAGAGAGGGCTCCAGgtgaggc
tccccaagcgctc...

---

fig 3.11.
Nucleotide sequence of exon III of the alpha CT/CGRP gene.

---

atctcattcttcccttgcagAGAGGTGTCATGGGCTTCCAAAAGTTCTCCCCCTTC
CTGGCTCTCAGCATCTTGGTCCTGTTGCAGGCAGGCAGCCTCCATGCAGCACCATT
CAGgtaagacagcctgaagccag...

---

fig 3.12.
Nucleotide sequence of exon II of the alpha CT/CGRP gene.

4.c. Exon II.

The probe used to identify exon II containing fragments was derived from the 'beta' gene (see next chapter) and was an 800 bp genomic Sau 3A 1 fragment containing both exons II and III. A 700 bp Sau 3A 1 fragment was identified hybridising with this probe but not with the exon III probe. This was cloned from the genomic clone lambda 2 and sequenced in one strand of the DNA. The sequence is shown in figure 3.12 and corresponds with that of the published sequence. Taken together, the sequences of exons II, III and IV provide the complete coding sequence for pre pro calcitonin and demonstrate that the gene encodes a normal precursor protein.

4.d. Exon V.

As has already been mentioned, the CGRP levels in our patient were normal. Although it is not certain which gene the peptide was derived from, it seems more likely that our patient did not have any defect in the CGRP coding part of the gene as he did not have any symptoms suggestive of loss of this peptide. Nevertheless, the CGRP-specific parts of the gene (exons V & VI) were also subcloned and sequenced. An exon V specific probe was subcloned from the clone pTB58 as a c 200 bp Pvu II / HinD III fragment. In the genomic clone, exon V was contained in an Alu/Bgl II fragment of 800 bp. This was sublconed in the Sma I/Bam HI site of M13 mp8 and the coding sequence was determined from one end only (Fig 3.13). It encodes a normal CGRP.

4.e. Exon VI.

An exon VI specific probe was subcloned from pHTB58 as a 450 bp Pvu II/Hind III fragment. In the genomic clone, exon VI was contained in an Rsa I genomic fragment of 600 bp. This was cloned in M13 and the nucleotide sequence was determined on both strands of the DNA. The sequence is presented in fig. 3.14. It corresponds exactly to the published normal sequence.

ttctccatcctgcaaatcagAATCATTGCCCAGAAGAGAGCCTGTGACACTGCCAC
CTGTGTGACTCATCGGCTGGCAGGCTTGCTGAGCAGATCAGGGGGTGTGGTGAAGA
ACAACTTTGTGCCCACCAATGTGGGTTCCAAAGCCTTTGGCAGGCGCCGCAGGGAC
CTTCAAGCCTGAGCAGCTGAATGACTCAAGAAGgtgactgcccttgtatgatg...

---

fig 3.13.
Nucleotide sequence of exon V of the alpha CT/CGRP gene.

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ctgttattttcttgctcctagGTCACAATAAAGCTGAACTCCTTTTAATGTGTAAT
GAAAGCAATTTGTAGGAAAGGCTCCATGGAAGACATACATATAGGCATCCTTCTTG
ATACTGAAAACTATCTTCTTTGTTTGAAAGGAACTATTGCTAAATGCAGAACAAGC
TCATTGCAGTTACCTATTGTGCATCTTTTTAAATACTTGATTATGTAACCATAAAT
CTGACAGCATGTCTCATTGGCTTATCTGGTAGCAAATCTAGGCCCCGTCAGCCACC
CTATTGACATTGGTGGCTCTGCTAAACCTCAGGGGGACATGAAATCACTGCCTCTT
GGGCATCTGGGGACACATGGTAATGCTGTGCCTTGACAGAAGTATTTGTTTAAAGA
AATGTCAATGCTGTCATTTGTGAACTCTATCAAAATTAAAAATGTATTTTCTATAC
CCTTcaatggaatctctgctgctat...

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fig 3.14.
Nucleotide sequence of exon VI of the alpha CT/CGRP gene.

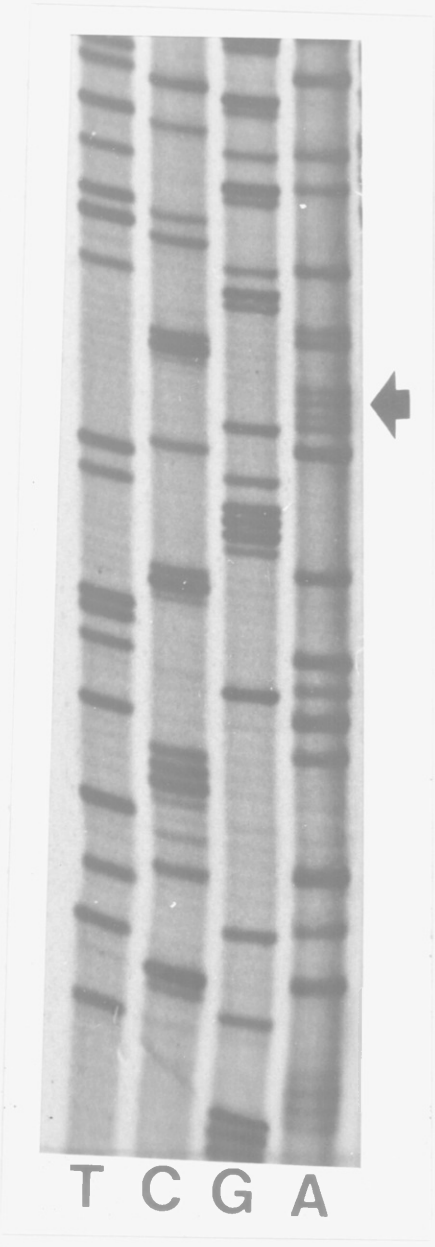

fig 3.15a. Intron IV sequence of patient's CT gene (sequence of complementary strand).

The arrow points to extra A (extra T in transcribed sequence) not present in previous reports of this sequence.

The exon I sequence was not isolated. It is possible that this region is not present in lambda 2.

4.f. Intron sequences.

Splice donor and acceptor sites flanking each exon were determined when the exons were sequenced (figs 3.10 - 3.14) and were found to be normal. The regions of greatest interest were introns III and IV surrounding the calcitonin coding exon since these may be important in influencing whether CT or CGRP mRNA is to be produced. No intron III sequence was available for comparison but the intron IV sequence had already been published (Edbrooke et al, 1985). Intron IV was isolated as a 700 bp Bgl II fragment from p3 and was sequenced (fig 3.15).

/aagatctgagctgtggtggtcattgctctgatctatgtcccaggcttcatagtgt
ctaagacctatgcttagaaatagccttaaccctaggctagctggacagaggatatg
gtgggtggtccctttgaccaagctcaagcaggaagaacaggggtcctaaggagcag
gtaagcacctctaggacttgatgctgcaaactccgctcctcttccaggtaagactg
aggaatttttatttcctaagaaagggtatttggtgcccgtgactggggtgtaga
ttttatagtcctttgtgaatggggctgggtgtgggaccataattcactccagtgtc
ataaacctccgctttgatttttagttaatttatacaggaaagattggctgttactg
ctccacattccatagccagtcatccagagtcaccttgggttt[t]ctgacacccct
gggaatatctatggggagtgatcatggcattttccctaatggccttgtgattttct
gctctgataattgtgtttaggagaaacacttaaagttaattggtgcctttcagcac
agcaactttaccatgaaggtcccatggggctgacctctctcccagcctctcactca
cagatcttctcttctttctccatcctgcaaatcag/

---

fig 3.15.
Nucleotide sequence of intron IV of the alpha CT/CGRP gene. The single nucleotide difference (T in position 432) from the published "normal" sequence (Edbrooke et al, 1985) is shown in brackets

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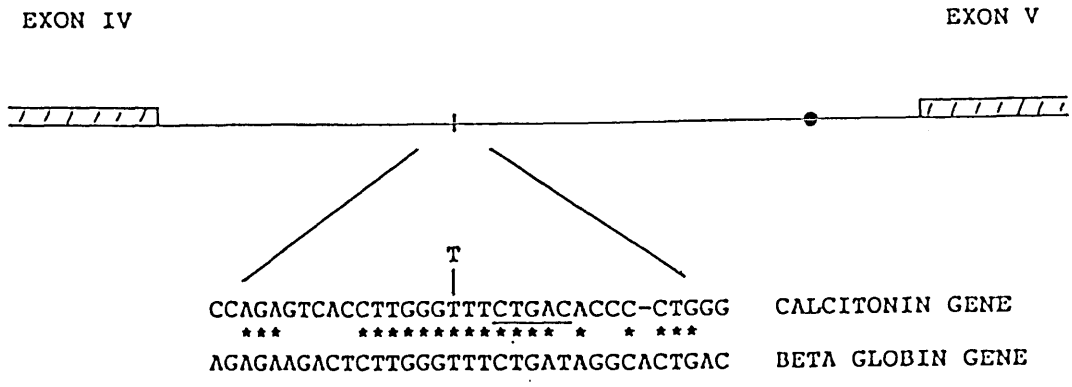

fig 3.16. Comparison of intron IV sequence with beta globin branch point sequence.

The altered sequence at position 432 of the fourth intron presented in fig 3.15. is compared with a sequence from the second intron of the beta globin gene containing the branch point. The consensus sequence for branch point formation is underlined. Asterisks indicate homology.

The only difference between our patient and the published "normal" sequence was a single base insertion at position 432. This is far from the exon-intron boundaries and was considered originally to be a neutral polymorphism, as these are not uncommon in non-coding regions of the genome. However, it was noticed that this mutation was located next to a CTGAC sequence, which is the consensus sequence for formation of a branch point during maturation of messenger RNA precursors (Konarska et al, 1985). During the process of splicing and after the precursor RNA is cleaved after one exon, the intervening sequence forms a looped structure at the branch point.

When the sequence surrounding this region was compared to the GENBANK database (1987 release, using IBI/Pastell sequence analysis programme), it was found that the best fit was with a highly conserved region of the 1st intron of the beta globin gene, which has been shown to become resistant to ribonuclease during in vitro splicing and is the site for formation of a branch point (Ruskin and Green, 1985) (fig 3.16).

The rules of splicing are still imperfectly understood so although this sequence is unusually far (212 bp) from exon V, it is possible that this altered sequence may be somehow interfering with this gene's expression (Alevizaki et al, 1989). The primary branch point site is in all probability much closer to the next exon and it is not clear how this change could be affecting the gene's expression. One possibility could be that the altered sequence takes on the properties of a branch point forcing an abortive splice which precludes the production of CT. This sequence lies in one of the introns flanking exon IV and these are of particular importance as they are the last ones to be removed during processing of the precursor RNA. Recent work has shown that the secondary structure of the precursor RNA is crucial for correct processing and introns III and IV probably interact with factors which determine whether CT or CGRP mRNA is to be produced (Leff et al, 1987, Emeson et

al, 1989).

However, it is necessary to consider the possibilities that the difference between the two sequences could represent a neutral polymorphism or a cloning / sequencing problem. The first author was contacted and was able to confirm that his sequence (like mine) was unambiguous on both strands. Nevertheless, the possibility of cloning artefacts cannot be ruled out.

Although it is possible to use allele specific oligonucleotide probes to whole genome Southerns, this is technically difficult and was not attempted. Such an analysis would show whether the sequence present in the patient was also present in normal individuals. At this time therefore, the possibility of a neutral polymorphism, widespread in the population, was not ruled out. However, with the introduction of the polymerase chain reaction, it became possible to amplify specific regions of the genome and thus simplify the analysis.

A pair of primers were synthesised which amplified a region of 250 bp around the altered sequence (Materials and Methods, section 1.8.). Genomic DNA was extracted from an individual with normal CT levels and no osteoporosis. The DNA was amplified, analysed on an agarose gel and the 250 bp band was eluted (fig 3.17) and cloned into the Sma 1 site of the plasmid Bluescript. The sequence of the DNA corresponded with that of the osteoporotic patient and not with the reported "normal" sequence. Although only one allele was sequenced, it was considered very unlikely that an individual selected at random would be a carrier for this extremely rare condition. If the frequency is one in a million, the carrier frequency would be one in a thousand. Nevertheless, the following procedure was followed to check if this individual was homozygous for this sequence.

The amplified DNA was again run on an agarose gel and blotted (fig 3.17). The membrane was then cut into two pieces. One was hybridised to an oligonucleotide probe

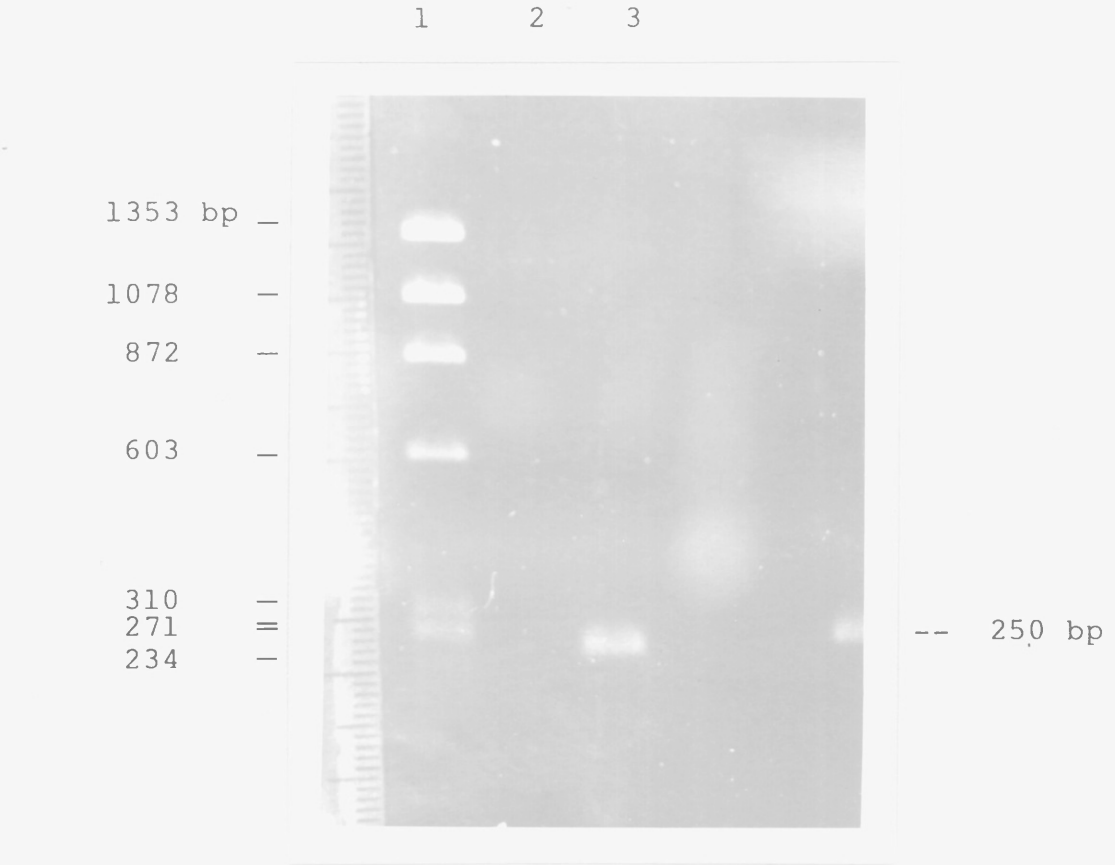

fig 3.17. Ethidium bromide stained gel showing amplification of intron IV region.

The intron IV region was amplified using PCR in the DNA from a normal individual. The two oligonucleotides used as primers for the amplification were: 1.GTGGGACCATAATTCACTCC and 2.AGTTGCTGTGCTGAAAGGCA. They flank a region of 250 bp containing the altered sequence. Lane 1 is a marker (Hae III of phi X153). Lane 3 is the PCR product and has the expected size.

fig 3.18. Southern blot of intron IV amplification.

DNA from a normal individual was amplified in the intron IV region and the gel (shown in fig 3.17) was blotted. The lane 3 region was cut into two identical strips (blots I and II). Blot I was hybridised to a patient specific oligonucleotide probe (AGGGGTGTCAGAAAACCCAAG), while blot II was hybridised to a "normal" sequence specific oligonucleotide probe (AGGGGTTGTCAGAAACCCAAG) (Materials and Methods, section 3.4). Panel A shows autoradiograph after a 40°C wash in 2X SSPE buffer. Both probes give a similar signal. Panel B shows autoradiograph after a 55°C wash in the same buffer. The "normal" sequence oligonucleotide probe (blot II) has melted off at the higher temperature showing that the control subject from whom the DNA was derived is homozygous for the patient specific sequence.

(Materials and Methods, section 3.4.) specific for the patient's sequence and the other was probed with an oligonucleotide specific for the reported "normal" sequence. After hybridisation the washing was done with a stepwise increase in the temperature. The "normal" sequence specific oligonucleotide melted off after the washing temperature was increased above 55 °C while the patient specific oligonucleotide still gave a strong signal (fig 3.18). This hybridisation experiment proved that the patient sequence was present in both alleles of an individual with normal CT levels (Alevizaki and Legon, 1989). It is therefore not responsible for the CT deficiency in the osteoporotic patient and probably represents a naturally occurring polymorphism. After these experiments had been completed one further report of the sequence of intron IV was published (Broad et al, 1989) and this too has the same sequence as our patient.

III.5. Conclusions.

The calcitonin gene of this patient has been isolated and studied in considerable detail. It is clear that it encodes a normal calcitonin peptide - the whole of the coding sequence for both CT and CGRP is entirely normal. The only difference between this and the "normal" sequence was found in intron IV. However, although the sequence which is involved has homology with a branch point sequence for splicing, it does not explain the patient's failure to produce CT as the same sequence is present in normal individuals.

The studies that have been described do not entirely rule out the possibility that the calcitonin gene is responsible for the patient's condition. There could be altered sequences in intron III which influence the structure of the precursor RNA and thus determine whether or not CT can be produced. However, in the absence of a normal sequence for comparison, it would not be possible to identify such

sequences. It is also possible that enhancer sequences active in the C-cells of the thyroid might be defective. Such changes would prevent expression in the thyroid but leave normal CGRP production in neural tissue unaffected. However, thus far the enhancer sequences of the CT/CGRP gene have not been characterised, so it has not been possible to investigate this possibility.

Similarly, there must be tissue specific factors regulating the expression and differential splicing of the gene but as these have yet to be characterised, little could be done to investigate this further. The absence of karyotypic abnormalities (Stevenson et al 1982b) rules out the possibility of gross changes to the genome.

CHAPTER IV

THE CALCITONIN RELATED SEQUENCE OF THE β CGRP GENE

CONTENTS:

Abstract: To investigate whether a second CT (possibly the salmon-like CT) was contained in the β CGRP gene, a genomic clone representing this gene was isolated from a human genomic library and studied. Modified hybridisation conditions were developed to allow identification of sequences with imperfect homology to an exon IV probe. A region was

identified which has the potential to code for a CT-like peptide, although this is probably not expressed in present day man. The predicted peptide has homology with the bovine as well as the fish and human types of CT and may be considered as a fourth distinct type. In situ hybridisation studies were performed which localised this gene close to the alpha gene on the short arm of chromosome 11, which suggests that it is probably the result of a local duplication. Finally, a region was isolated from the genomic clone representing exon VI of the gene. This can be used as a probe hybridising specifically to β CGRP.

IV A. INTRODUCTION

As was described in chapter I, section E.7., in addition to the alpha CT/CGRP gene, there is a second gene coding for a CGRP molecule, the beta gene. As soon as this became known, the obvious question arose as to whether this gene also contained a CT coding sequence. Although original reports suggested that the β CGRP gene did not contain any CT related sequences, the structure of the mRNA for this peptide was identical to its α gene counterpart (Steenbergh et al, 1985), suggesting that the structure of the gene might be similar too. Later, it was reported by a different group that CT hybridising regions might be present in genomic clones containing the gene (Jonas et al, 1985). It was therefore considered likely that a beta CT was present in the beta gene, but that it had diverged considerably and could not be detected under standard hybridisation conditions. If this was the case, then it was possible that this beta CT might prove to be the salmon-like CT which has long been reported to circulate in man both under normal conditions as well as in patients with lung carcinoma (Tobler et al, 1984, Gropp et al, 1985). Identifying a salmon CT-like peptide of human origin would have important therapeutic implications. It would probably be more potent than human CT and at the same time it would lack the antigenicity that salmon CT has in man.

To investigate this possibility a human genomic library was screened with a CGRP specific probe and clones whose restriction map was different to that of alpha, and probably represented the beta CGRP gene, were isolated to study in detail. To look for CT related sequences, modified hybridisation conditions were developed, which would allow the identification within this clone of areas with imperfect homology to the CT coding exon of the alpha gene.

The clone pCTI representing this exon was used as a probe for the screening.

Using these conditions a region was identified in the beta gene which has a 67% homology to exon IV of the alpha gene. This region does not appear to code for a protein despite its similarity to the CT coding sequence. The technique of in situ hybridisation was then used to see whether the beta gene was adjacent to the alpha gene on the short arm of chromosome 11. Such a finding would indicate that the beta CGRP gene probably arose as a local gene duplication.

IV B.RESULTS AND DISCUSSION

IV.1. Screening the genomic library for beta CGRP.

The CGRP coding region of the beta CGRP gene (exon V) has a very strong homology to exon V of the alpha gene (>90%). Probes specific for alpha CGRP are therefore expected to cross hybridise with beta CGRP coding sequences. The α CGRP specific probe (containing exons V and VI) was therefore used to screen a human genomic library (see chapter III). Fifteen positive clones were isolated and plaque purified in addition to the four clones positive with the CT specific probe, which were mentioned in the previous chapter (section III.B.3).

DNA was prepared from all the positive clones and digested with Eco RI which separates the genomic DNA from the vector DNA. The restriction patterns of the various clones were compared. (Fig 4.1). When DNA from various lambda clones was compared to identify identical ones, the plaque purification step was often not pursued further and DNA was prepared from a mixture of two to three clones. Provided there was pure DNA preparation from at least one clone, it was usually easy to identify the ones which were identical. This procedure allowed faster processing of clones. Occasionally similarities were not immediately apparent using this method in cases where the clone DNA was underrepresented in the mixed stock (fig 4.1).

The genomic clone DNA was probed with exon IV of the alpha gene under stringent hybridisation conditions (see Materials and Methods, section 4.3.) to exclude any clones containing the alpha gene. To exclude clones which lacked sequences 5' to exon V, an exon III specific probe was also used. Exon III of the alpha gene is also highly homologous to its beta gene counterpart. When a clone was positive for exon III and V probes, but negative for IV under strin-

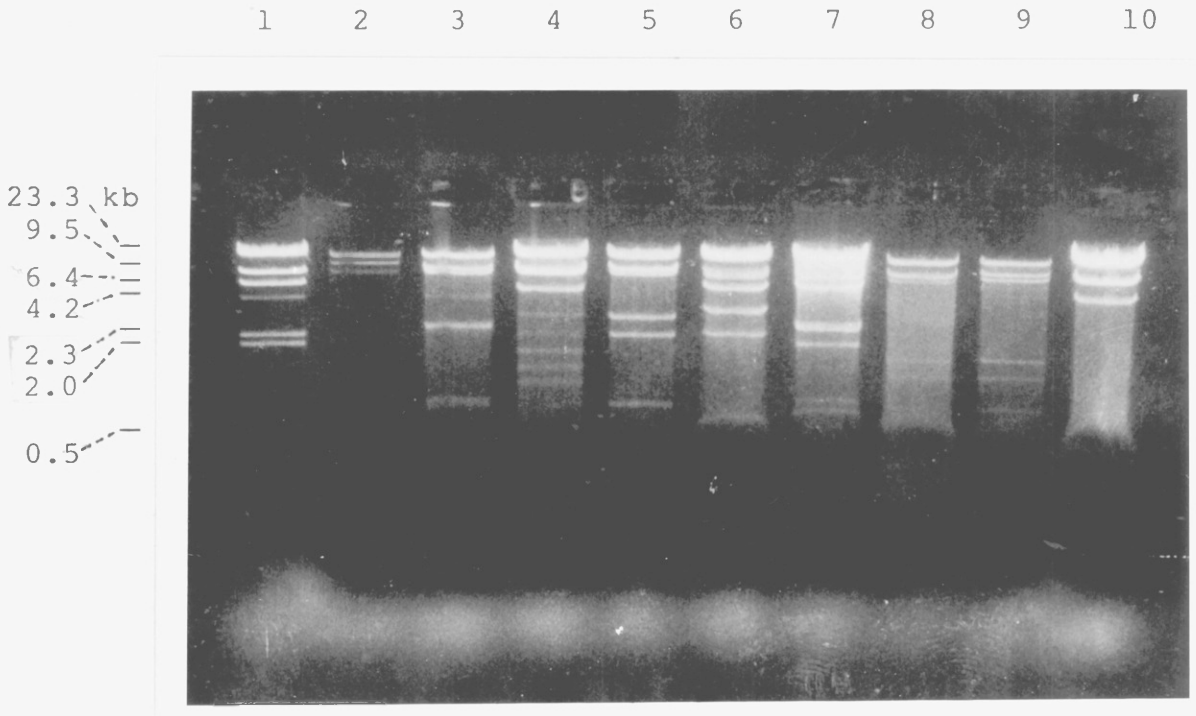

fig 4.1. Eco RI digests of genomic clones detected with a CGRP specific probe.

Lane 1 is a marker (Hind III of lambda) and 2 is an Eco RI diggest of the cloning vector EMBL4. Clones are: In lane 3:lambda 2, lane 4:lambda 3, lane 5:lambda 5, lane 6:lambda 6, lane 7:lambda 8, lane 8:lambda 14, lane 9:lambda 23, lane 10:lambda 24. [Lanes 3,4,6 and 7 contain mixed phage DNA coming from at least two different clones]. Clones 3 and 24 are identical as are clones 14 and 23. Clones 3, 5, 14, 23 and 24 all hybridised to exon III and exon V specific probes and were negative for exon IV under stringent hybridisation conditions. They therefore represent beta gene clones. Clones 2,6 and 8 represent alpha gene sequences as they all hybridised to a CT specific probe under stringent conditions.

gent conditions, it was considered as a possible candidate to represent the beta gene. Two independent genomic clones having these characteristics were analysed further (lambda 5 and lambda 23).

IV.2. Development of non stringent hybridisation conditions.

If CT sequences were contained in the beta gene and were homologous to salmon CT, they would be expected to have rather poor homology to an α calcitonin probe. The salmon CT gene had not been cloned at that time, but the peptide is very similar to the chicken CT, whose coding sequence has been reported (Lasmoles et al, 1985a). Chicken and human CT genes have a 60 % homology in the exon IV region (Lasmoles et al, 1985b) and this might be an indication of the extent of homology to be expected.

From the experiments with genomic Southern blots, which were described in the previous chapter, it was known that under standard conditions, an exon IV specific probe would only detect the band corresponding to the alpha gene, even after long exposures (fig 3.5). Since the degree of homology between the probe and the putative exon IV of the beta gene was not known, it was not possible to calculate the hybrid melting temperature and design the hybridisation and washing conditions. Modified conditions were therefore chosen with the following guidelines in mind.

$$Tm = 81.5 + 16.6\ (\log [Na^+]) + 0.41\ (\%G+C) - 0.65\ (\%formamide) - 500 / \text{length of duplex} - \%\ \text{mismatch}.$$

Thus, increasing the salt concentration or reducing the amount of formamide should stabilise mismatched hybrids (Anderson and Young, 1987).

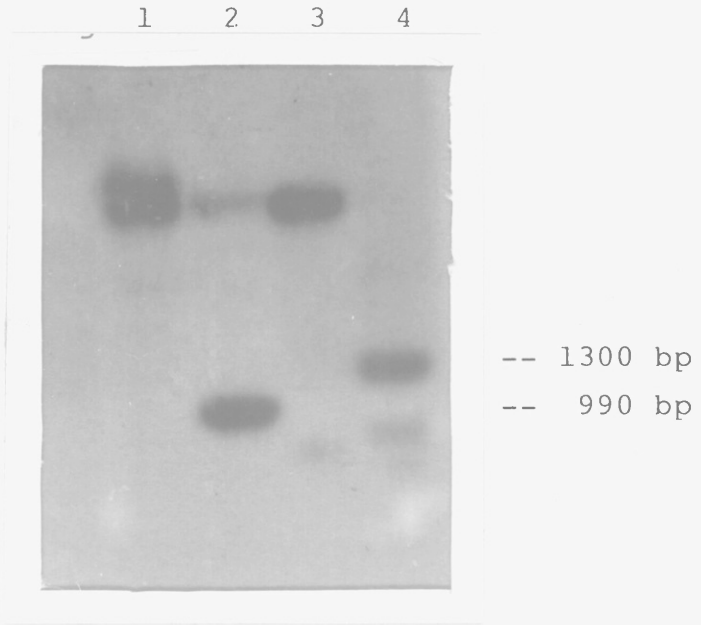

fig 4.2. Non-stringent hybridisation of clone λ23 with a CT specific probe.

An exon IV specific probe derived from pCTI was hybridised to the blot under non-stringent hybridisation conditions as described in text. The same filter was negative for CT under ordinary - stringent- hybridisation conditions. Enzymes are: Lane 1:Eco RI, 2:Hind III, 3:Bgl II and 4:Sau 3 A.

This experiment proved that CT related sequences are present in the beta gene clone.

In the first control experiment the stringency of hybridisation was reduced by lowering the percentage of formamide in the hybridisation buffer from 50% to 40%. However, a very high background was seen and these conditions were therefore not used for the actual experiment.

The other approach was :

a) To lower the temperature of hybridisation from 42 °C to 35 °C, while keeping the formamide concentration buffer at 50% and

b) To lower the washing temperature to 50 °C while keeping an increased concentration of salts (2xSSPE, i.e.300 mM NaCl) in the washing buffer.

Using these conditions a region was identified in the beta gene which hybridised strongly with the exon IV specific probe; this was previously undetectable with the standard hybridisation conditions (fig 4.2).

IV.3. Cloning and analysis of the CT related sequence.

From Southern blot analysis of the beta gene clones it could be seen that the hybridising region was contained in a large PstI fragment of about 3 kb and in a 780 bp Hae III fragment, which gave a relatively weak signal. The strongest signal was obtained from a Hind III fragment of 950 bp (fig 4.3). The Hind III fragment was thus chosen for further study.

A Hind III digest of lambda 23 was ligated into M13 and clones were selected by hybridisation with an exon IV probe under the low stringency conditions. The Hind III fragment was thus cloned in both orientations and the recombinant was named subclone φ11. A sequence of several hundred bases was determined from both ends of the clone. No homology was immediately obvious. When however the sequence was compared with the EMBL sequence data base, a high score was given for the human CT sequence. The hybridising region was thus identified. Restriction fragments of lambda 23 extending further downstream from the CT-like

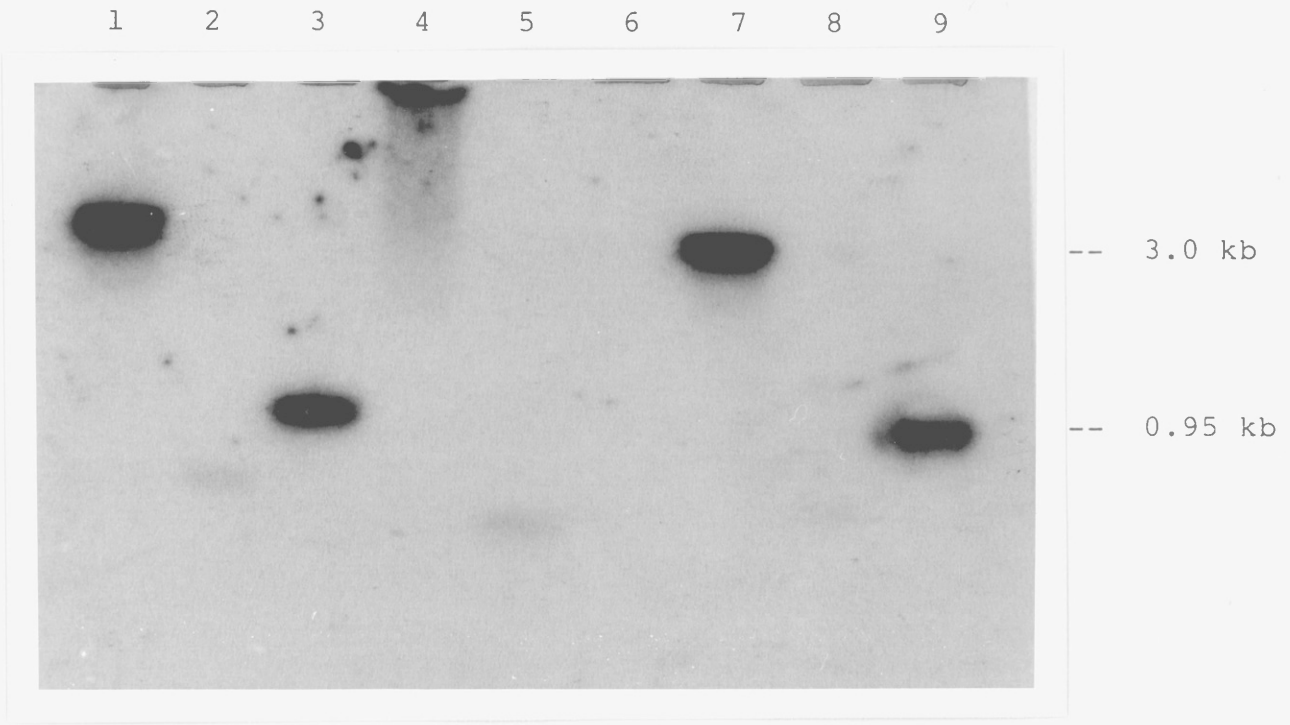

fig 4.3. Southern blot analysis of beta gene clones.

Lanes 1-4: λ5 DNA, lanes 5-9: λ23 DNA. Enzymes are Pst I (lanes 1 and 7), Hae III (lanes 2 and 8), Hind III (lanes 3 and 9), Alu I (lanes 4 and 6 - lane 4 is a partial) and Hind III/Sau 3 A (lane 5). Although the two clones have different restriction maps they must overlap as the fragments hybridising to exon IV are identical between the two clones. The 950 bp Hind III fragment was subcloned from λ23 for further analysis.

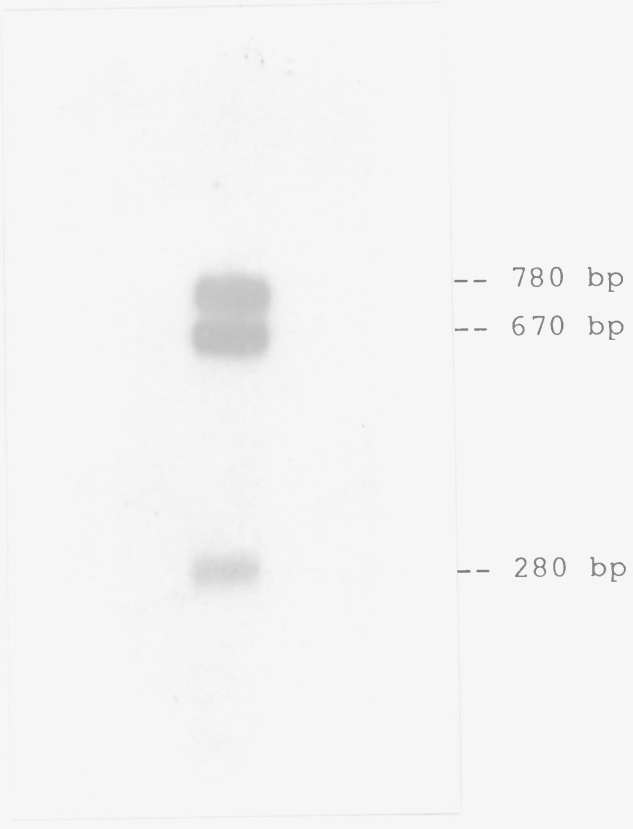

fig 4.4. Autoradiograph of a Hae III restriction digest of genomic clone λ23.

The 950 bp Hind III fragment (subclone φ11) was used as a probe. All three hybridising Hae III fragments were sub-cloned and studied.

Footnote to page 160

* The sequence of the 280 bp fragment was determined in its entirety together with the relevant ends of the 670 and 780 bp fragments. Appropriate subclones were then made from the larger fragments to allow the other strand to be determined.

sequence also contained the exon V sequence showing that this region is located 5' to the CGRP coding region. This indicates that the structure of the beta gene is very similar to that of the alpha gene.

Because the Hind III clone was too large to sequence, smaller Hae III fragments were identified in the same region by using the Hind III clone (φ 11) as a probe to a Hae III digest of the genomic clone. Three bands were identified (fig 4.4) one of 280 bp, one of 670 bp and one of 780 bp. All these Hae III fragments were also cloned and sequenced.* The nucleotide sequence of the hybridising region was determined on both strands of the DNA and is shown on fig 4.5. This sequence has a 67.6% overall homology to exon IV alpha. The calcitonin coding region does not seem to have been conserved to a greater degree than the rest of this exon. The potential of this sequence for expression has been examined.

The longest open reading frame (ORF) in this region comprised 65 amino acids. From position 3 to position 35 of this ORF, a 32 a/a peptide is encoded which has very strong homology to the known CTs (fig 4.6). The putative beta gene peptide has closer homology to the human/rat type (17/32 common a/a) and to the bovine type of CT (18/32 a/a in common) and is more distantly related to the salmon one (14/32 common a/a). It appears to be of a fourth distinct type, but lacks the 1-7 disulphide bridge and the invariant leucine at position 9 (Alevizaki et al, 1986).

However, a number of features of this sequence indicate that this is not part of a functional gene (fig 4.5). Although the sequence preceding the exon IV -like region appears to conform to the consensus sequence for splice acceptor sites (Mount et al, 1982), any splice from exon III to this sequence would lead to the termination of translation after only 8 further amino acids. Moreover, even if some novel type of rearrangement allowed this termination codon to be bypassed, the CT like sequence is not flanked by the paired dibasic amino acids typical of a peptide

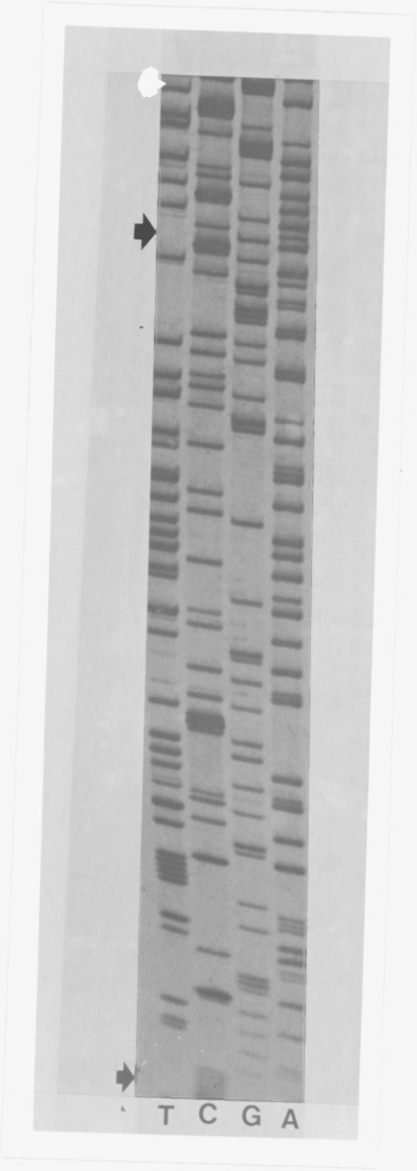

fig 4.5a. Sequencing gel of exon IV-like region of the beta gene.

The sequence shown here is in the complementary strand of a HaeIII subclone starting from the relevant recognition site at position 51 in the third line of the sequence in fig 4.5. The arrow points to the cloning site in M13. The sequence in this strand reads CCCGAAGTTGA..... The second arrow points to the end of sequence which corresponds to the first nucleotide shown in fig 4.5.

fig 4.5. Nucleotide sequence of the exon IV-like region of the beta CGRP gene. All three reading frames are blocked in the beta sequence (highlighted stop codons). ➧ :ORF begins. Comparison with the corresponding region of the alpha CT/CGRP gene. Gaps have been introduced to maximise homology. The overall degree of homology is 67%.

(I) exon/intron boundaries, (+++) termination codon of a gene, (^^^) codons for paired dibasic aminoacids, (---) poly A addition signal.

COMPARISON OF CALCITONIN PEPTIDES

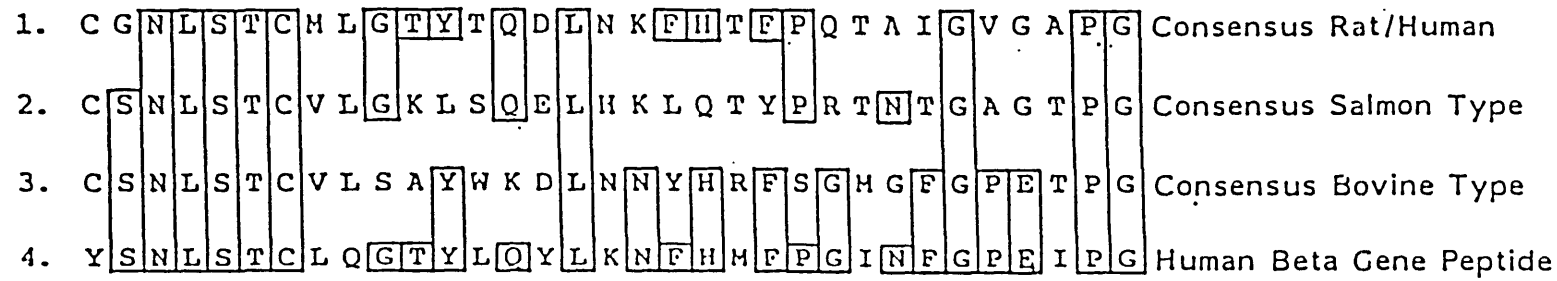

fig 4.6. Comparison of the beta gene CT-like peptide with the three types of CTs. Consensus sequences are presented.

hormone precursor protein. At the 3' end of the exon IV-like sequence, the polyadenylation signal (AATAAA) is not present, so any transcript of this region would be unlikely to be stable. Surprisingly perhaps, the first 35 bases of intron IV have 87% homology with the corresponding region of the alpha gene. This might be further evidence of an important role for this region in the processing of the precursor RNA. However, this high degree of homology concerns only the sequence shown in fig 4.5 and then decreases to 25-30%.

IV.4. Expression of CT-like sequence.

The most interesting question about this region is of course whether a second CT mRNA may be produced. The data available make it rather unlikely that this region is functional in a way similar to the alpha gene. However, recently a post-transcriptional alteration to an mRNA sequence (a C to U base change) has been reported in the Apo B gene (Chen et al, 1987, Powell et al, 1987) giving rise to the intestinal type of the protein. One cannot, therefore, rule out the possibility that this sequence might be expressed in a similar fashion.

The CT-like sequence was thus used as a probe on mRNA extracted from various tissues, where CT gene expression would be expected (Materials and Methods, section 5.). The tissues studied were medullary carcinomas of the thyroid (both familial and sporadic forms), a phaeochromocytoma as well as a leukaemia and a bronchial cell carcinoma cell line, which are known to produce CT. No message from this region was identified after 3 weeks of exposure (Fig 4.7). The same Northern blot was subsequently stripped and re-probed with a probe from the alpha gene and CT expression was detected after a two days exposure in three of the samples. This suggests that this sequence is probably not expressed at all.

After completion of these studies a paper was published

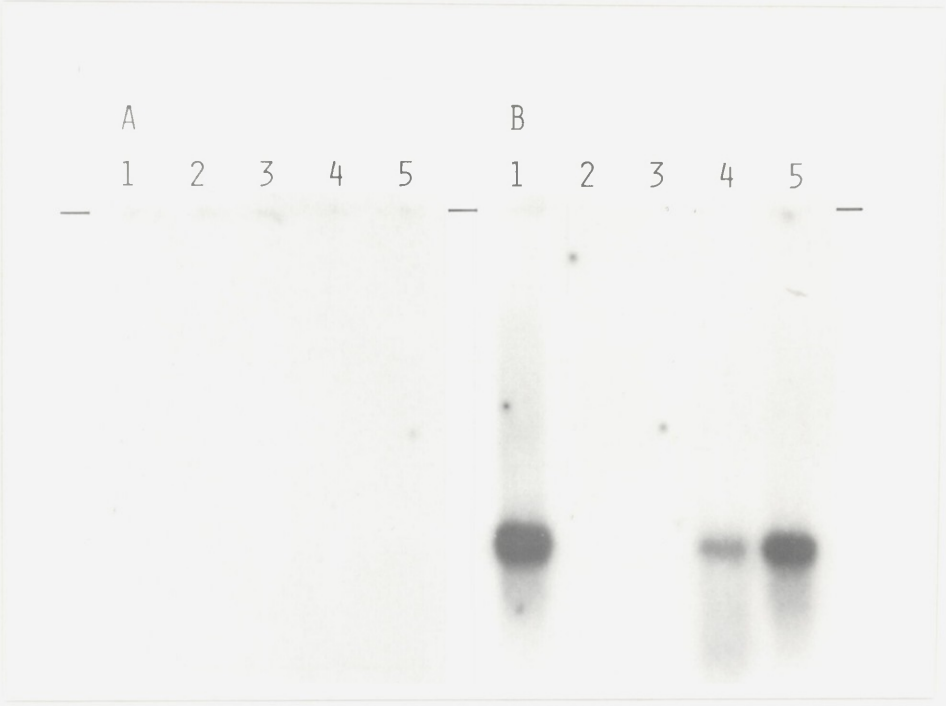

fig 4.7. Northern blot analysis of mRNA from human tissues.

Lane 1: 3µg from bronchial carcinoma cells, lane 2: 3µg from HL60 leukemia cell line, lane 3: 3µg from phaeochromocytoma, lane 4: 0.5 µg from sporadic type MTC, lane 5: 0.5 µg from familial MTC. Panel A shows hybridisation with φ11, panel B shows hybridisation with an exon IV specific probe derived from pCTI.
No expression of the CT-related sequence of the beta CT/CGRP gene is therefore detected.

by Lipps' group describing the cloning and nucleotide sequencing of this region (Steenbergh et al, 1986). Their data is in agreement with mine, so far as the predicted peptide is concerned. There are single base differences in positions 158, 167 and 169 of the exon IV-like region [G to T, A to G and A to G respectively], which perhaps represent sequence polymorphisms. These positions are not within the reading frame of the hypothetical beta calcitonin.

Although no longer functional, the CT-like sequence must have been active at some stage in evolution. It was therefore considered possible that the predicted peptide would still retain some useful biological properties. Three peptides were therefore synthesised- beta CT itself and two close homologues: one having the 1-7 disulphide bridge considered necessary for biological activity and one with the conserved residue leucine in position 9. These peptides were tested for biological activity in the isolated osteoclast system (Chambers and Moore, 1983), this being the biological property of CT which is of potential therapeutic importance (Materials and Methods, section 11.). These experiments were performed by Dr M Zaidi. All three peptides were found to be inactive, at least at the concentration at which human CT is active.

IV.5. Sequence analysis of coding regions of the beta CGRP gene.

Other regions of the beta gene were also characterised. It was necessary to confirm that the clone was indeed the beta gene by sequencing the CGRP coding region. This will be referred to as exon V although it is presumably the fourth functional exon of the gene. Exons II and III were also sequenced as the latter was only partially known and the former had not been reported at all. Finally, exon VI was characterised. Exon VI is only 65% homologous with alpha gene exon VI and would therefore provide a probe sequence specific for beta CGRP mRNA.

..ttctctatcttgcaaatcagCTCCGCTGCCCAGAAGAGAGCCTGCAACACT
GCCACCTGTGTGACTCATCGGCTGGCAGGCTTGCTGAGCAGATCAGGGGGCATGGT
GAAGAGCAACTTCGTGCCCACCAATGTGGGTTCCAAAGCCTTTGGCAGGCGCCGCA
GGGACCTTCAAGCCTTAGCAGATGAATGACTCCAGGAAGAAGgtaactaccctaa
tgctatg...

---

fig 4.8.
Nucleotide sequence of the exon V region from the beta CGRP gene.

..agtttgcttcccttccacagGTCTGCCCTGGAGAGCAGCCCAGACCCGGCCAC
ACTCAGTAAAGAGGACGCGCGCCTCCTGCTGGCTGCACTGGTGCAGGACTATGTGC
AGATGAAGGCCAGTGAGCTGAAGCAGGAGCAGGAGACACAGGGCTCCAGgtgaggt
tccccaagcgccc...

---

fig 4.9.
Nucleotide sequence of the exon III region from the beta CGRP gene.

5.a. Exon V.

This was cloned as a 500 bp Hae III fragment from lambda 23 using the exon V specific part of phTB58 as probe. The sequence is shown in figure 4.8 and corresponds with the published sequence of the cDNA clone isolated by Steenbergh et al (1985). This proves that lambda 23 does indeed contain the beta CGRP gene.

5.b. Exon III.

This was cloned as an 800 bp Sau 3A 1 fragment from lambda 23 using the exon III specific subclone from pCTI. The sequence from one end was found to include the complete exon III sequence. The other strand was sequenced from two Hae III subclones. This is shown in figure 4.9 and extends the previously reported sequence of Steenbergh et al (1985). The complete exon III sequence has 92% homology with exon III of the alpha gene.

...taacgtcatccttcctttacagAGAGGCGGCATGGGTTTCCGGAAGTTCTCCC
CCTTCCTGGCTCTCAGTATCTTGGTCCTGTACGAGGCGGGCAGCCTCCAGGCGGCG
CCATTCAGgtgagacagcctggagccag...

---

fig 4.10.
Nucleotide sequence of the exon II region from the beta CGRP gene.

5.c. Exon II.

When the other end of the 800 bp Sau 3A 1 fragment was sequenced it was found to contain a sequence homologous with exon II of the alpha gene. The sequence was confirmed on the other strand of the DNA from an Alu I subclone and is presented in figure 4.10. Exon II of the beta gene has 92% homology with the corresponding region of the alpha gene.

5.d. Exon VI.

Because exon VI has only 65% homology with the alpha gene sequence, non stringent hybridisation conditions were used to try and localise smaller fragments from the beta gene which contained exon VI sequences. This however proved more difficult than the identification of exon IV in the genomic clone. Although definite signals were obtained on the Southern blots of the beta gene clones, when M13 plaques were screened no positive signals were seen.

A different strategy was followed, which was to elute the hybridising DNA fragments directly from an agarose gel, and then directly clone into M13. The enzyme chosen for this approach was Hae III. A restriction analysis of the genomic clone λ23 with this enzyme revealed the presence of an 800 bp fragment, which hybridised to exon VI alpha (fig 4.11). Four different Hae III fragments very similar in size were present in the digest of the clone and it was very difficult to distinguish the hybridising fragment.

The experiment was done by picking 5 recombinants and using these DNAs as probes to a Southern blot of the exon VI specific part of phTB58. One of the clones was thus identified as a possible exon VI clone. However, as sequencing from one end was not informative and it proved difficult to "turn around" the clone, an oligonucleotide was synthesised complementary to part of exon VI beta. This was used to confirm that exon VI was in fact present in our genomic clone and in the above mentioned M13

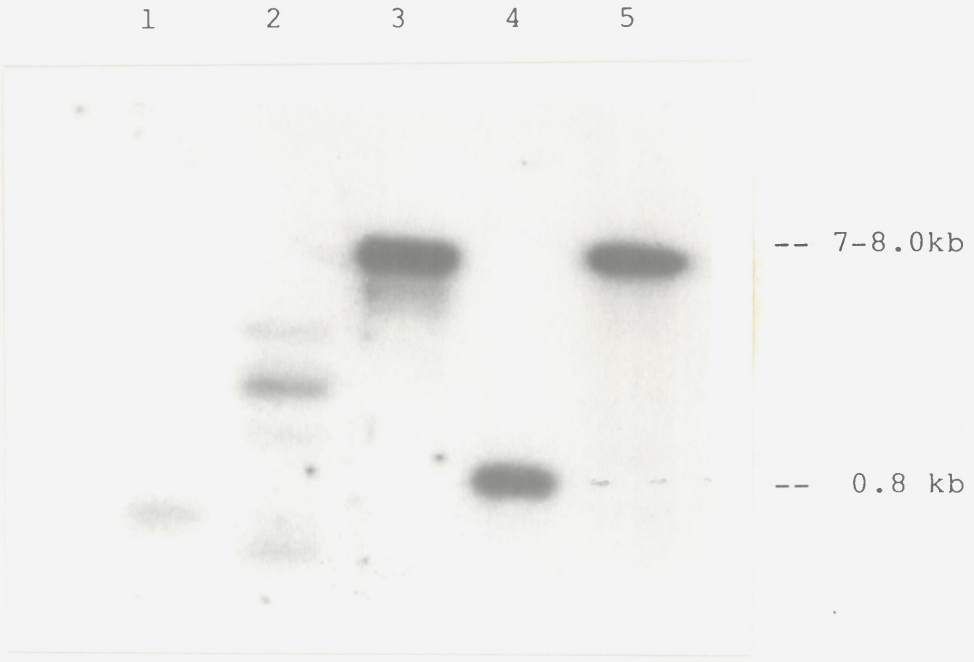

fig 4.11. Southern blot of genomic clone λ23.

Enzymes are lane 1: Hind III/Sau IIIA, 2:Alu I(partial), 3:Pst I, 4:Hae III, 5:Hind III. The probe is a fragment derived from phTB58 representing exon VI of the alpha gene. In the position of the hybridising 0.8 Kb HaeIII fragment there were four fragments visible in the ethidium bromide stained gel, all similar in size and it was difficult to identify directly the hybridising one. This was however subsequently subcloned and the nucleotide sequence determined (see page 168).

subclone. The sequence of this region was finally obtained on one strand using the synthetic oligonucleotide as primer and on the other strand when it finally proved possible to "turn around" the clone.

From this sequence analysis it is clear that the coding sequences, exons II, III and V, have been highly conserved while the non-coding sequences of exons IV and VI have diverged from the alpha gene sequence by approximately 35% (fig 4.12). The two genes have similar structures and it is interesting to note that the degree of divergence of the exon IV-like region is similar to the degree of divergence of exon VI, which, although present in the mRNA, is non-coding in both genes. This indicates that all parts of the precursor protein are important for the functioning of this gene and that the other regions do not have functions which are dependent on the retention of a precise nucleotide sequence.

IV.6. Localisation of the beta CGRP gene.

From study of human / rodent cell hybrids it was already known that the beta gene was present on chromosome 11 (11 q1 - pter) but the resolution of this method is somewhat limited (Hoppener et al, 1985). It was therefore decided to use the method of in situ hybridisation to determine whether the beta gene was adjacent to the alpha gene on the short arm of this chromosome (11 p13-p15 (Przepiorka et al, 1984)). Probes suitable for in situ hybridisation need to be at least 500 bp in length and, ideally, should not contain repetitive sequences. The 950 bp Hind III fragment representing the exon IV like sequence was therefore used as a probe on a whole genome Southern blot and gave a single band indicating that no repeats were present. In collaboration with Dr Feyruz Rassool, this was then used as a probe for in situ hybridisation. The probe was labelled by nick translation with ^{125}-I dCTP and hybridised

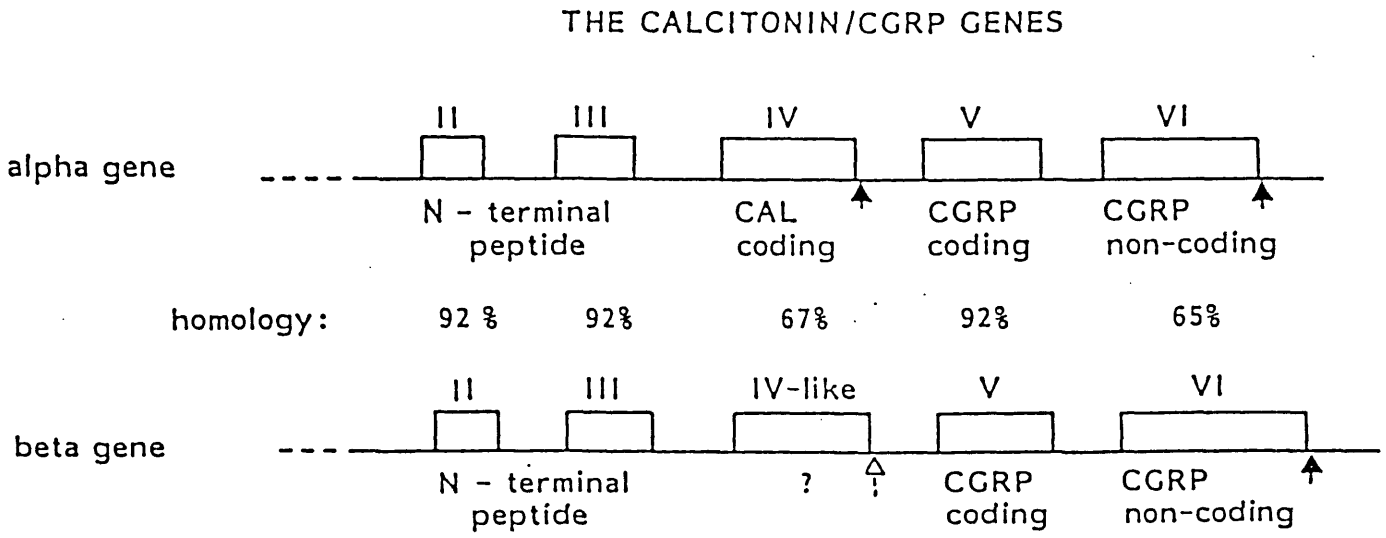

fig 4.12. Comparison between alpha and beta genes. (↑)=pA site.

* Several methods have been described which would allow a more precise localisation to be made. There are now extensive panels of man : mouse hybrid cell lines which could be used to refine the mapping. Alternatively two groups have described improved in situ techniques which allow high resolution gene mapping (Lawrence et al 1988, Lichter et al 1988)

** Chromosome 12 is evolutionarily related to chromosome 11, for instance the PTH gene is on chromosome 11 and the related humoral hypercalcaemia of cancer peptide gene is on chromosome 12. The duplication of the primitive CGRP-like gene may therefore have been a consequence of a chromosome duplication (Comings D.E. 1972).

Comings D. E. (1972) Nature 238 455 - 457.

Lawrence J. B., Villnave C. A. & Singer R. H. (1988) Cell 52 51 - 61.

Lichter P., Cremer T., Borden J., Manuelidis L. and Ward D.C. (1988) Hum Genet 80 : 224-234.

to human metaphase chromosomes (Materials and Methods, section 10.). The grains were counted on 50 metaphases. The average grain count was 1.4 per metaphase and 28% of the grains were localised on chromosome 11. The histogram of the distribution of grains is shown in fig 4.13. The gene was thus localised to the short arm of chromosome 11 to the region p12-p14.2, which roughly corresponds with the position of the alpha gene (11p13-15, Przepiorka et al, 1984) to within the limits of resolution attainable with a ^{125}I labelled probe. This localisation agrees with the findings of Hoppener et al (1985). Subsequently, it has been reported that alpha and beta genes are not present in the same cosmid clones (Steenbergh et al, 1985) indicating that they are not immediately adjacent.*

IV.7. Evolution of the beta CGRP gene

Before considering the evolution of the beta CGRP gene it will be helpful to consider what has recently been described about a further member of the CGRP family : amylin (Leighton and Cooper, 1988). This peptide, which has also been referred to as Islet Amyloid Polypeptide (IAPP) (Westermark et al, 1987), or Diabetes Associated Peptide (DAP) (Cooper et al, 1987), is also a 37 amino acid peptide with a 46% homology with beta CGRP. The amylin gene appears to lack the equivalent of exons III, IV and VI of the CT/CGRP gene and is located on chromosome 12 (Mosselman et al, 1988 and 1989).*

All of these point to a distant relationship and the separation of amylin and CGRP genes should be considered as the earliest identifiable event. The ancestral gene must have duplicated with one member giving rise to the modern amylin gene and the other giving rise to the CGRP genes. At this stage, it is simplest to imagine that no CT like sequence was present in the gene.

It has been suggested that the CT and CGRP coding exons may have arisen as a result of exon duplication and diver-

gence (Jonas et al, 1985). This possibility becomes apparent when the CGRP peptide is compared to CT peptides and in particular to salmon CT (fig 4.14). If 5 amino acids are deleted from the CGRP sequence, the resultant peptide bears a considerable resemblance to CT. As a result of this exon duplication, one would have the type of CT /CGRP gene which is seen in fishes and has been retained in birds.

At some stage during the evolution of the mammals, selective pressure on the CT sequence may have changed. Either pressure was relaxed for a period and the sequence drifted, or new pressures were applied forcing changes to the CT sequence. In either case, the result has been the emergence of the bovine and human types of CT.

The alternative view would be that there are in fact three types of CT gene - fish, bovine and human types, all of which may co-exist in present day species. This would be consistent with the various observations concerning the existence of a salmon type CT in man. This question remains unresolved although it has to be said that the evidence supporting these claims is far from conclusive.

Where does the beta gene fit into this scheme? The non-coding exons of the beta gene have diverged from the alpha sequences by 35%. When there is no selective pressure, sequences tend to drift by approximately 0.5 - 1% per million years (Britten R, 1986). This would indicate that the beta gene may have appeared at about the time of the mammalian radiation. Further study could easily clarify at what stage in mammalian evolution this event occurred or whether it preceded the emergence of the mammals. The remaining question is why this duplication should have occurred. Three possibilities seem most likely :

1) It is possible that alpha and beta CGRPs have slightly different biological activities and the benefit this confers has caused this duplication to be fixed in the human and rat genomes. However, no difference in biological acti-

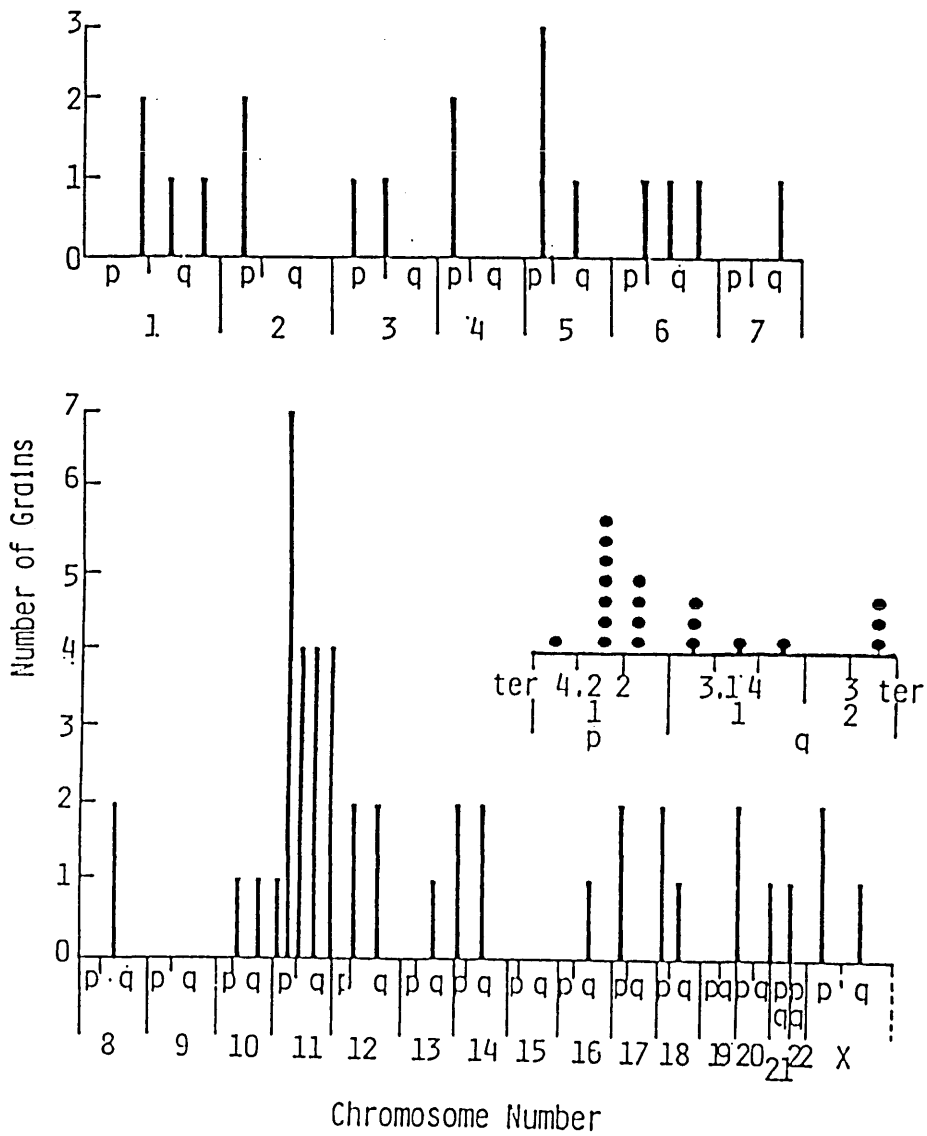

fig 4.13. Localisation of beta gene.

Histogram showing the clustering of grains during in situ hybridisation. 50 metaphases were analysed. 28% of grains were localised on chromosome 11. The grain distribution along chromsome 11 is shown in the inset. Hybridisation is seen specifically in the region p1.2-p1.42 of the short arm of chromosome 11.

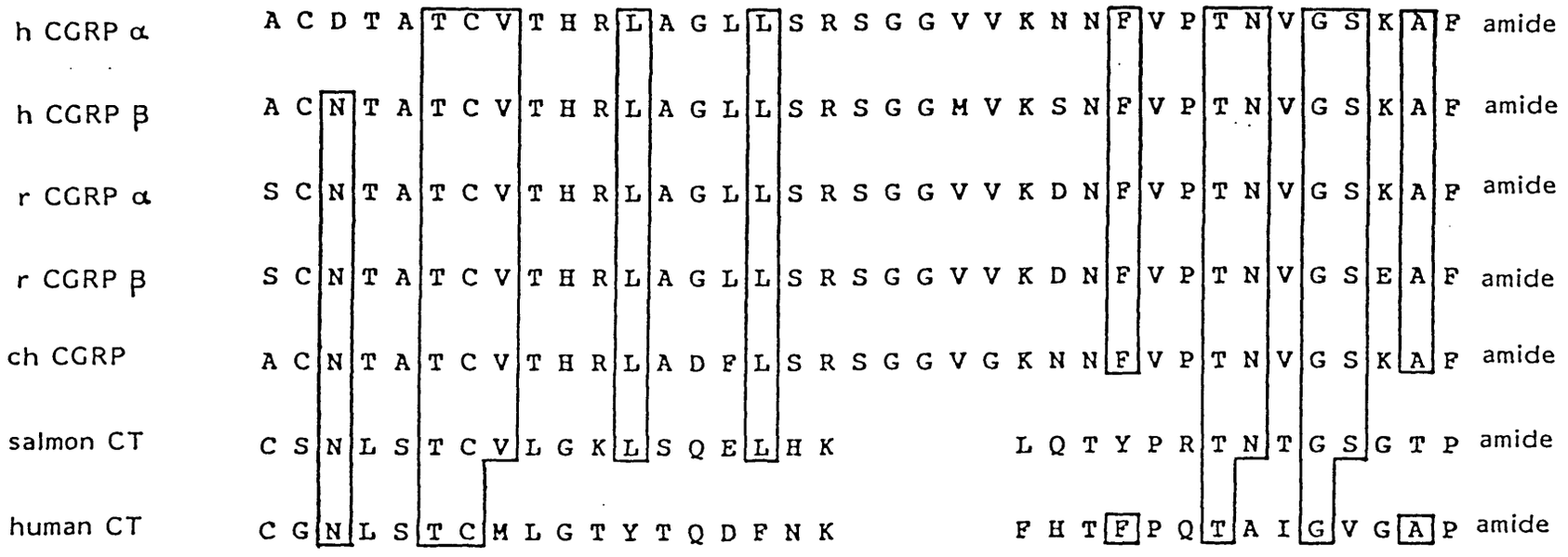

fig 4.14. Comparison of CGRP a/a sequence with salmon CT.

vity has been reported so far, although there have been reports about differences in biological potency which were discussed in chapter I, section E.7. (Holman et al, 1986, Tippins et al, 1986).

2) The possession of two genes may have made possible the evolution of a more flexible pattern of gene expression. It is known that the distribution of alpha and beta CGRPs in neural tissues is slightly different. For instance, in the enteric autonomic neurones only beta CGRP is found (Mulderry et al, 1988).

3) As was discussed in chapter I section E, non-neuronal tissues appear to lack a factor which is necessary for efficient expression of CGRP from the alpha gene. The possession of a second gene, lacking a functional CT exon, might allow CGRP expression in tissues lacking this factor. In support of this view is the observation that the beta gene is expressed in Ewing's sarcoma at relatively high levels in the absence of expression of alpha CGRP (Hoppener et al, 1987).

CHAPTER V

THE "GAMMA" SEQUENCE - FURTHER CT-RELATED SEQUENCES

CONTENTS:

Abstract. The N-terminal peptide coding region of the CT/CGRP gene is highly conserved suggesting that it has an important function to serve. When the relevant coding region (exon III) was used as a probe to identify related sequences in the human genome, a band was seen in a genomic Southern blot, which did not correspond to the alpha or beta genes. The genomic library was screened under non-stringent hybridisation conditions to characterise this sequence, which might contain one of the other members of the CT gene family which have been postulated to exist. Several weakly hybridising clones were identified some of which corresponded to this third sequence. This genomic

locus was analysed and was found to contain regions with a very high homology to exons II and III of the CT/CGRP genes (>90%). Although the initiation codon at the beginning of exon II is mutated, and there is a frameshift at the end of exon III, this sequence has the potential to code for an N-terminal peptide similar to that of the other genes. However, no CT or CGRP related sequences were identified in this clone even under extra-low stringency hybridisation conditions.

A unique sequence from this region was used as a probe on human chromosomal metaphases and was found to hybridise to the short arm of chromosome 11 to roughly the same region where the beta gene was localised. The gamma sequence is probably the result of a relatively recent duplication of part of the beta gene on the short arm of chromosome 11, as the whole of the exon II-intron II- exon III region is conserved to a similar degree between beta and gamma genes. One cannot exclude the possibility that this sequence may now be used by a different gene, using an initiation codon in an upstream exon, but it seems more likely that it is non-functional.

The hybridising sequences of some of the other genomic clones were also analysed. Some of them were found to represent artefacts coming from G-C rich regions hybridising to the C tail which had been added to the cDNA probe during the cloning procedure. In one case a stretch of 27 nucleotides identical to part of exon III alpha was identified in one of the clones while no other similarity was obvious. No CT related sequences were identified in any of the isolated clones. It is thus very unlikely that any of them contains the salmon CT-like peptide, which has been postulated to exist in man; it appears that if this sequence exists at all, then it must have diverged significantly and cannot be detected under the low stringency hybridisation conditions used.

V A. INTRODUCTION

As was discussed in the previous chapter, there are a number of reports which indicate that there may be a second CT coding sequence in the human genome. When genomic Southern blots were probed with the CGRP coding sequence, two bands were seen indicating the presence of two CGRP coding sequences (chapter III) but the CT like sequence associated with this second gene proved to be non-functional.

When genomic Southern blots were probed with an exon III probe, three bands were seen in a Taq digest, one of which did not correspond to either of the characterised CT/CGRP genes. This band was identified even under standard, stringent hybridisation conditions which suggests that it has a rather high degree of homology to the probe. The probe represents the N-terminal flanking peptide of the CT/CGRP gene. It has been believed that this part of the prohormone is important for the correct processing of the CT and CGRP precursors, although recent reports have suggested that it may have a biological function of its own (Burns et al, 1989, Roos et al, 1989). It is thus possible that this sequence may have been conserved in a third member of this gene family, while the sequences of the active peptides have diverged. Alternatively, the sequence may have become incorporated into an unrelated gene where it might serve a similar function in the processing of a precursor protein or have some other biological function.

The genomic library was therefore screened to isolate a clone containing this sequence. This was named the "gamma" sequence. To identify further sequences with homology to the CT/CGRP genes, both N-terminal peptide specific as well as CT specific probes were used for library screening. The hybridisation conditions were modified to reduce still further the stringency of hybridisation so as to allow detection of distantly related sequences.

V B.RESULTS AND DISCUSSION

V.1. Genomic Southern blots with exon III sequences.

The signal and amino-terminal peptide coding regions (exons II and III) of the CT/CGRP genes are very well conserved between alpha and beta genes having a homology of 92%, which is of the same order as that observed for the CGRP coding region. This suggests that the N-terminal peptide has an important function to serve and is therefore highly conserved. For this reason, when genomic DNA was screened for further CT related sequences, as well as exon IV and exon V probes, an exon III probe was also used. If a second active CT exists, then it would be expected to be associated with an N-terminal peptide, which might be better conserved than the other regions of the gene.

When a Taq I genomic Southern blot was probed with pCT I (exons III + IV alpha) three bands were seen (fig 5.1). The enzyme Taq 1 has recognition sites in the alpha and beta genes which are located outside the exon III-intron III- exon IV region; the probe used is therefore expected to hybridise to two bands only, one from the alpha gene containing exons III and IV and one from the beta gene containing exon III beta (which has 92% homology with alpha). The third band was originally thought to represent a Taq polymorphism. However it was also present in genomic Southern blots from two further idividuals. The three identified bands were 6.5, 3.2 and 2.3 kb respectively. When the alpha gene exon III was used as a probe, the bigger Taq fragment hybridised most strongly. This was expected from the published map of the alpha gene which predicts a 6.5 Kb Taq 1 fragment containing exons III and IV (Steenbergh et al, 1985). To identify which of the other two Taq fragments corresponded to the beta gene, an exon

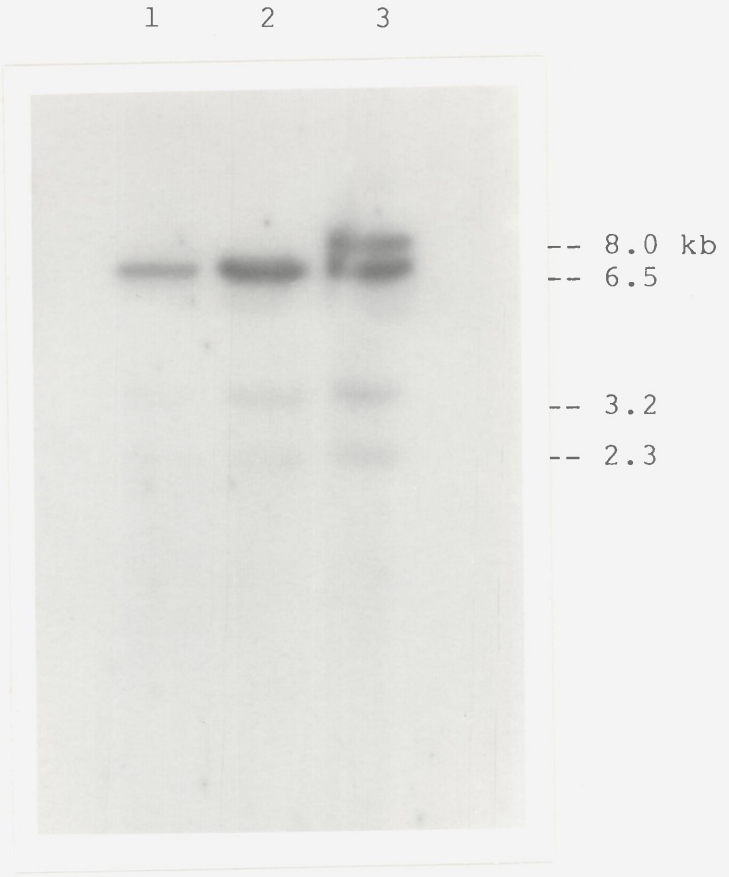

fig 5.1. Genomic Southern blot.

DNA was prepared from three unrelated individuals and 10 µg from each was digested with the enzyme Taq I. The probe is pCTI containing exons III and IV of the alpha gene. In lane 3 a Taq polymorphism of the alpha CT/CGRP gene is present (Hoppener et al, 1984).

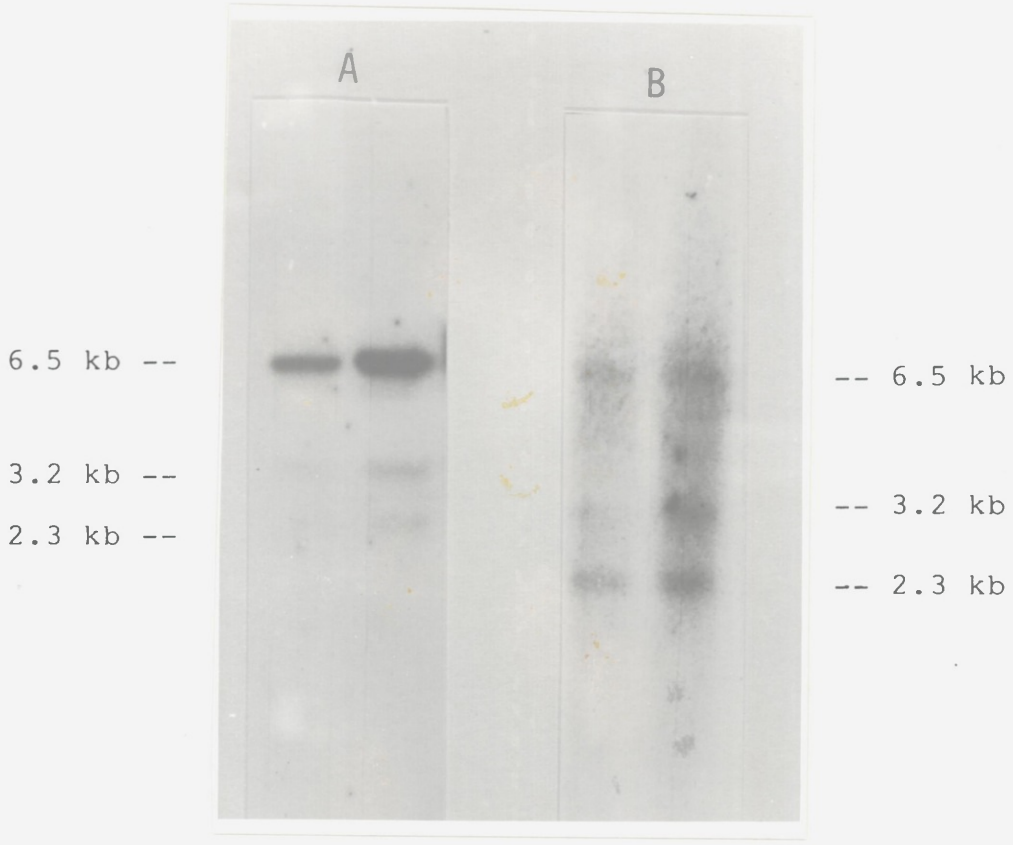

fig 5.2. Genomic Southern blot probed with exon III.

Genomic DNA was digested with Taq I. Panel A shows hybridisation with an exon III probe derived from the alpha gene. The strongest hybridisation is seen with the 6.5 kb fragment. Panel B shows hybridisation with an exon III specific probe from the beta gene. The strongest hybridisation is seen with the 2.3 fragment. The high background seen in lane 2 is due to "aging" of the membrane which had been reprobed several times.

III specific probe from the beta gene was used (see chapter IV, section 5.2). The strongest signal came from the smaller, 2.3 kb fragment (fig 5.2). The middle fragment presumably contained the third CT related sequence, which was named the "gamma" sequence.

V.2. Low stringency screening of genomic library.

To isolate clones representing the gamma sequence, the genomic library was screened with a probe representing exons III and IV of the alpha gene. The reason for using a probe containing exon IV was to permit the isolation of any further clones distantly related to CT itself. The hybridisation conditions used for the screening were similar to those developed for the cloning of exon IV beta which were described in chapter IV.2. The hybridisation was done in 50% formamide but the temperature was reduced to 32 °C. The washing of the filters was done with 2XSSPE at 50 °C. Under these conditions any sequences with >60% homology are expected to hybridise. Several clones giving strong signals with the probe were identified, presumably representing alpha and beta gene clones as well as clones containing the gamma sequence. Apart from these however, many more clones were identified hybridising weakly to the probe. Many of these were picked and plaque purified for further analysis. A total of 80 clones were picked.

V.3. Identification of "gamma" sequence clones.

The "macroplaque" method (Materials and Methods, section 4.7.) was originally used to screen for clones representing alpha and beta gene. Both these genes contain CGRP specific sequences. A CGRP specific probe was therefore used for the screening of the macroplaque lifts using high stringency conditions (fig 5.3). The clones hybridising to

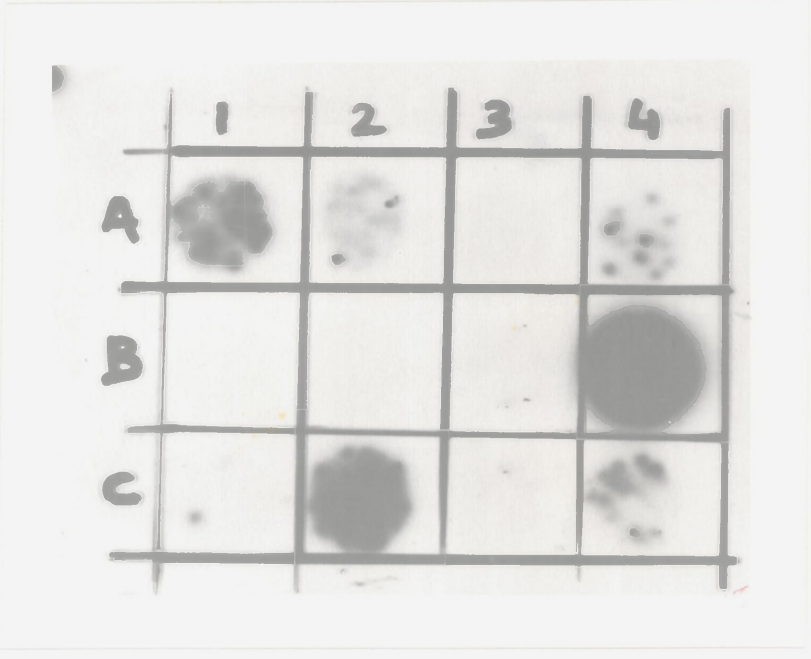

fig 5.3. Hybridisation of plaque dots (macroplaques) with phTB58.

Phage stock from the various genomic clones identified during the screening of the genomic library was dotted on a lawn of cells on a plate. A lift was taken and this was originally hybridised with an exon III specific probe. All clones were positive. The same filter was subsequently hybridised with a CGRP specific probe under stringent conditions (shown here) to exclude any alpha or beta gene clones. Each square represents one clone. The clones A3, B1, B2 and C3 are negative and therefore represent "gamma" sequence clones.

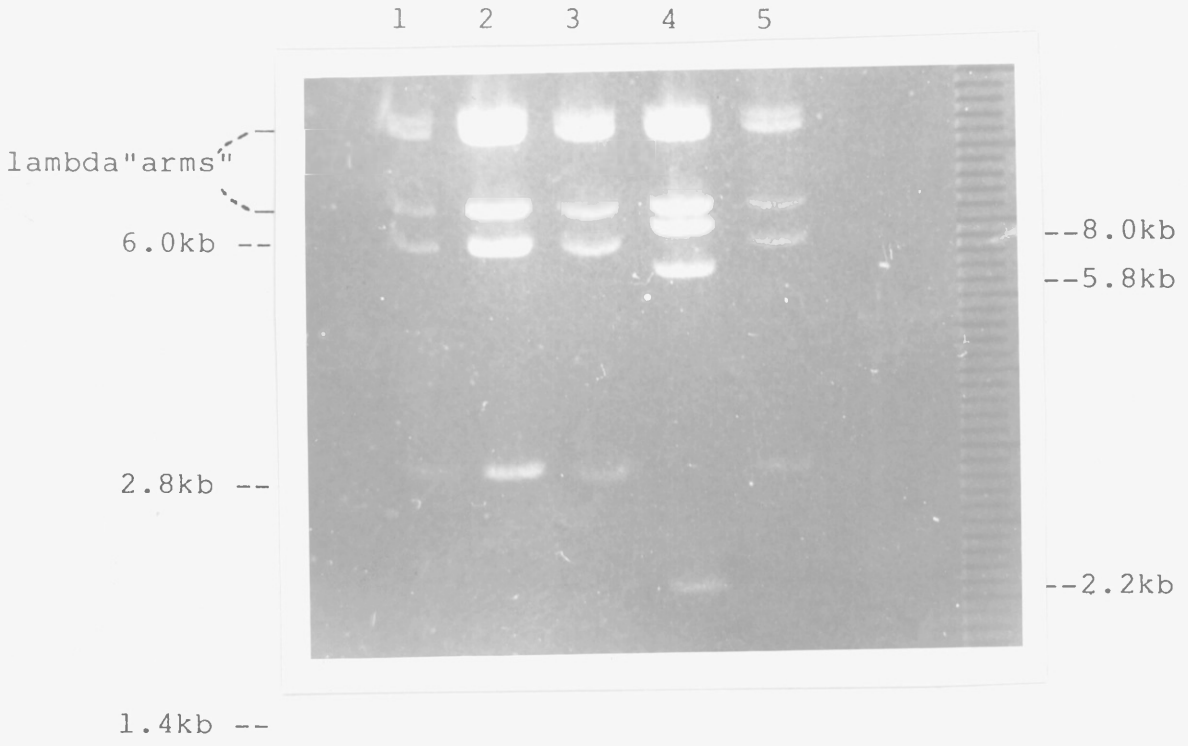

fig 5.4. Restriction analysis of gamma clones.

DNA was prepared from 4 clones containing the gamma sequence and digested with Eco RI. Lanes 1,2,3 and 5 (corresponding to clones 1,5,10 and 44) are identical. Lane 4 (clone 26) shows a different restriction pattern. Eco RI fragments from the four identical clones are 6kb, 2.8 kb and 1.4 kb (not shown in photo). Insert fragments from λ26 are 8 kb, 5.8 kb and 2.2 kb.

Hybridisation experiments showed that the gamma sequence was contained in the 6kb fragment in lanes 1,2,3 and 5 and in the 8kb fragment in lane 4.

this probe were excluded from further analysis. The clones which hybridised strongly to the exon III probe, but were negative for CGRP, were chosen as possible gamma sequence candidates. Restriction analysis of DNA prepared from these clones with the enzyme Eco RI showed two different patterns suggesting that there were at least two different sets of clones with these characteristics (fig 5.4).

V.4. Sequence analysis of "gamma" clone.

One clone from each group of 'gamma sequence' clones was chosen for further analysis, lambda 10 and lambda 26. Southern blot analysis with various restriction enzymes showed that the hybridising sequence was contained in a Sau 3 A fragment of 750 bp (fig 5.5). When used as a probe to Taq 1 digested genomic DNA, this hybridised to the 3.1 kb band that had been previously identified and was thus shown to represent the gamma sequence.

The 750 bp Sau 3A fragment was subcloned into M13 and the nucleotide sequence was determined. Three smaller Hae III fragments containing parts of this sequence were also sub-cloned separately, so that the sequence could be read in both strands of the DNA and the coding potential of this region could be determined. The entire sequence of this 750 bp fragment is shown in figure 5.6 together with the corresponding region of the beta gene. There is a very high degree of homology, not only with exon III (92%) but also with exon II (86%) and intron II (89%). When all three sequences were compared (fig 5.7), it was clear that the gamma sequence was more closely related to the beta gene than the alpha gene. Intron II of the alpha gene is approximately 600 nucleotides longer, while it is very similar in size between beta and gamma genes. The homology between gamma sequence and alpha and beta genes is shown schematically in figure 5.8. The high degree of homology ends abruptly 230 bp after the end of exon III.

fig 5.5. Southern blot of restriction digests of clone λ10.

Lane 1: Hae III, lane 2: Sau 3A, lane 3: Rsa I. The probe is an exon III specific probe derived from pCTI. Both the Hae III as well as the Sau 3A fragments were subcloned for sequencing.

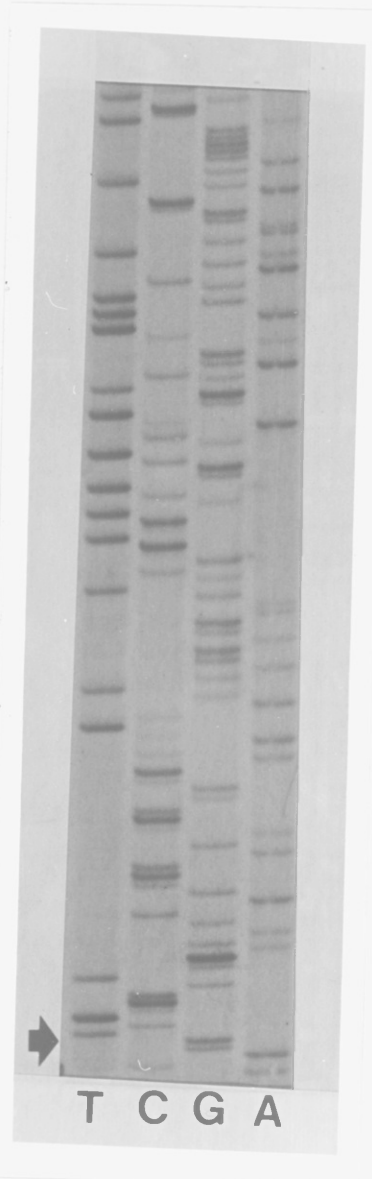

fig 5.6a. Sequencing gel of the exon III region of the "gamma" sequence.

The sequence shown here was obtained from an Alu1 subclone of the exon IV region. The arrow points to the first nucleotide of exon III and the sequence reads GTCTCCC... (see figs 5.6 and 5.7).

beta sequence: *taacgtcatccttcctttacagAGAGGCGGCATGGG*
gamma sequence: *catcattttacttgttacatag CAAACAAAGG*

TTTCCGGAAGTTCTCCCCCTTCCTGGCTCTCAGTATCTTGGTCCTGTACCAGGCG
CTCCCAGAAGTTCTCCCGCTTGCTGGCTCTCAG TCTTGGTCCTGTGCCAGGCA

GGCAGCCTCCAGGCGGCGCCATTCAGgtgagacagcctggagccagcggc cttc
GGCAGCCTCCAGGCAGCGCCATTCAGgtgagacagcttggaaccaggggctcctc

tgctcccactgcccctaggaccagacagctctgtgcctctgaactcacgcgtggc
tgctcccactgcccctgggaccaggcagctctgtgcctctgaaaccatgcatggc

tcctggtgaatcagtgcccacaggtggaccctggcctat gccc gtcccctggg
tcccggtgaagcagtgcccacaggtggaccctgacctcatgccctgtcccctggg

agtcgcggtgcc acatccccaggggaagaagcagagaccaggaagcctggctgc
agtcgcagtggccacatcctcaggggaagaagcagataccagggagcctggctgt

ctatcctggggagggtcagtcaggggctcacagccttgcaaggagtttgcttccct
ttatcctggggaggggcaggcagggactcacagcct gcattgagtttgcttcccc

tccacagGTCTGCCCTGGAGAGCAGCCCAGACCCGGCCACACTCAGTAAAGAGGAC
tccacagGTCTCCCGTGGAGAGCAGCCCAGACCCGGCCACACTCAGTGAGGAGGAA

GCGCGCCTCCTGCTGGCTGCACTGGTGCAGGACTATGTGCAGATGAAGGCCAGTGAG
GTGCGCCTCCTGCTGGCTGCACTGGTGCAGGACTATGTGCAGATGAAGGCCAGTGAG

CTGAAGCAGGAGCAGGAGACACAGGGCTCCAGgtgaggttcccc...
CTGGAGCAGGAGCAGGAGACAGAG CTCCAGgtgaggctcccc...

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fig 5.6.
Nucleotide sequence of the exon III positive region of the gamma gene clone compared with highly homologous region of the beta gene.

fig 5.7. Nucleotide sequence of the exon II and III regions. Asterisks indicate homology.

The two base deletions in the gamma sequence which alter the reading frame in positions 48 and 135 are highlighted.

There are a number of significant features in this sequence (fig 5.7). There are potential splice sites at each supposed intron/exon boundary although the sequence preceding exon II is not perfect. There is an open reading frame in both exons which corresponds largely with those of the alpha and beta genes. However, the reading frame is altered at two points. A two base deletion at position 48 in exon II means that there is no signal peptide and no initiation codon in the gamma sequence. If this sequence is utilised, then it must be initiated at an upstream exon. The second frame-shift is a two base deletion at position 135 in exon III. This affects only the last three amino acids but, since the splice site is conserved, means that this frameshift must extend to any downstream exons. This reduces the likelihood that these sequences might be associated with an exon IV-like CT coding sequence, although it is possible that such an exon might itself contain a compensating frameshift prior to its CT-coding sequence.

The genomic clones were further analysed for the presence of CT or CGRP related sequences. Low stringency hybridisation conditions were used but no evidence for the presence of related sequences was found. Very weak signals could be seen with the exon V probe after very long exposures. However, the most prominent of these proved to be vector sequences. From restriction mapping and Southern blot analysis of the two clones, it was evident that a nucleotide sequence of at least 4.2 kb downstream of the exon III region was present in the two clones (fig 5.9). In both alpha and beta genes the exon IV region is located approximately 1 kb downstream of exon III so it is reasonable to suppose that if there was a similar region associated with the gamma sequence, then it would be present in the clones.

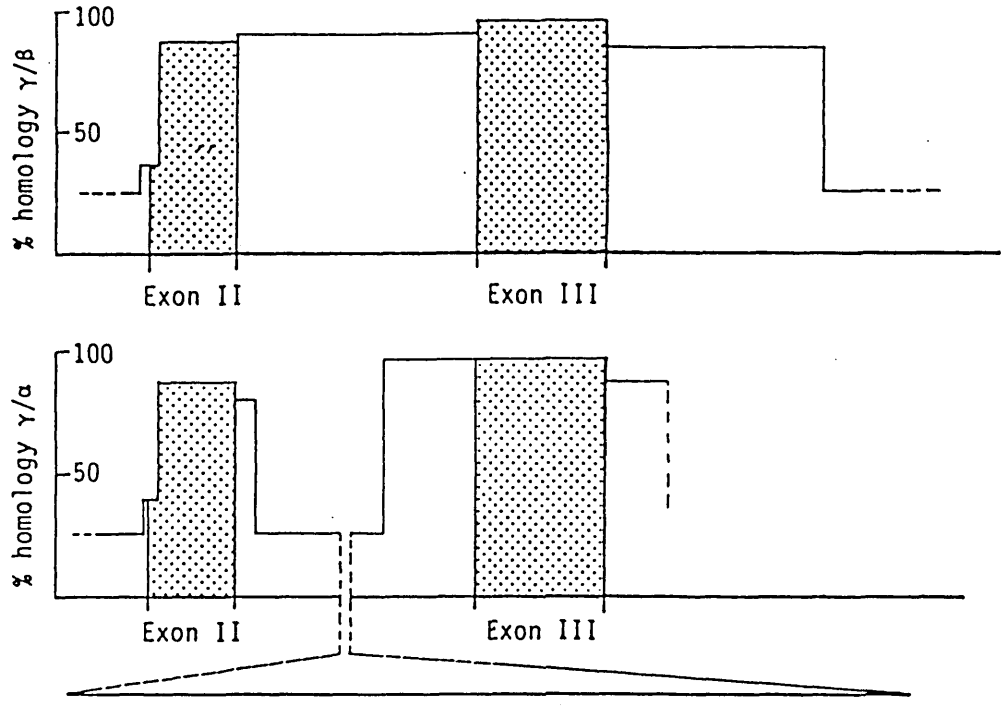

fig 5.8. Homology chart between gamma sequence and alpha and beta genes.

The intron II of the alpha gene is much longer than gamma and is shown at the bottom of the figure as an insert. Homology in this region is roughly 25%.

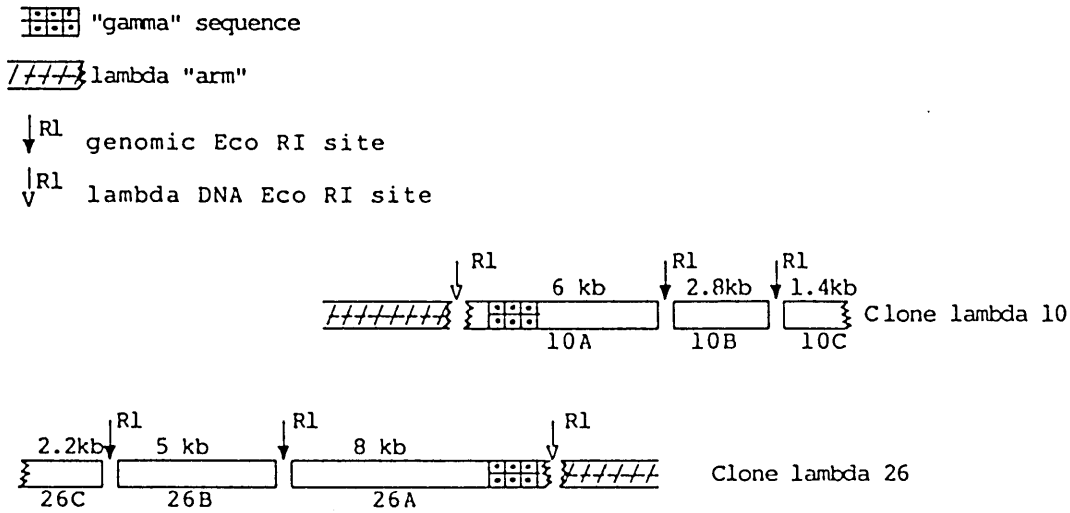

fig 5.9. Eco RI restriction map of the two clones containing the gamma sequence.

The "gamma" sequence (exons II and III) is contained in fragments 10A and 26A which therefore overlap. Fragment 10A, being 2kb shorter than 26A, must have been cut by Sau 3A during the cloning process and must therefore be adjacent to lambda sequences in clone 10. It follows therefore that fragments 10B and 10C must both lie on the other side of 10A. Fragments 26B and 26C, which are not represented in clone 10, must therefore lie on the other side of 26A. Wherever the hybridising region lies within 10A/26A, there must be at least 4.2 kb on one side and 7.2 kb on the other.

V.5.Localisation of "gamma" gene clone.

A region of lambda 26 was chosen for use as a probe for in situ hybridisation. A suitable restriction fragment was first identified which did not cross hybridise with the alpha or beta genes and which did not contain any repetitive sequences. A Southern blot of restricted lambda 26 was hybridised successively with three nick-translated probes. One was an alpha gene lambda clone, one was a beta gene lambda clone and the third was total genomic DNA. As no individual fragment of sufficient size appeared to be suitable, two small Sau 3A 1 fragments (300 & 400 bp) were used together. These were eluted from a gel and labelled with ^{125}I dCTP using the random primer method (Materials and Methods, section 3.2.). As previously (chapter IV), in situ hybridisations were done in collaboration with Dr Feyruz Rassool and the methodology is described in Materials and Methods, section 10.. Although there was a clustering of grains on the short arm of chromosome 11, for this to be statistically significant, analysis of a larger number of metaphases would be required. As by that time sequencing data had indicated that this region is probably not part of a functional gene, it was decided that further studies for exact localisation of this sequence were not justified.

V.6. Evolution and possible "function" of gamma sequence.

As can be seen from the comparison of "gamma" sequences with the corresponding region of the other two genes, the strongest similarity is with the beta rather than alpha gene. The homology (fig 5.8) starts abruptly at the beginning of exon II, then continues throughout intron II of the beta gene and finally ends equally abruptly at 230 bp downstream of exon III. The intron separating exons II and III is much longer in the case of the alpha gene (890 bp)

than in the beta and gamma genes (256 bp). The extra sequence in the alpha gene includes an 'Alu' repetitive sequence and has presumably been inserted into the alpha gene since the separation of alpha and beta genes.

It is apparent from the homology chart that the "gamma" sequence must have been derived from the beta gene by a local duplication of part of this gene on the short arm of chromosome 11. This duplication must have occurred relatively recently. An 89% homology in a non-coding sequence indicates that the gamma sequence separated from beta some 5 - 10 million years ago. The sequences surrounding the duplicated region do not provide us with any information as to how this duplication may have occurred. However, the in situ data show localisation of this sequence on the short arm of chromosome 11 near the other two genes, which is consistent with a duplication occurring through unequal cross-over.

After completion of these studies (Alevizaki et al, 1987) cloning of the "gamma" gene has been reported by Lips' group (Hoppener et al, 1988). Their data largely agree with mine, although there is a size difference in the intron II-like region, which is larger in their clone. However, since complete sequence of this region is not shown in the publication, it is not possible to localise the differences.

All the information so far obtained about the gamma sequence suggests that it is a non-functional duplication of part of the beta gene. However one cannot totally exclude the possibility that it now forms part of another gene. It contains an open reading frame which encodes a polypeptide having extensive homology with the N-terminal flanking peptide of the CT/CGRP genes. The remarkable conservation of this region between alpha and beta genes indicates that it must serve some important function. It has long been believed that its function is important for the transport and/or processing of the precursor polypeptides, although there was not much evidence to support this speculation.

Such functions must be required by other regulatory peptides and it is by no means impossible that these two exons have become inserted into another gene, thus conferring on it the ability to present its peptides to common elements of the processing machinery of the cell. However, recent work by Roos and colleagues, showing that the N-terminal peptide is a secretory peptide in the rat, which appears to have some important biological activity when administered to osteoblast-like cell lines (Burns et al, 1989, Roos et al, 1989), points to its role as a possible regulatory peptide.

This secretory peptide may prove to be very important, in which case, one possible "function" of the gamma sequence may be that it provides the organism with a third copy of an "important" regulatory peptide. There have been several reports of bioactive peptides residing in the N-terminal and the C-terminal regions of prohormones such as that of POMC and LHRH (Estivariz et al, 1988, Nikolics et al, 1985). It is also interesting to note that if one compares the CT precursors in various species, one sees that the N-terminal peptide is much better conserved than the C-terminal cleavage peptide (Burns et al, 1989).

V.7. Sequence analysis of three further related sequences.

45 of the clones which gave weaker signals during the screening of the library were picked and purified by secondary screening. Because the clones were numerous and the signals were rather weak, several were lost during the secondary screening. However, DNA was prepared from 20 of these using the plate lysate method (Materials and Methods, section 1.5.5.). Restriction analysis with the enzyme Eco RI was performed to separate the vector arms from the insert and the gels were blotted. They were subsequently hybridised with the pCT I probe under the same low stringency hybridisation conditions.

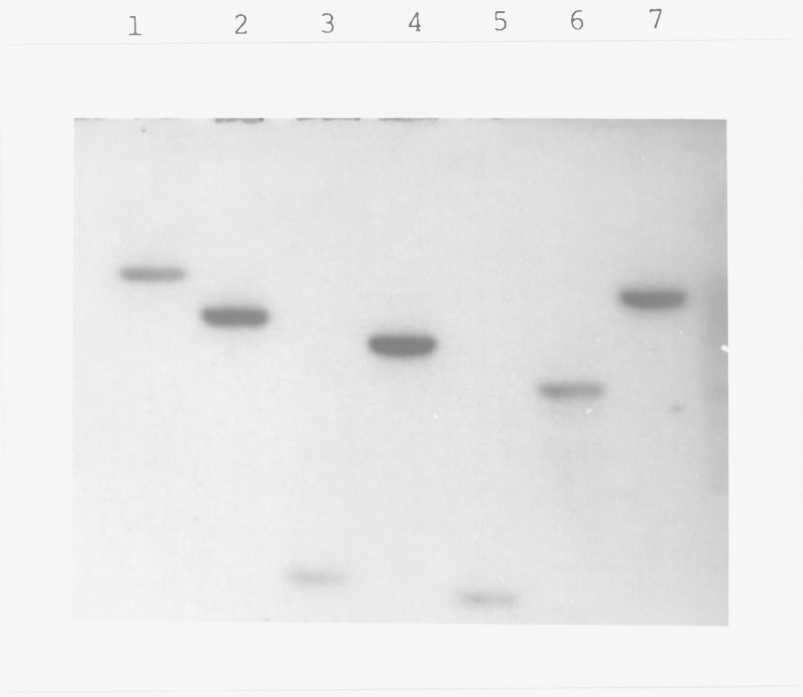

fig 5.10. Analysis of a distantly related clone (λ40)

Enzymes are lane 1: Bam HI, 2:Hind III, 3:Pst I, 4:Pvu II, 5:Hae III, 6:Sau 3A, 7:Bgl II. The probe is pCT I (CT cDNA) and the hybridisation conditions are as described in text-section V.2. The Pst I fragment was subcloned and partially sequenced (see text). When the filter was reprobed with genomic exon III and exon IV probes under the same conditions, no hybridisation was detected.
Size markers were not included in this gel. Suitable small restriction fragments were identified by reference to mobility of the dye marker.

Full sequence analysis of all twenty genomic clones showing weak hybridisation to the CT cDNA probe was unrealistic. However, three of the clones were analysed to give an idea about the nature of the hybridising sequences and to see if CT related sequences might also be present. These were the clones giving the strongest signals one of which was represented 3 times in the 20 clones. These were digested with various restriction enzymes, blotted and probed with pCT I under low stringency conditions to identify suitable fragments for cloning.

V.7.1. Lambda 40.

Lambda 40 was one of the three identical clones (fig 5.10) and a 600 bp Pst 1 fragment was subcloned into M13 for sequencing. A long stretch of Gs (14) was identified in this sequence. This was considered as a possible source of cross hybridisation, because the probe which was being used (pCT I) contained C tails which had been added to the cDNA during the cloning procedure. The hybridisation experiment was then repeated using exon III and exon IV specific probes which had been derived from genomic DNA (chapter III) and therefore contained no C tails. No hybridisation was detected with these probes!

Following this observation, a new probe was made by cutting the cDNA sequence of pCT I with Hae III, which eliminated the ends -and thus the C tails - from the probe. This new probe (pCT III), was made by cloning the sequence between the Hae III restriction sites at positions 1793 and 1348 of the published sequence of clone B3 (Edbrooke et al 1985) into M13. The hybridisation experiment was then repeated and again no signal was seen. It was therefore concluded that the C tails were indeed the source of the cross-hybridisation.

V.7.2. Lambda 12.

A 500 bp Hae III fragment was subcloned from lambda 12 and sequenced. The partial sequence of the λ12 subclone is

shown in fig 5.11. A stretch of 27 bp was identified which is identical to a sequence at the end of exon III in the alpha gene. However, no suitable open reading frame was present in the surrounding region, nor were there appropriate splice sites. No explanation can therefore be offered for the origin of such a sequence.

TATAAAGAAATACCTAAGACTGGGTAATTTATAAAGGAAAGAAGTTTAATTGGCTCA
CAGTTCCACAGGATGCATAGAAAGCATGATGCTGGCATCTGCTTGGTTTCTGGGGCA
GTCTCAGGAAACTTACAGTTCATGGTGGAAGGTGAAGGGAGAGTTGGC

ACTTCACATG/GCTGGAGCAGGAGCAAGAGAGAGAGGG/GTGAGGTGC λ12
****
....TGA GCTGGAGCAGGAGCAAGAGAGAGAGGG CTC.. [exon III]

ATCCCATTTTTAAATGACCAGAGCTCATGAGAACAGCACCAAGGGGATGGTAGTAAACC
ATCAAGAAATCTGCCTCCATGATCTAGTTACCTCCCACTAGGTCCCACCTCTAACAGGA
GGGATTACAATTTGACATGAGAGTTTGG

---

fig 5.11.
Partial nucleotide sequence of genomic clone λ12.
The region hybridising to exon III is underlined.

CCAGGTGTGGTGGCTCACGCTGTAATCCCAGCACTTTGGGAGGCTGAGGCGAGTTG
ATCACCTGAGGTCAGGTGTTCGAGACCAGCCTGACCAATATGGTGGAACCCCGTCT
CTACTACAAATACAAAAAAATTAGCCAGGCGTGGTGGTGGGCGCCTATAGTCCCAG
CTACTGGGGAGGCTGAGACAAGGAGAATTGCTTGAACCTGGGAGGCAGAGGTTGCA
GTGAGCCAAGATCCACGCCACTGCACTCCAGCCTGGGCGACAGAGCACAACTCCAT
CTCAAAAAAAAAAAAAACCCCAATTTGGGA ....approx. 50 bases.....
GGGACTCTCCTGGATTATACACTTTAAATTTGTAAGCTGTTATGTCAATAAAGCTG
TTTGAAGAAAAAAAAAAGGATAGTTGAATCACAAGGCCACAAACTGTCAATGGTACT
TAATGATAAACTGATAGGAAACTGTTGACAGGCACTTTTGAGAAAGCAAAGCTCTA
GGCTAGATATTATCATTCATTCAAAATTATAAATCATAAAGTGAAACAACAAAATT
CAGTCAAGCAGGGTTAAATTTCCTCCTCCACAGATTGTAACCCTTAATCTCCAGGC
TACAGTGATTGTAATGCTTGATGTAGAAATTCTTTTTCATTCACTGTCAAAATAAA
TGGAATTTCTCGAAAACCCATTAACCCATTTGACC

---

fig 5.12. Partial nucleotide sequence of genomic clone lambda 78.

The subcloned fragment is 700-750 bp, therefore the uncharacterised sequence in the middle is a maximum of 50 nucleotides long.

V.7.3. Lambda 78.

A 700 bp Hae III fragment was subcloned from lambda 78 and sequenced on one strand only from both ends (Fig 5.12). No homology could be found between this sequence and the pCT I sequence, although there was a sequence of 50 bases in the middle of this fragment which was not characterised. The fragment did not give a hybridisation signal when probed separately with the exon III and exon IV (genomic) probes but did give a signal when pCT III was used. The only sequence present in pCT III which is not also present in either exon III or IV is the junction sequence formed when the two are spliced together so it seems likely that this region is giving rise to the hybridisation signal.

This analysis of three clones is sufficient to provide an indication of the type of homology which can be detected under the low stringency hybridisation conditions. The sequence in lambda 12 with 27 bp matching part of the alpha gene is quite interesting. It would be surprising to find a perfect match of this length in a random sequence the size of the human genome. However, this does not necessarily imply that the lambda 12 sequence is derived from the alpha CT / CGRP gene. The sequences surrounding the 27 bases are not similar and the lambda 12 sequence does not seem to be part of a coding sequence.

The signals seen in lambda 40 came from a homopolymer region which was hybridising with the G/C tails in the original probe. Clearly it is important to remove any such tails before attempting low stringency hybridisations. In lambda 78, the hybridising region could not be identified, although clearly there was not a region of extensive homology.

The general conclusion of this series of experiments has to be that the very weak signals which can be detected in low stringency hybridisations are very often due to short

sequences with good homology to the probe. If this type of approach is to be successful then it will be necessary to devise conditions in which long imperfect hybrids give stronger signals than those due to short but perfect matches.

However, it should be apparent that the type of homologies which have been detected using the present conditions should be more than sufficient to detect the putative salmon-CT in the human genome. The exon IV-like sequences of the beta gene give very much stronger signals on Southern blots than any of the three clones considered above. As the beta CT peptide is no closer to alpha CT than is salmon CT, one would expect that an alpha CT probe would detect a salmon CT sequence very easily.

CHAPTER VI

GENERAL DISCUSSION

VI A. The calcitonin gene and osteoporosis.

One of the underlying assumptions behind these studies has been the notion that there is a major genetic component in the aetiology of osteoporosis. This is based on the observation that patients with osteoporosis often give a positive family history. However, it is possible that other factors such as environmental and nutritional ones, which are often shared by members of the same family, affect peak bone mass.

A review of all publications during the period 1983-89 failed to reveal any study in which the presence of a genetic factor was adequately proven. In a Belgian study, 30 pairs of twins (16 monozygotic pairs) were investigated (Dequeker et al, 1987). Both single photon absorptiometry (SPA), as well as dual photon absorptiometry (DPA) were used to evaluate bone mineral content (BMC). The conclusion was that bone mass was affected by a genetic determinant to some degree but that environmental factors were more important. In another study, mother daughter pairs were studied (Sowers et al, 1986); no evidence for a resemblance in bone mass was found. A different approach was taken by Seeman et al (1989), who measured BMC in the daughters of osteoporotic women. BMC of this group was significantly lower than controls in the lumbar spine indicating there may be some genetic component in peak bone mass.

Thus, it is by no means clear that the development of os-

teoporosis in general is determined to a significant degree by genetic factors. However, this does not mean that individual cases might not be caused by gene defects. It was considered worthwhile to investigate a young male osteoporotic patient with no detectable calcitonin.

It was recognised at the outset that there is no proof that the lack of calcitonin and the osteoporosis are causally related since there is a small percentage of the healthy population in whom calcitonin is not detectable. However CT levels are higher in men than women and it has been this department's experience that after stimulation CT has always been detectable in men. Therefore, if the sequence of his CT gene revealed that he could not make CT, and this defect could not be found in normal "zero CT" individuals, this would constitute strong supportive evidence for the hypothesis that CT deficiency can cause osteoporosis in adolescence.

Despite sequencing over 2,000 bases of the CT gene, it is still not clear why it does not appear to be expressed in this patient. The coding region of the gene is entirely normal and the only difference seen between our patient's sequence and the previously published 'normal' sequence proved to be present in both alleles of an unaffected individual. It seems appropriate to ask what might be the cause of the patient's condition and how one might best investigate it.

One possibility would be that he lacks C cells, either because they never developed or because they were destroyed through some form of auto-immune reaction. If anti C cell antibodies were available, then it is possible that some form of in vivo imaging technique could be used to identify any C cells in the patient. The possibility of an autoimmune response could be checked by challenging normal thyroid tissue sections with serum from the patient. However, this was not a standard procedure in the department and no suitable collaboration could be arranged. The possibility that the patient had antibodies directed

against CT itself had been checked and his satisfactory long-term response to CT therapy confirms that finding.

The other possibilities that have been considered concern the CT gene. The most likely of these was a homozygous defect in the CT coding sequence and that can now be ruled out. Other ways in which CT production could be disrupted without preventing alpha CGRP production might include mutations affecting an enhancer or other control sequence active only in the thyroid. Such a defect would eliminate CT and alpha CGRP in the thyroid but would leave unaffected CGRP production in the nervous system. It is known that circulating CGRP is mostly derived from perivascular nerve endings in the rat (Zaidi et al, 1985), so such a defect might account for the findings with this patient, who, as was mentioned, had normal CGRP levels.

Still further possibilities which have been postulated are defects in whatever factors interact with the gene in the C cells. The investigation of such possibilities would be extremely difficult as such sequences or factors have not so far been identified.

In many ways, studies of this type have been transformed by the introduction of the polymerase chain reaction (PCR). If one were faced with the same problem today, the most sensible strategy would be to amplify the CT coding sequence and clone this for sequence analysis (as was done for the normal intron sequence, chapter III). If this proved to be normal, the rest of the known sequence could be checked by amplifying 500 bp regions from the patient and from normal individuals, forming hybrids between the 2 DNAs and cleaving at any mismatched sequences with hydroxylamine or osmium tetroxide (Cotton et al, 1988). This method would provide rapid screening for all sequenced parts of the gene though until it has been used more widely, there must be some reservations about whether it detects all possible mismatches.

The ability of the polymerase chain reaction to detect single molecules opens up a further means of analysis. It

would appear that white blood cells express every potentially active gene at a level (albeit very low) that can be detected using PCR on reverse transcripts of mRNA (Sarkar and Sommer, 1989). These authors were able to clone copies of the mRNA from tissue specific genes from heterologous tissues. If CT mRNA could be detected in the patient's leucocytes at roughly the same level as in controls then one could conclude that there was no absolute barrier to transcription of the gene although of course this would provide no information about tissue specific gene expression.

One of the intentions in these studies had been to investigate the expression of the CT gene. It is of course not possible to take thyroid samples from normal individuals so the proposed experiments had to be restricted to thyroid samples taken during operations - samples of normal thyroid adjacent to thyroid nodules which were removed for histological examination. Preliminary experiments were done on thyroid samples from patients undergoing surgery for parathyroid adenomas looking for any effect of chronic exposure to mild hypercalcaemia on the expression of the gene.

However, the distribution of C-cells within the thyroid is unequal. When a normal thyroid obtained post mortem was examined for CT expression, CT mRNA was detectable in the middle part of the thyroid but not in the upper and lower parts of the gland (Fig 6,1). Since it was not possible to control which parts of thyroid the samples would come from, it would be impossible to obtain satisfactory results without a very large number of samples. These studies were therefore discontinued.

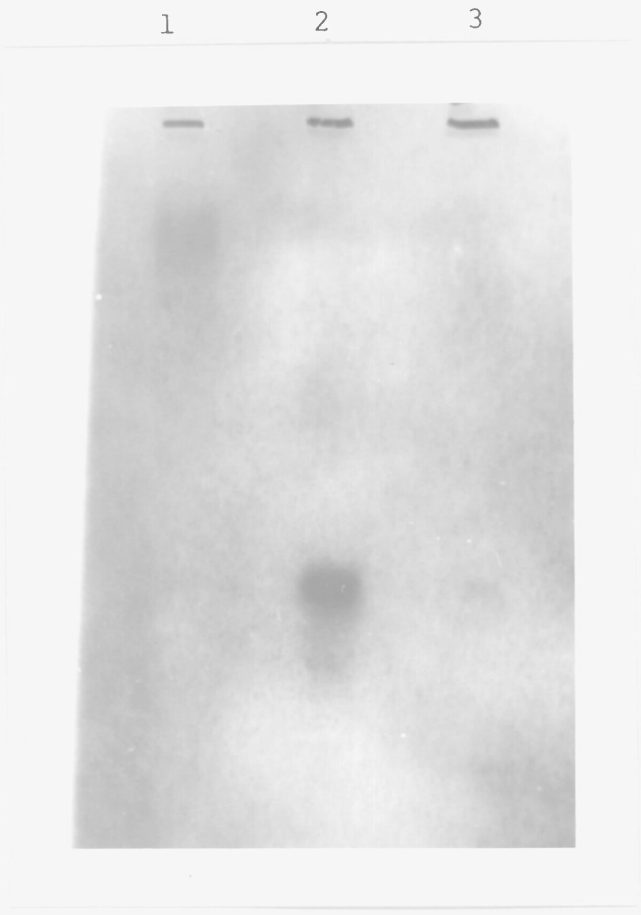

fig 6.1. Northern blot analysis of normal thyroid tissue.

Total RNA was extracted from thyroid tissue obtained postmortem from a normal individual. Lane 1: upper part, lane 2: middle part and lane 3: bottom part of the thyroid. 10 µg were run on each lane. The probe is pCTI.

VI B. Evolution of the calcitonin/CGRP genes.

The greater part of this thesis has been the study of further members of the CT/CGRP gene family. In chapter IV, the CT-like region of the beta CGRP gene was described and in chapter V, the gamma sequence was investigated. These studies have provided a further illustration of the way in which genes evolve by repeated duplication and divergence. There appear to be two principal mechanisms by which gene sequences multiply in the genome - retroposition and unequal crossover.

In the former, it appears that polyadenylated RNAs are copied by a reverse transcriptase activity in germ line tissues and that these DNA copies then integrate into the genome. Many of the most abundant repetitive sequences such as the human 'Alu' repeat, show evidence of having been amplified in this way. The characteristics of this type of multiplication are the dispersal of the sequence randomly around the genome and the appearance of short directly repeated sequences to either side of the integrated sequence. When this type of event happens to an mRNA sequence, the newly integrated sequence lacks introns and also lacks the regulatory sequences normally found upstream of the parental transcription unit. For both reasons, retroposition is unlikely to result in the generation of a new active gene but many examples have been found of "pseudogenes" which appear to have been generated in this fashion. It has been suggested that this mechanism of sequence multiplication may be an important factor in the creation of new species by promoting genetic isolation.

Unequal crossover during meiosis appears to be the more important mechanism by which genes duplicate. When homologous chromosomes pair during meiosis, it is always possible that an error will occur such that the two

GENE DUPLICATION FOLLOWING UNEQUAL CROSSOVER

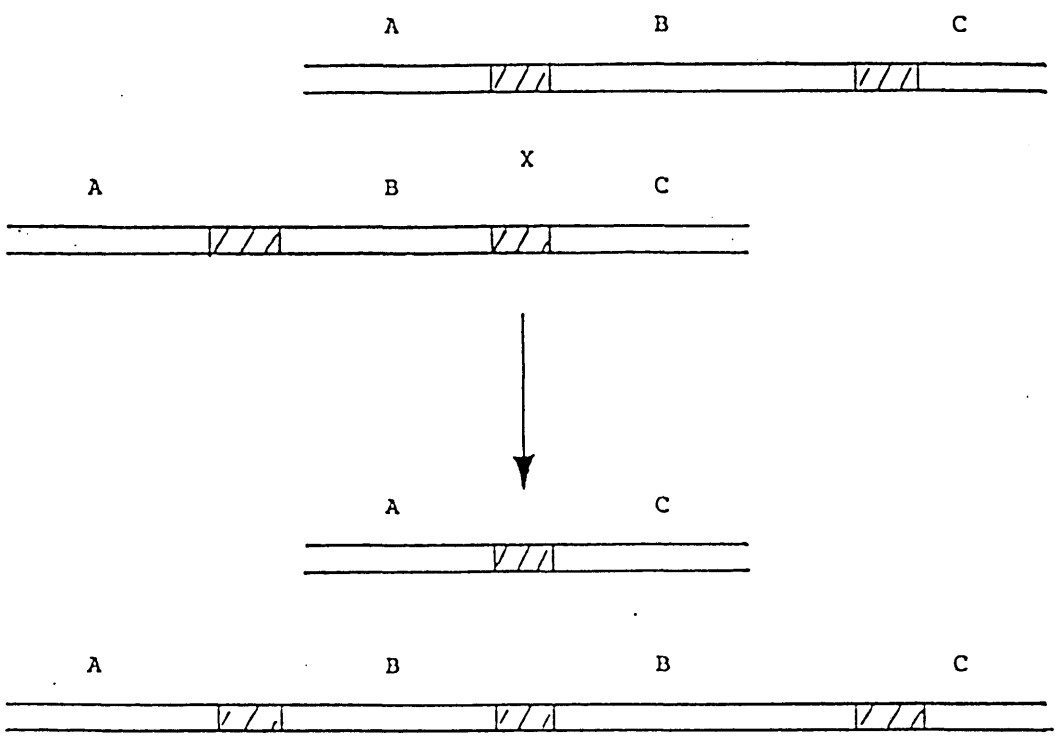

fig 6.2. Unequal crossing over during meiosis.

Homologous regions are misaligned through a repetitive sequence (shaded). After cross-over gene B is duplicated on one chromosome and eliminated from the other.

footnote to page 209

* An interesting example of a cardiac myosin heavy chain hybrid gene created as a result of unequal crossing over has recently been published (Tonigawa et al, 1990). This type of genetic abnormality is thought to underlie some cases of familial hypertrophic cardiomyopathy.

Tanigawa G, Jarcho JA, Kass S, Solomon SD, Vosberg HP, Seidman JG and Seidman CE (1990) Cell 62:991-998.

chromosomes are not in perfect alignment (Fig 6,2). Recombination then occurs resulting in the formation of two chromosomes - one with the duplicated sequence and one in which this region has been deleted.* It is often supposed that highly repetitive sequences facilitate this misalignment and recombination, but there is in fact little evidence to support this idea. A good example of a gene family which appears to have been arisen by this mechanism is the growth hormone / prolactin family (Hirt et al, 1987).

Growth hormone and prolactin peptides are distantly related (34% homology) and their genes are found on chromosomes 17 and 6 respectively in man (Owerbach et al, 1980, Owerbach et al, 1981). It is assumed that there was an ancient gene duplication and that the two genes remained active, drifting in sequence until they took on separate functions. At some stage, major chromosomal rearrangements have led to the separation of the two genes. A similar duplication and drift must be assumed to have taken place with the respective receptor genes (Edery et al, 1989).

Evolution must always proceed by the most economical route and this mechanism is much "cheaper" than "designing" a wholly new hormone / receptor pair. The growth hormone (GH) family also illustrates how duplications need not always lead to the appearance of useful new activities. In a region of some 48 Kb on chromosome 17 there are two growth hormone like genes and three somatomammotropin (CS) genes, which have been created more recently by the same mechanism. One of the somatomammotropin genes cannot be expressed. Structural analysis of the hGH gene cluster has shown that there are at least 27 Alu type repetitive sequences in this genomic locus (Hirt et al, 1987). Three closely homologous unique sequences have been identified in the vicinity of hGH/hCS genes possibly defining the breakpoints of the duplication units. These homologous sequences may predispose towards unequal recombinational events probably providing the molecular basis for some

cases of GH gene deletions (Vnencak-Jones et al, 1988).

It should be stressed that genes do not duplicate in order that new activities might develop. The process is considered to be accidental and confers no advantage on the immediate progeny. Over the course of subsequent generations, random mutations may either lead to the complete inactivation of the extra sequence or may occasionally lead to the generation of useful new activities or patterns of regulation. Although the process of duplication may be accidental, the overall process is beneficial to the species, so this tendency is preserved by evolution.

This type of mechanism has probably been responsible for the generation of diversity in the CT/CGRP gene family. Just as in the case of prolactin and growth hormone, a very early event can be identified, in which a primitive gene duplicated and gave rise to the amylin and CGRP series of genes. A duplication of the CGRP coding exon then gave rise to an additional exon which later became the CT-coding exon.

The beta gene which was studied in chapter IV appears to have been derived by a gene duplication of the alpha CT/CGRP gene, possibly about 70 million years ago. The CGRP coding exons do not appear to have diverged significantly as the 2 peptides seem to have very similar structures and activities. However, the regulatory elements associated with the gene have been subject to changes resulting in a distinct pattern of expression for the two genes. The CT coding sequence in the beta gene seems to have lost the capacity to be expressed soon after the duplication as judged by the degree of sequence divergence. It may be that the reason for the fixation of this duplication has been that it permits the expression of a CGRP sequence in non-neural tissues without accompanying CT production. It is possible that "leaking" CT expression might be harmful to some tissues.

To date, the various studies which have been reported on

the beta gene have provided no clues as to the precise mechanism of the duplication. Alpha and beta genes map to the same locus by in situ hybridisation (Chapter IV) but this is an imprecise measure. It has been reported that the two genes are not present in the same cosmid clones (Steenbergh et al, 1985) suggesting that they may be at least 20 Kb apart. If true, this would suggest that the duplicated region was unusually large. For instance, only 12 kb separate the arginine vasopressin from the oxytocin gene (Sausville et al, 1985). These two genes are very similar in their exon-intron structure (Sausville et al, 1985). Further studies using the technique of pulsed field gel electrophoresis might shed further light on this question.

In chapter V, the gamma sequence was investigated. This is clearly a recent duplication of part of the beta gene. The degree of homology is around 90% throughout a region which includes exons II and III together with a small portion of the flanking introns. Exons II and III code for the signal peptide and N-terminal flanking peptide of the CT/CGRP precursors and, despite 2 frame shifts, the coding sequence remains largely intact in the gamma sequence. It is possible that this region has become inserted into some other gene and is still functional, providing these important regions for the processing of some other precursor protein. Another possibility is that this might be a functional gene by itself and that the N-terminal peptide is the principal peptide of the gamma sequence. The rat N-terminal peptide has recently been shown to have some potentially important biological properties (Roos et al, 1989). However, it seems more likely that the gamma sequence is non-functional and that the preservation of splice sites and an intact open reading frame is a matter of chance.

The sequences at the boundaries of the duplicated region* provide no clues as to the mechanism by which it was created. However, a simple sequence duplication caused by

* either from my own data or from those reported by others,

unequal cross-over can be ruled out since this would lead to the retention of the duplicated region between exons I and IV of the beta gene. It is not clear how close the gamma sequence is to the beta gene - the localisation by in situ hybridisation described in chapter V suggested no more than that it was also on the short arm of chromosome 11.

If a simple unequal cross-over can be ruled out, the mechanism by which gamma was generated may have something in common with the generation of Philadelphia negative cases of chronic myeloid leukaemias, in which rearrangements of the BCR gene can be identified (Kurzrock, Gutterman and Talpaz, 1988). In these cases, a portion of chromosome 9 including the Abl oncogene has been inserted into chromosome 22 to create a hybrid gene which is believed to be involved in the pathogenesis of the malignancy. The mechanism by which this takes place has not been elucidated. However a similar (non-lethal) event in the germ line would lead, in subsequent generations, to a duplication of this region.

VI C. Further members of the CT/CGRP gene family.

There have been a number of reports which suggest that there may be further members of this gene family in the human genome. One group has described finding salmon-type immunoreactivity in thyroid tissue from normal and MTC patients as well as in brain and, in a second report, in the plasma of both normal individuals and MTC patients (Fischer et al, 1983, Tobler et al, 1984). In both these reports, the salmon CT-like immunoreactive material was found to co-elute with synthetic salmon CT in HPLC. However, a different group had not detected any salmon-CT like immunoreactivity in thyroid tissue in man (Perez Cano et al, 1982b). More recently, another group reported finding salmon CT-like material in the plasma of patients with various forms of lung cancer (Gropp et al, 1985). Again there was no detailed characterisation. However, although it is now more than 5 years since the original report and the therapeutic / commercial potential of the discovery is considerable, the work has not proceded to the stage of peptide purification and sequencing. One therefore remains somewhat sceptical about these reports.

Further indications that there might be a salmon-like CT expressed in human MCT were obtained by a French group (Lasmoles et al, 1985b). These workers had characterised a cDNA clone representing chicken CT. They then used this cDNA as a probe to MTC mRNA and detected hybridisation. Two experiments were described. In one, hybridisation was seen to dot blots of human MTC mRNA where no hybridisation was seen with the control which was a dot of human alpha CT cDNA. The conclusion was that there was an mRNA hybridising with the probe and that the control cDNA dot showed that this was not alpha CT mRNA. The washing temperature was 72 °C - an unusual choice for this type of work. It should be borne in mind that DNA : RNA duplexes are more

stable than DNA : DNA hybrids. It seems possible that at this temperature, hybrids formed with the human alpha CT cDNA were unstable whilst those formed with alpha CT mRNA were stable.

The other hybridisation experiment which was described was a Northern blot where the chicken CT cDNA probe was shown to detect a species exactly the same size as the species detected by a human alpha CT probe. The suspicion remains that the chicken probe was simply detecting human alpha CT mRNA.

Further experiments were described in which human MTC mRNA was translated in vitro and evidence was obtained that salmon CT precursor was synthesised. As before, no satisfactory evidence was obtained that this was a CT precursor and the 4 years which have ensued have not seen the publication of any further characterisation of either the mRNA or the peptide.

To summarise, none of the evidence in favour of the presence of a second human CT is in any way compelling. However, it is true that salmon CT is more active in man than is human CT and there is some evidence that there are binding sites for salmon CT in human brain, although they cannot be distinguished from those for human CT (Fischer et al, 1981). Thus, it remains possible that there is a second CT, but none of the evidence is at all persuasive. When the human genomic library was screened with pCTI, many weak signals were seen but analysis proved that these came from clones with very little homology with the probe (chapter V). If there is a second human CT and if it was present in the library then one would expect that it would have been detected in this screening. However, there could be a more diverged form of CT sharing some epitopes with salmon CT or the sequence might be difficult to clone. The possibility therefore remains.

As was discussed in chapter V, it seems unlikely that further members of this family will be revealed by low

stringency screening of genomic libraries. Some improvement might be obtained by screening cDNA libraries from MTCs but one would still anticipate a large number of false positives. Perhaps the best approach to use is one we recently used to obtain a probe for human amylin (Girgis et al, 1988) - mixed primer PCR. If the primers are designed to accomodate all possible codons for 2 conserved regions of the CTs then it should be possible to amplify whatever CT coding sequences might be present in the genome. At relatively low cost, a series of a dozen primers might be made which would probably ensure that any CT like sequence could be amplified. If the second CT is assumed to be expressed in MTC then one would amplify MTC cDNAs and look for bands of the predicted size which were not present when, for instance, liver cDNA was amplified. Given the potential importance of such a discovery, such experiments would seem well worth doing.

ADDENDUM TWO.

One of the more interesting points raised by this work concerns the handling of my finding of a single base difference between the intron IV sequence of the osteoporotic patient and a published normal sequence. The issue concerns not so much the interpretation of the data but the steps which should be taken to confirm the significance of the findings before presentation of the results.

The base difference was identified during the course of the gene sequencing and was largely discounted on the grounds that it was more than 200 bp from exon V and that such intron sequences frequently contain neutral polymorphisms. After completion of this work the possibility of screening populations with sequence specific oligonucleotides was discussed and in this context, a database was screened to exclude the possibility that this sequence might be part of a repetitive sequence. Although no perfect match was found, the most similar sequence was a region of the rabbit beta globin gene which is implicated in splicing. The author of the original 'normal' sequence was contacted and he confirmed that his sequence was unambiguous. A letter was then written to the British Medical Journal, describing this finding and suggesting that it might be responsible for our patient's acalcitoninaemia. At about this time the polymerase chain reaction (PCR) was being introduced into the laboratory, enabling me to study this region from control subjects. Using both sequence specific oligonucleotides and DNA sequencing, the amplified region was shown to have the same sequence as the patient. A note was then sent to the B.M.J. withdrawing the suggestion that the reported sequence difference could be responsible for the lack of calcitonin.

This raises two important questions about how one should deal with this type of situation. The first concerns the

reporting of experimental findings. One has to be aware that a statement that the data suggest "the change observed may be responsible for the patient's calcitonin deficiency" will be widely taken as meaning that it most probably is responsible. This understandable interpretation must be taken into account particularly in a case such as this where one's work might have important consequences for the understanding of this disease. The second question concerns the control experiments which should be done before considering publication of such findings.

I will discuss this first in the context of the techniques which were available when the sequencing was done and then go on to consider the present-day options. It would seem unrealistic to double the work involved by cloning and sequencing a normal gene in parallel with the patient's calcitonin gene when the coding sequence had already been reported by several groups. However, when a difference was noted in an intron where there was only one reported sequence, this option should be considered. However it is clearly not possible to screen populations in this fashion. The simplest screening technique is possible in cases where the affected sequence forms part of the recognition site of a restriction endonuclease. A simple Southern blot of restricted DNA would then reveal the extent of the sequence difference. Unfortunately, in this case, the affected sequence (TTGGGTTTTCT) does not form part of any restriction site. The approach which I was considering was to make use of sequence specific oligonucleotides to detect one or other sequence. Although appealing in theory, this technique can be difficult in practice and the results are not always wholly unambiguous. However, in retrospect, it is now clear to me that I should have taken this approach before considering publication.

With the techniques which are available today, the control experiments are now much easier. Making use of PCR to amplify the relevant region, all three of the above

approaches are made much simpler. When taking this approach one has to be aware of the possibility of sample contamination. It is necessary to perform 'no DNA' controls and, more importantly perhaps, to ensure that the DNA which is being amplified cannot be contaminated. If these precautions are taken then PCR means that the analysis can now be completed in a matter of days or weeks. In this particular case, since restriction enzyme analysis was not applicable, the amplified region was cloned and sequenced from a normal individual and Southern blots of PCR products were probed with sequence specific oligonucleotides. Since it was then clear that the 'patient' sequence was in fact the normal sequence, the question of population screening did not apply.

In the general case, it would be preferable to sequence control samples in parallel with the patient's DNA. This should prevent the possibility of having the amplified product of the first reaction contaminating the control DNA. An additional important control with PCR is to sequence from two independent amplifications. This is because the PCR technique does introduce errors and one has therefore to guard against the possibility that sequence differences are in fact PCR errors.

Although inevitably there will be occasions when scientists reach the wrong conclusions despite taking all reasonable precautions, the controls discussed above should be sufficient to minimise this possibility.

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The calcitonin-like sequence of the β CGRP gene

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We have identified a region within the β CGRP gene which has the potential to encode a novel calcitonin-like peptide. The gene is located on the short arm of chromosome 11 (11p 12–14.2) and we suggest that it resulted from a local duplication of the α gene. We have been unable to detect the corresponding mRNA in a variety of tissues which express α-calcitonin. It is not clear whether this sequence can be expressed in man.

Calcitonin gene related peptide *βCGRP gene* *Calcitonin* *Gene duplication*

1. INTRODUCTION

Study of calcitonin gene expression in the rat revealed that two different mRNA species can be produced from the same primary transcript, one encoding calcitonin (CT) and the other calcitonin gene related peptide (CGRP) [1]. The 2 mature mRNAs share a region at their 5′-end (exons 1–3); exon 4 is calcitonin-specific and exons 5 and 6 are CGRP-specific. Similar findings were soon reported for the human calcitonin gene [2,3]. Recently a second gene generating an mRNA related to CGRP has been identified in both rat and man [4,5]. This is known as the β CGRP gene – the organisation of its mRNA appears to be similar to that of α CGRP [4]. While no mRNA related to calcitonin has yet been identified from this gene, evidence both for and against the existence of calcitonin related areas within the β gene has been published [4,6]. The possibility of a second calcitonin arising from the β gene seemed particularly interesting in view of reports that a salmon calcitonin-like peptide can be detected in normal individuals as well as in patients with small cell carcinoma of the lung [7,8].

We report here the isolation of a genomic clone containing most of the β CT/CGRP gene. We have performed in situ hybridisation studies with regions lacking strong homology with the α gene and have shown that it is located close to the α gene on the short arm of chromosome 11 (11p 12–14.2). We have detected an area of the β gene which is related to the CT-specific exon of the α gene. Despite a 67% homology with exon 4 of the α gene it does not appear to code for a protein similar to the calcitonin precursor and we have been unable to detect any mRNA containing this sequence. The CT-like region of the β gene does however contain an open reading frame of 65 amino acids. Although this is probably not translated it does have considerable homology with the mammalian calcitonins. Of particular note is the finding that this potential peptide has features characteristic of both the bovine and human type of calcitonin.

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2. MATERIALS AND METHODS

A human genomic library was constructed in λ EMBL-4 using 15–20 kb *Sau*3A fragments. The library was screened with a probe derived from phTB58 [9] containing exons 5 and 6 of the α gene. The chain-termination method of Sanger et al. [10] was used for sequencing using ^{35}S-labelled dATP. Both strands were sequenced in multiple determinations. Plaque screening and DNA transfers were done on nitrocellulose filters hybridising in 50% formamide, 5 × SSPE (1 × SSPE: 180 mM NaCl, 10 mM Na phosphate buffer, pH 7.7, 1 mM EDTA), 0.1% dried milk, 0.2% SDS and 10% dextran sulphate at 42°C. DNA probes were nick-translated to a specific activity of 10^8–10^9 dpm/μg DNA using [^{32}P]dCTP. Filters were washed in 0.1 × SSPE/0.2% SDS at 65°C. For non-stringent hybridisation the hybridisation temperature was reduced to 35°C and the washing was done in 2 × SSPE at 50°C. mRNA was prepared using guanidinium/CsCl [11] and poly(U)-Sepharose columns. RNAs were electrophoresed on formaldehyde/agarose gels, transferred to Pall filters, and hybridised as for DNA. To reprobe, the filter was washed at 80°C for 15 min in 2 mM Tris-HCl, pH 8.0, 2 mM EDTA with 0.5% SDS.

In situ hybridisation: female metaphase chromosomes were prepared from peripheral lymphocytes [12] and hybridised with ^{125}I-labelled probes [13]. Metaphases were banded according to Zabel et al. [14], photographed and analysed.

3. RESULTS

A library of human genomic clones was constructed and screened with a probe representing human α CGRP. Three clones were identified which did not correspond with the known restriction map of the α CT/CGRP gene and one of these, MGL23, was selected for detailed analysis. Areas hybridising strongly with exons 3 and 5 of the α gene [6,9] were detected in this clone. We have now determined the nucleotide sequence of its CGRP coding region (not shown) and have confirmed that it is indeed β CGRP. Restriction fragments lacking strong homology with the α gene were labelled with ^{125}I by nick-translation and hybridised to human metaphase chromosomes. Specific hybridisation was found to the short arm of chromosome 11 in the region 11p 12–14.2 (fig.1). This agrees with the findings of Hoppener et al. [15] (11 q1–p ter) and corresponds with the position of the α CT/CGRP gene [16] (11 p13–15) to within the limits of resolution attainable with a ^{125}I-labelled probe.

Under stringent hybridisation conditions restriction fragments were identified within the genomic clone that gave a weak signal with probes specific for the calcitonin coding exon of the α gene. Non-stringent hybridisation conditions were therefore used subsequently and strong signals were obtained. The nucleotide sequence of this region was determined and compared with the corresponding sequence of the α gene (fig.2). The overall

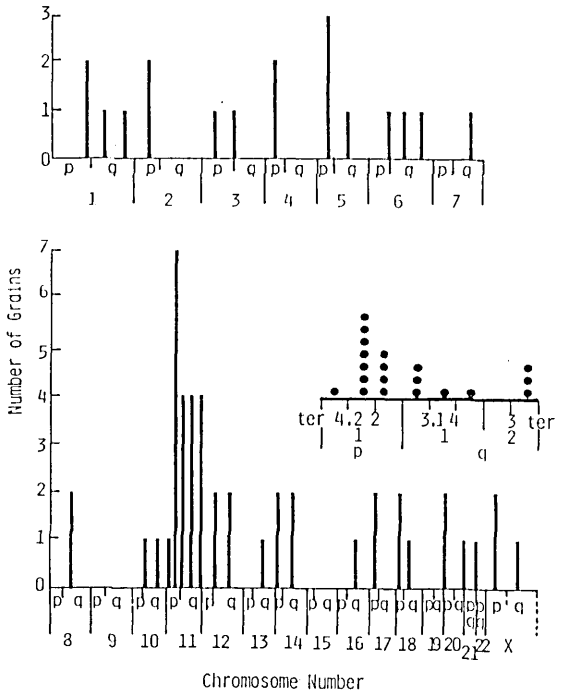

Fig.1. Localization of the β CGRP gene. 50 metaphases were analysed. The average grain count was 1.4 per metaphase and 28% of grains were localised on chromosome 11. The main histogram shows the grain count in the major regions of each chromosome. Inset: grain distribution along chromosome 11. Chromosome 11 has been divided into 8 regions. On the p arm, cent–p12, p12–p14.2 and p14.2–p ter; on the q arm, cent–q13.1, q13.1–q14, q14–q20, q20–q23 and q23–q ter.

```
   CCATGGGGACAGTCCCTAGTGCATGGTACTGTCTGGCATGTCC TTCCCTTGCAG |
                                          ** ****   ******  *** |
                                         GGTATGTGTTTTCCCTC CAG |

CTTGAGCAGTCCTAGATTTAAGTAGCATATAGTAATCTGAGTACCTGCTTGCAGGGCACA
* **   *** ** **** ****  *      ************ *** *** *******
CCTGGACAGCCCCAGATCTAAGCGGTGCG  GTAATCTGAGTACTTGCATGCTGGGCACA
                    ^^^^^^

TACTTGCAGTACCTGAAAAACTTTCATATGTTCCCTGGCATCAACTTCGGGCCTGAAATT
***  **** ** * ** ** ***** * ******    *         **** ** *
TACACGCAGGACTTCAACAAGTTTCACACGTTCCCCCAAACTGCAATTGGGGTTGGAGCA

CCTGGCAAGAATAGGGACATAGTCAACAGC   TTGCAGAGGGACCACTACCCG ACTCC
***** ***** ***** ** ***  ****   *** **** *****   ***  *
CCTGGAAAGAAAAGGGATAT GTC  CAGCGACTTGGAGAGAGACCATCGCCCTCATGTT
      ^^^^^^

AGGGTCCCCCAGA TGGCAGCTG AACTTCTCTCAACTCT CCTGATTCCCCTTCTTGC
**  * ******* ** ** **  **** *** *    ** *** *** **********
AGCATGCCCCAGAATGCCAACT AAACTCCTCCCTTTCCTTCCTAATTTCCCTTCTTGCA
                       + ++

TCCACTTTAT GAACCTGATGCATGTGGATT    CCTCTCTGATTTGTCTTCATGCTGG
***  **  *  *** ************ **    ******** *   ****   *****
TCC  TTCCTATAACTTGATGCATGTGGTTTGGTTCCTCTCTGGTGGCTCTTTGGGCTGG

TATTGGTATTTTTGCTTATGACAGAGAATGTTTTGAAGACCTCAGGATGGAAGGGAAGAC
*******   *** *** ** ***** **** *  ** ** *** ***** *** ****
TATTGGTGGCTTTCCTTGTGGCAGAGGATGTCTC AA ACTTCAAGATGGGAGGAAAGAG
    T
AGCAGGACTTACTGAACACGTT   AGAGA                TAAAAGAAAATAAGGG
*********       *** ***   *****                **  ******* ****
AGCAGGACTC      ACAGGTTGGAAGAGAATCACCTGGGAAAATACCAGAAAATGAGGG

AAGCTTCTTGAGACTGT AGAGGGTGTTATGACAGAGGCATCCAATTT CTGCTTCTAAAT
  ***  ***** *    ****  *** **  *****   ***  * * ******** ***
CCGCT  TTGAGTCCCCCAGAGA TGTCAT  CAGAGC  TCCTCTGTCCTGCTTCTGAAT

GTACTACGATAAAATAAGCACGTCCTTAATGCCTTCGGATTAGATGAATCAT CTATTTTT
** **  ***                                *** ** **** *   *******
GTGCT  GAT                              CATTTGAGGAATAAAATTATTTTT
                                                 ------

CTAA | AAGGA CTGAGCTGCGGTGCTCATTGCTCTGGTAC = BETA
*    | ** ** ******** **** ********** *
CCCC | AAAGATCTGAGCTGTGGTGGTCATTGCTCTGATCT = ALPHA
```

Fig.2. The β gene calcitonin-like region. The nucleotide sequence of exon 4 of the α gene [6,9] is shown aligned with the corresponding region of the β gene. Important features are indicated as follows: (|) limits of exon 4; (^^^) codons for paired dibasic amino acids bounding calcitonin sequence; (+++) termination triplet of α gene; (---) poly(A) addition sequence; (*) common residues. Residue 407 in the β gene sequence is uncertain, giving clear but conflicting data when the 2 strands were sequenced.

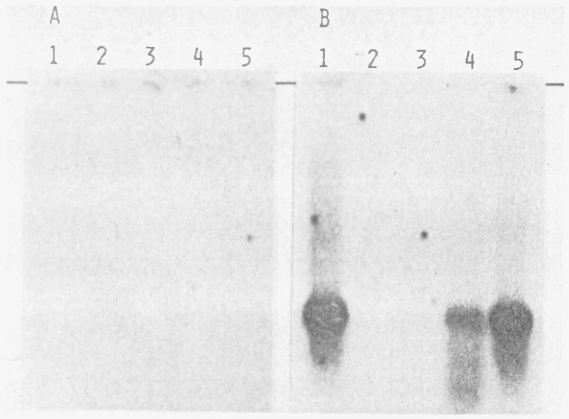

Fig.3. Northern blot analysis of human mRNA samples. (A) Probing with β gene sequence; (B) probing the same filter with α-calcitonin probe. Tracks: 1, 3 μg BEN cell mRNA (bronchial carcinoma [21]); 2, 3 μg HL60 mRNA (leukaemia cell line); 3, 3 μg phaeochromocytoma mRNA; 4, 0.5 μg sporadic type medullary thyroid carcinoma; 5, 0.5 μg familial type medullary thyroid carcinoma.

homology is 67.6%. The calcitonin coding sequence has not been conserved to a greater degree than other regions and in fact, over its first 200 bases, the 3′-non-coding region shows slightly more homology with its β counterpart.

As the β gene sequence contains an open reading frame of 65 amino acids (see below) the possibility that it might be expressed could not be ignored. A Northern blot was performed with mRNA from various tissues where expression might be expected. No signal was detected on the filter after 3 weeks exposure (fig.3A). The same filter was then reprobed with an α-calcitonin-specific probe (fig.3B). Calcitonin message was detected after 2 days exposure.

4. DISCUSSION

We have presented the structure of the calcitonin-like region of the gene encoding β CGRP. The principal interest in this region lies in the possibility that it encodes a previously unidentified regulatory peptide. Our attempts to detect an mRNA representing this region were unsuccessful (fig.3) but this must be treated with caution since we cannot know where or at what level such an mRNA might be expressed.

If this sequence is to be expressed in the manner of the calcitonin coding exon of the α gene then it would be excised from a much longer transcript. It is known that the β gene has a high degree of homology with exons 3 and 5 of the α gene [4] and that these are spliced together to produce β CGRP. The splice donor signals at the 3′-end of exon 3 are therefore functional and if suitable signals exist around the exon 4-like sequence then an alternate mRNA could be produced. The consensus sequence preceding a splice acceptor site is $(Y)_{11}$NYAG (Y = pyrimidine, N = any base) [17] and sequences conforming precisely to this paradigm are found before both α and β gene sequences in fig.2. The other likely requirement for the production of a message would be the conservation of sequences involved in the formation of the polyadenylated 3′-terminus. Such sequences are believed to comprise both the region preceding the polyadenylation site (including the hexanucleotide AATAAA) and sequences beyond this point [18]. The former is a region of strikingly low homology between α and β genes whilst the latter shows 87% homology. It seems unlikely that normal processing can occur at this site but the possibility cannot be ruled out.

If a calcitonin-like mRNA were somehow produced then the reading frame of the protein would be defined by the 3/4 splice and, since β CGRP would share the same splice donor site in exon 3, we can deduce the reading frame from that of β CGRP [4]. This reading frame terminates after only 8 amino acids. There is an open reading frame of 65 amino acids which starts at position 76 of fig.2. We have considered the possibility of its expression from an alternative splice acceptor site but there is no other suitable sequence. It therefore seems unlikely that this sequence is expressed in man. However, it is possible that the predicted peptide or a close homologue would have useful biological properties. A stretch of 33 amino acids shows homology with the calcitonins though this region is not flanked by paired dibasic amino acids as would be expected if it were to be cleaved from a larger precursor protein.

Three types of calcitonin (salmon, bovine and human) have been described [19,20] and a comparison with the β gene peptide is presented in

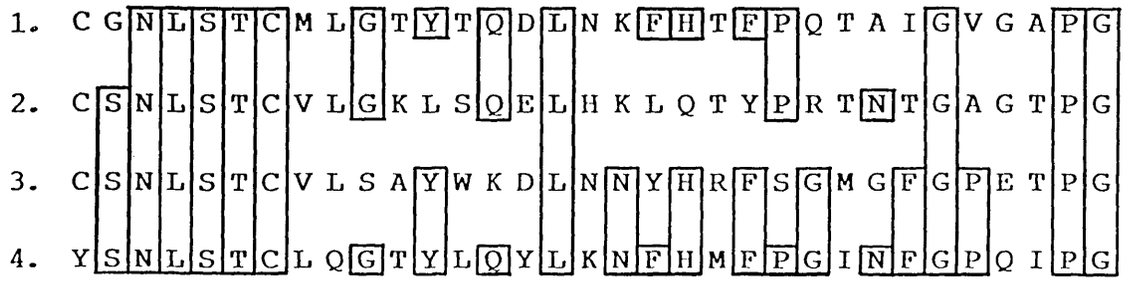

Fig.4. Comparison of β gene peptide with calcitonins. The predicted β gene peptide (4) is compared with the 3 known types of calcitonin sequence. Consensus human type (1), salmon type (2) and bovine type (3) calcitonin sequences were compiled from published sources [19,20], selecting the most abundant residue within each group. Where 2 amino acids were equally frequent, the one found in other types was chosen and in the one case where this did not permit assignment, the human amino acid was chosen rather than the rat.

fig.4. The β gene peptide has specific features in common with both mammalian-type sequences and might be considered as a separate type. The homology of the exon 4-like DNA with the α gene is roughly constant at 67% throughout its length (fig.1). This is comparable with the CGRP 3′-non-coding sequences (65% homologous) and in marked contrast to the CGRP coding sequences which are 92% homologous [4]. We suggest therefore that the β gene may have arisen from a gene duplication on the short arm of chromosome 11. The exon 4-like region of the new gene then lost its potential to code for a calcitonin. Whether the β gene is now committed only to CGRP production or has some other alternative product is a question whose answer must await complete sequencing of the gene.

ACKNOWLEDGEMENTS

We thank Dr R. Craig for providing the cDNA clones pHT B3 and pHT B58 from which our probes were derived. M.A. is in receipt of The Edmund Davis Scholarship in Medicine of The University of London; A.S. was a Sankyo Training Fellow. This work was supported in part by The Armand Bartos Foundation.

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The Calcium-Regulating Hormones: Advances in Cell and Molecular Biology

29 REGULATION OF OSTEOCLASTIC BONE RESORPTION.

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Resident bone cells form a communicating network of osteocytes, surface osteocytes and osteoblasts, that seem well placed to monitor the structure and performance of bone, and to judge where bone formation or resorption is appropriate. These cells probably initiate bone resorption, in response to local and systemic influences, by secreting neutral proteases, which remove the unmineralised organic material that lines bone surfaces. This exposes mineral to osteoclastic contact: mineral contact induces resorptive behaviour in osteoclasts, which proceed to resorb both the organic and mineral phases of bone. Resident cells can then increase the rate of osteoclastic resorption by releasing a factor that we have found is secreted by osteoblastic cells in response to agents that stimulate resorption, that directly stimulates osteoclastic bone resorption. This factor may also be chemotactic, and may stimulate precursor recruitment and differentiation. Some of the agents that induce factor-production are clearly systemic hormones; others may be the means through which non-osseous cells influence bone, or may be the agents through which resident bone cells (such as osteocytes) influence other resident bone cells (such as surface osteocytes); others may have a role only in pathology. Osteoclastic resorption may be terminated by prostaglandins of the E group and PGI_2, which are known to be produced by osteoblastic cells, and are inhibitory to osteoclasts at very low (nanomolar) concentrations.

30 THE MOLECULAR BIOLOGY OF THE CALCITONIN GENES.

M. Alevizaki, I MacIntyre & S. Legon. Dept of Chemical Pathology, Royal Postgraduate Medical School, Ducane Rd, London W12 OHS.

The application of molecular biology techniques to the study of calcitonin (CT) has led to the identification of a second regulatory peptide encoded by the CT gene. The CT gene is located on the short arm of chromosome 11. As a result of alternative processing of the primary transcript, two distinct mRNAs can be produced. These share a common region at their 5' ends encoding identical N-terminal peptides. One of the mRNAs codes for the CT precursor and is the main product of the gene in the thyroid ; the other codes for a previously unidentified neuropeptide, CGRP (CT gene related peptide) which is the main product in neural tissue. The precise mechanism by which this differential expression is achieved has not been fully elucidated yet.

Recently a related gene referred to as the beta CGRP gene was described which is also located on chromosome 11. Using in situ hybridisation we found that it is located on the short arm of this chromosome close to the alpha gene. Although original observations suggested that this gene contained only CGRP related sequences we have recently identified a CT-related sequence as well which could code for a CT-like peptide. Existing data show that this beta CT-like sequence is not expressed in man; however the predicted peptide could nonetheless have useful biological properties.

These two genes do not seem to be the only CT-related areas in the human genome. We have recently isolated a human genomic clone which has a region with very close homology to the amino terminal peptide coding region of the known genes. We are currently investigating the possibility that a third CT-like peptide may be encoded by this genomic sequence.

Calcified Tissue

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OP9

A THIRD CALCITONIN RELATED SEQUENCE IN THE HUMAN GENOME. M. Alevizaki, F.V. Rassool, K.L.S. Collyear, I. MacIntyre & S. Legon. Dept of Chemical Pathology, Royal Postgraduate Medical School; London, England.

The study of the calcitonin (CT) gene in both rat and man revealed the existence of a previously unidentified alternative peptide product of the gene, CT gene related peptide (CGRP). Further studies showed that there was a second gene, the beta gene, encoding another CGRP-like molecule. We have shown that the beta CGRP gene also contains a CT-related sequence diverging by 35% from the alpha gene. Although this has the potential to code for a CT-like peptide, it is probably not expressed in man.

However these two genes are not the only CT-related genes in the human genome. We have recently isolated a third (gamma) human genomic sequence with a very close homology (90%) with exons II and III which code for the amino-terminal peptide that CT and CGRP share within their precursors. As detection of a salmon CT-like molecule has been repeatedly reported in man, the possibility that this is encoded by this genomic locus has been investigated. Two overlapping genomic clones spanning an area of 20 kb around this sequence were anlysed using modified hybridisation conditions which should allow cross-hybridisation between two sequences whose encoded peptides differ by about 50%. No CT or CGRP related sequences were detected in our clones. Furthermore, sequence analysis of the amino-terminal peptide related region indicates that this has probably been derived from the beta gene as the whole of exon 2- intron 2-exon 3 region is conserved to the same degree. Using in situ hybridisation we have localised this sequence to the short arm of chromosome 11. These data suggest that it is probably the result of a relatively recent duplication.

We suggest that if a salmon-like CT gene exists in the human genome at all, it is very unlikely that it is associated with the gamma sequence.

OP10

CALCIUM ADMINISTRATION PROVOKES TRANSCRIPTIONAL AND POST-TRANSCRIPTIONAL MODIFICATIONS OF CALCITONIN mRNA IN THE RAT.

Marie-Christine Delehaye, Nadine Segond, Jacqueline Taboulet, Gérard Milhaud, Mohsen S. Moukhtar and Annick Jullienne.
INSERM U113 and CNRS UA 163, Laboratoire d'Endocrinologie Moléculaire, CHU St. Antoine, 27 Rue Chaligny, 75571 Paris cedex 12, FRANCE

Calcium, administered in rats, stimulates the secretion of calcitonin (CT). We have previously shown that calcium elicits a rapid increase in translatable calcitonin mRNA. In this work, plasma and thyroid glands were obtained at 2, 30, 60, 120 and 240 minutes after Ca injection and CT levels estimated by a specific radioimmunoassay for the hormone. Poly A rich RNA were extracted from thyroids and purified by oligo dT cellulose, and CT mRNA quantified by its ability to direct the synthesis of calcitonin (CT) precursors in a cell free system and by hybridization to a ^{32}P cDNA probe specific for CTmRNA. We report here that the levels of hybridizable CT mRNA remained constant up to 30 minutes and then increased from 1 to 4 hours. Translational activity of CT mRNA varied during the experiment: a sharp and transient rise, as previously reported, occured at 2 minutes and a second increase after 30 minutes. CT plasma levels reached peak values at 2 minutes and then declined rapidly, reaching basal levels at four hours. CT tissue contents decreased significantly, two minutes after the injection, normal levels were recovered by 30 minutes and remained unchanged to 4 hours. This suggests that the actions of Ca ions are complex, as the ion exerts a dual action on calcitonin mRNA levels in the thyroid gland: an early post-transcriptional rise only in translatable calcitonin mRNA, presumably by the activation of inert mRNA pools and a late transcriptional increase in both translatable and total poly A^+ calcitonin RNA. This action results in the biosynthesis of new hormone and thus to a restoration of tissue levels of calcitonin. Subsequently the pools of calcitonin mRNA are restored by an increase in the transcription rate.

OP11

MOLECULAR CLONING, EXPRESSION, AND APPLICATION OF cDNA ENCODING THE VITAMIN D RECEPTOR. J. Wesley Pike. Baylor College of Medicine, Houston, Texas USA.

Vitamin D receptors (VDR) are intracellular transcription factors that mediate the hormonal action of 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$). The recent molecular cloning of these proteins from chicken, rat, and human sources has revealed a common structural architecture comprised of two distinct domains whose primary sequences are virtually conserved from species to species, and which show a striking degree of homology with similar domains found within other members of the steroid receptor gene family. Two cDNAs representing the entire coding region of human VDR were ligated at a common restriction site and cloned into an adenovirus major late promoter-driven mammalian expression vector (p91023b). Acute transfection of this construct into COS-1 cells resulted in high expression (250,000 copies/cell-average) of a recombinant protein displaying wildtype characteristics of molecular mass 52,000, an equilibrium dissociation constant (K_d) for $1,25(OH)_2D_3$ of $5x10^{-11}M$, and ability to interact with immobilized DNA. Expression and assay of a series of 3' VDR cDNA deletion mutations reveal that DNA binding activity resides in N-terminal domain I and steroid-binding is associated with C-terminal domain II--the functional characteristics of these domains correspond to similar regions found in other steroid receptors. Several human vitamin D-regulated target genes are currently being developed in which to test and localize the transcriptional activity of recombinant VDR. VDR cDNA probes are presently being exploited to define the structural organization of the natural human VDR gene and to identify its unique promoter elements. Simultaneously, these probes are being utilized to evaluate the genetic basis for the resistance syndrome vitamin D-dependent rickets, type 2. Both physical and functional evidence has accumulated to implicate aberrant VDR genes as the underlying cause of this disease. Further application of VDR gene probes should prove essential in defining the physiology and pathophysiology of vitamin D action.

OP12

STRUCTURAL ANALYSIS OF THE GENE FOR RAT VITAMIN D-DEPENDENT CALCIUM-BINDING PROTEIN (9K CaBP).

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The structural organization of the entire rat 9KCaBP (chocalecalcin or calbindin) gene was determined by analysis of overlapping genomic clones isolated from a rat genomic library using the rat 9KCaBP cDNA (Desplan et al J. Biol. Chem. 258, 13502, 1983). These clones together span 30 kbp of rat genomic DNA with the rat 9KCaBP gene lying in the middle. The 9KCaBP gene is 2.5 kbp long and contains 3 exons interrupted by 2 introns. The first exon contains almost the entire 5' untranslated region. The second exon, which is separated from the first by one intron of 306 bp, contains the end of the 5' untranslated region and codes for the calcium-binding site I. The third exon codes for the calcium binding site II and the 3' untranslated region. A 1806 bp intron separates the two last exons. Therefore each of the calcium-binding domains is encoced by single, separate exons. The transcription initiation site was identified by SI nuclease mapping and primer extension. A consensus sequence TATAAA was localized 31 bp upstream from the cap site and a "CAAT-box" (CAAAG) lies 72 bp upstream from the transcription start. One $(AC)_{25}$ and one $(AG)_{23}$ repeats are present in the second intron together with an Alu-like sequence. Repetitive elements are present 5 kbp upstream from the cap site and in the 3' flanking region. Comparison of the known rat CaBP sequences (9KCaBP, 28KCaBP, S_{100} protein) shows that the 9KCaBP is more closely related to the S_{100} protein than to the 28KCaBP. There is no evidence to indicate that 9KCaBP has arisen from the 28KCaBP.

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Altered calcitonin gene in a young patient with osteoporosis

M Alevizaki, J C Stevenson, S I Girgis, I MacIntyre, S Legon

Abstract

To assess whether calcitonin is important in maintaining the integrity of bone the calcitonin gene of a young male patient with osteoporosis and no detectable plasma concentrations of calcitonin was studied. Genomic Southern blots with various restriction enzymes showed no large abnormalities in his calcitonin gene. Genomic clones representing his calcitonin gene were then analysed. His gene encoded normal precursor polypeptides for calcitonin and calcitonin gene related peptide; the only abnormality identified was a single base insertion in the intron separating exons IV and V of the gene. The affected sequence is homologous with an intron sequence from β globin that is implicated in splicing and forming a crucial intermediate structure during the maturation of messenger RNA.

The change observed may be responsible for the patient's calcitonin deficiency and consequently for his condition, suggesting that calcitonin is important in preventing bone loss.

Introduction

Whether calcitonin has an important role in maintaining skeletal integrity is not known.[1,2] One finding supporting such a role was the identification of a young male patient with severe osteoporosis who was completely deficient in calcitonin and has responded well to calcitonin replacement treatment over the past nine years.[3] We therefore considered the possibility that his calcitonin gene might be defective. As the C cells of the thyroid are not accessible for direct analysis of calcitonin gene expression we instead carried out a detailed structural study of the gene looking for any changes that might explain this failure to produce calcitonin.

Materials and methods

DNA was extracted from the patient's peripheral lymphocytes[4] and after restriction enzyme digestion was analysed on Southern blots probed with labelled restriction fragments specific for either calcitonin or calcitonin gene related peptide. Probe fragments were derived from the clones phTB3[5] and phTB58[6] and contained only exon IV or exon V and VI sequences from the human α calcitonin-calcitonin gene related peptide gene (see fig 1). DNAs were labelled with phosphorus-32 by random primed synthesis[7] to 10^9 disintegrations per minute/μg and blots were hybridised and washed under stringent conditions as previously.[8] Genomic DNA was cloned into the Bam H1 site of phage lambda EMBL4 and two independent clones were identified with a probe specific for calcitonin. Suitable regions were subcloned into M13 vectors and sequences were determined with the chain termination method. In all cases both strands were sequenced.

Results and discussion

THE CALCITONIN GENE

The calcitonin gene is complex and many details about how its expression is regulated remain unclear. Calcitonin is produced by proteolytic cleavage of a pre-prohormone, which also includes N and C terminal flanking peptides and a short signal peptide (fig 1). In the calcitonin gene the sequences representing calcitonin messenger RNA are found in four blocks (exons): exon I contains non-coding sequences from the beginning of the messenger RNA, exon II encodes the signal peptide, exon III encodes the N terminal flanking peptide, and exon IV encodes calcitonin itself, the C terminal flanking peptide, and the non-coding sequences from the end of the messenger RNA. When calcitonin messenger RNA is being made the synthesis of RNA starts at exon I and continues through the entire gene, ending several thousand bases beyond the end of exon IV. This primary transcript is then cut and reassembled in a complex series of splicing reactions, which result in the formation of the mature messenger RNA, which includes only the sequences of the first four exons (fig 1).

This type of organisation is common to almost all human genes. The calcitonin gene is unusual in that it can give rise to a second messenger RNA encoding another regulatory peptide known as calcitonin gene related peptide.[9] When this peptide is produced the primary transcript undergoes an alternative series of splicing reactions, which result in the assembly of a messenger RNA comprising exons I, II, and III joined to two further exons, V and VI (fig 1). Exon V encodes calcitonin gene related peptide and its C terminal flanking peptide, and exon VI is non-coding.

A second calcitonin gene related peptide exists in the human genome: β calcitonin gene related peptide is encoded by a related gene in which the equivalent of the calcitonin coding exon is no longer functional.[8] Plasma calcitonin gene related peptide concentrations in our patient were normal, and he showed no symptoms that might suggest loss of this peptide, which is believed to have an important role in maintaining vascular tone. Although our immunoassay cannot distinguish between the two forms of calcitonin gene related peptide, both are probably present because differing distributions in tissue suggest complementary rather than duplicated activities. In considering the type of changes that might cause our patient's condition, changes that would prevent the correct expression of calcitonin while leaving the

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Br Med J 1989;298:1215-6.

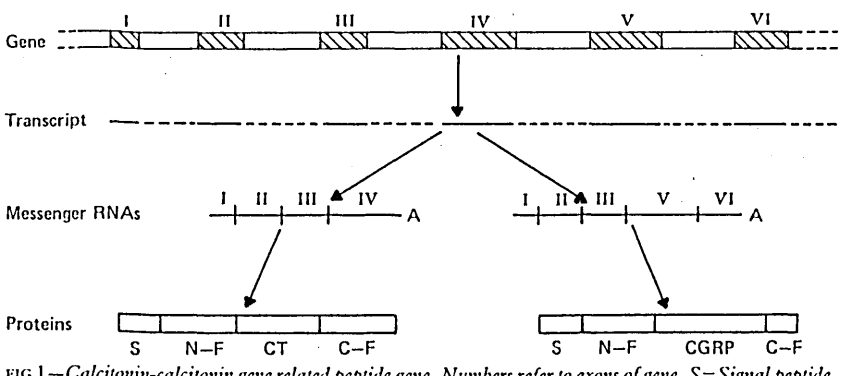

FIG 1—*Calcitonin-calcitonin gene related peptide gene. Numbers refer to exons of gene. S=Signal peptide, N-F=amino terminal flanking peptide, CT=calcitonin, C-F=carboxy terminal flanking peptide, and CGRP=calcitonin gene related peptide*

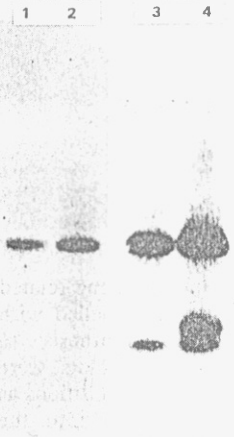

FIG 2—*Southern blot analysis of calcitonin-calcitonin gene related peptide gene. Samples of DNA from patient (lanes 1 and 3) and control subject (lanes 2 and 4) were digested with restriction enzyme Taq I and analysed by Southern blotting, probing the filters with probe specific for calcitonin (1 and 2) or calcitonin gene related peptide (3 and 4). Lower band in track 4 is doublet. Control subject has a previously unreported polymorphism in this part of gene*

expression of calcitonin gene related peptide largely unaffected seem more likely. The simplest such change would be an alteration to the DNA sequence of exon IV.

DNA SEQUENCE OF CALCITONIN GENE

As a first step in the analysis of our patient's calcitonin-calcitonin gene related peptide gene we looked for large structural changes in the region of exon IV by using the technique of Southern blotting (fig 2). With this procedure changes can be seen in the lengths of restriction fragments of the DNA that may result from insertion or deletion of sequences in the region being analysed. The method provides a rapid analysis but is limited in that changes of fewer than 20 bases are not detectable. No differences, quantitative or qualitative, were seen between our patient's calcitonin gene and that of controls. For a more detailed analysis we isolated the calcitonin-calcitonin gene related peptide gene from DNA prepared from our patient's peripheral lymphocytes. The entire DNA sequence encoding pre-procalcitonin was determined and corresponded exactly with the published gene sequence. Further analysis showed that the parts of the gene specific for calcitonin gene related peptide (exons V and VI) were also entirely normal.

We then focused on the regions important for the differential processing of messenger RNAs for calcitonin and calcitonin gene related peptide. During the process of splicing the precursor RNA is cleaved after one exon, and the intervening sequence (intron) then forms a looped structure before cleavage at the second exon, joining of the two exon sequences, and elimination of the intervening sequence (fig 3).

FIG 3—*Formation of looped structure during maturation of messenger RNA precursors. First step in eliminating intervening sequence is cleavage after first exon and looping back to branch point*

The important regions are those at the ends of the intervening sequence and at the branch point where the looped structure forms.[10] In the particular case of the calcitonin-calcitonin gene related peptide gene cleavage of the precursor RNA at the end of the calcitonin exon (IV) is important in preventing the splice that leads to production of calcitonin gene related peptide[11] and further evidence suggests that the secondary structure of the precursor RNA is important in allowing its interaction with exon specific splicing factors.[12]

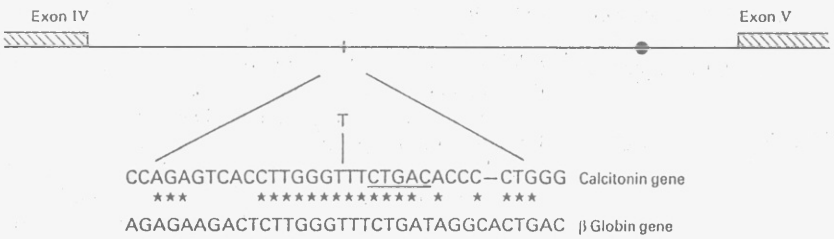

FIG 4—*Altered calcitonin gene sequence from patient with osteoporosis. Sequence from intervening sequence between exons IV and V shows single base insertion, which is next to consensus sequence for formation of branch point (CTGAC (underlined)) and has homology with more extensive sequence from branch point in the β globin gene. Asterisks show identical residues in calcitonin and β globin genes*

When our patient's DNA was sequenced no changes were found in these critical regions. The only difference between our sequence and the normal sequence was a single base insertion at position 462 in the intervening sequence between exons IV and V (fig 4). Such a finding was not unexpected as non-coding sequences such as these commonly show differences (neutral polymorphisms) between individual people and these differences have no effect on the expression of the neighbouring genes. The extra base is, however, next to a CTGAC sequence, which is the consensus sequence for formation of a branch point during splicing.[10] We searched the GENBANK database of DNA sequences for similar sequences. Out of more than 10 million bases in the database, the best fit was with a short sequence from the β globin gene, which has been shown to become resistant to ribonuclease during in vitro splicing and is the site for formation of a branch point (fig 4).[13]

This homology indicates that the altered sequence in our patient may participate in splicing reactions. It is, however, unlikely to be the primary branch point site in this intervening sequence as these are generally much closer to the following exon sequence. Further studies with the gene in an in vitro expression system would be necessary to determine how any effect is produced. One possibility is that the altered sequence takes on the properties of a branch point, forcing an abortive splice which precludes the production of calcitonin.

Establishing a role for calcitonin in the aetiology of this patient's osteoporosis is of considerable importance. Although his condition is undoubtedly rare, the results suggest that calcitonin plays an important part in maintaining skeletal integrity, strengthening arguments in favour of its use in treating osteoporosis in general.

We thank the Hammersmith and Queen Charlotte's Special Health Authority for its support of this work.

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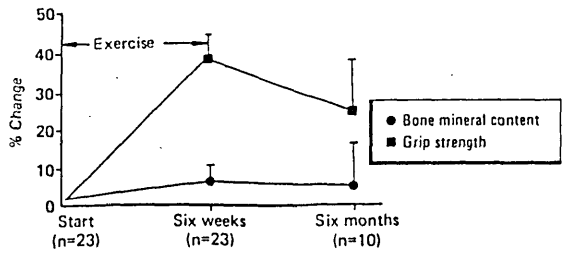

FIG 4—*Percentage changes in grip strength and bone mineral content in the exercised arm for women who had a gain in grip strength of >50 mm Hg at six weeks. Bars are 95% confidence intervals*

content (13·3%, p<0·02; 2·6 to 24·0%) in their uninjured arm. Comparison of both forearms at six months showed that the exercised arm had 29·1% (p<0·004, 11·5 to 46·7%) greater grip strength and 4·9% (p<0·14, −1·9 to 11·7%) higher bone mineral content than the arm that had been injured. The best exercisers (n=10) conserved some of their increases in strength and bone density at six months, but neither was significant.

The results at six weeks for the 33 women who were seen at six months showed a 20·6% (p<0·0001, 13·5 to 27·7%) improvement in grip and a 3·4% (p<0·036, 0·2 to 6·5%) improvement in bone mineral content in the exercised arm; their initial grip strength was 261 mm Hg (231 to 290 mm Hg) and bone mineral content 36·9 units (33·9 to 39·9 units). The initial grip strength in the exercised arm of the 66 women who did not reach the end of the study was 275 mm Hg (249 to 301 mm Hg) and their initial bone mineral content 33·9 units (31·4 to 36·4 units). Those who did not attend at six months were older (mean age 60·5 years) than those who did (mean age 56·8 years).

Discussion

As expected, our results showed that both grip strength and bone mineral content in the forearm fall with age, but we also found a close correlation between the two variables irrespective of age. In addition, the small but significant initial difference in both variables between the dominant and non-dominant arms of the 69 volunteers supports our hypothesis that a forearm's bone mineral content is related closely to the physical demands made on it by activities that require grip strength. We had assumed that a grip of maximum power stresses the skeleton of the forearm as much as any other activity that is performed commonly. We had not expected the change in grip strength in the unexercised arm, which was associated with a non-significant increase in bone mineral content. There was, however, insufficient emphasis in our instructions to prevent volunteers exercising both forearms, and several admitted to doing this.

We had also assumed that after a Colles' fracture the uninjured forearm is used more. This may be wrong: a manual worker might avoid physical stress when off work, whereas an independent retired person might be obliged to undertake more stressful activity with the intact arm. Women with fractures achieved a significant increase in bone mineral density by three weeks. Several of those with minor fractures were out of plaster before six weeks and were unlikely to attend hospital again, which may account for the non-linear results over three to six weeks. Similarly, frail volunteers were less likely to attend follow up clinics, and it was not feasible to retest some of those in old people's homes at six months. Those who attended six months after the exercise regimen ended were therefore likely to be the more motivated women who had originally done well with the exercises.

As numbers of women fell with succeeding visits the mean strength and bone mineral content rose. Measurements at six months showed a loss of the previous gains in grip strength and bone mineral content in all of the women except those who had had a fracture. It may be that after a fracture patients with apparently normal function continue to protect the injured arm from peak stresses by using the uninjured wrist for activities that require a powerful grip. They may thereby continue to increase both grip strength and bone mineral content in the non-fractured arm.

Changes in grip strength were mirrored by changes in bone mineral content at all stages, and this supports our contention that mineral content reflects quickly the physical demands made on bone. Muscle may be trained for great endurance without bulk by prolonged exercise, as in marathon runners, or for strength and bulk by isometric exercise, as in weight lifters. A similar analogy might apply to bone, in which short periods of skeletal stress may trigger osteogenesis more effectively than hours of gentler exercise.

Several questions remain—for example, Can this principle be applied to the entire skeleton? and What is the minimum daily requirement of skeletal stress? A few seconds of grip exercise each day may stress the forearm skeleton sufficiently to stimulate a local gain in bone mineral content. The best method of regularly stressing the whole skeleton of aging and reluctant women, however, remains to be shown.

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Retraction in the light of new data

Altered calcitonin gene in a young patient with osteoporosis

From M Alevizaki and S Legon: We recently reported the sequence of the calcitonin gene of a young male osteoporotic patient in whom no calcitonin could be detected (M Alevizaki, J C Stevenson, S I Girgis, I MacIntyre, S Legon. *Br Med J* 1989;298:1215-6 (6 May)). We noted a difference of a single base between this sequence and the previously published "normal" sequence in a region which may be important for the maturation of the mRNA, and we speculated that this might have caused his osteoporosis. However, using the polymerase chain reaction to screen normal subjects we now find this sequence to be widespread in the general population. We therefore conclude that this is a neutral polymorphism and is not responsible for the patient's condition. The cause of his calcitonin deficiency thus remains to be established.