PHYSICAL MAPPING OF A 2-Mb REGION CENTERED AT D10S94, A LOCUS VERY TIGHTLY LINKED TO THE MULTIPLE ENDOCRINE NEOPLASIA TYPE 2 GENE(S)

by

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ABSTRACT

The dominantly inherited cancer syndrome multiple endocrine neoplasia type 2A (MEN 2A) is characterized by medullary thyroid carcinoma (MTC), pheochromocytoma and parathyroid hyperplasia. The related syndrome MEN 2B comprises MTC, pheochromocytoma and mucosal neuromas. Genes responsible for these cancers map to the pericentromeric region of chromosome 10, as do loci responsible for dominantly inherited MTC without additional clinical features, and MEN 2A associated with a skin disorder, cutaneous lichen amyloidosis. The relationship between the genes responsible for these diseases is not known.

Nothing is known of the biochemical basis for MEN 2; a positional cloning strategy to identify MEN2 gene(s) is therefore warranted. This thesis makes contributions to several steps of a strategy to identify MEN2.

The first objective was to clone DNA markers more closely linked to MEN2A than the known flanking markers, FNRB and RBP3. Repeat element-mediated polymerase chain reaction (REM-PCR), a method of cloning human DNA fragments from hybrid DNA sources, was developed for this purpose. Primers directed to the 3' ends of human Alu and L1 elements provided the basis for amplification of human-specific DNA fragments; DNA from a somatic cell hybrid highly enriched for the MEN2A region was used as template. This technique yielded thirty-three REM-PCR clones, two of which mapped to intervals near MEN2A. One of these, pCl1/A1S-6-c23, defines a polymorphic locus, D10S94.

Somatic cell hybrid mapping of pCl1/A1S-6-c23, combined with the results of linkage studies conducted by Drs. Nancy Simpson and Paul Goodfellow, served to localize D10S94 to 10q11.2 between the centromere and RBP3. D10S94 is very tightly linked to MEN2A and was not
demonstrated to recombine with the disease locus in these studies. It was used as a starting point for large scale cloning and mapping efforts directed to the identification of genes. Long range restriction mapping by pulsed field gel electrophoresis revealed a dense cluster of CpG islands at D10S94. Genes potentially associated with these CpG islands represent candidate genes for MEN2.

To determine whether relatively large sequence alterations, which could serve to direct gene cloning efforts to a small region, were associated with D10S94, a survey of the 180-kb CpG island-rich region at this locus was undertaken in the genomes of MEN 2A and MEN 2B patients. Long range restriction mapping by field inversion gel electrophoresis demonstrated that no alterations of 3.3 kb or more were present at D10S94 in these patients.

Yeast artificial chromosomes (YACs) identified in collaboration with Drs. Ken Kidd and Jay Lichter, expanded D10S94 and, with genomic long range restriction mapping, established physical linkage to six other DNA markers near MEN2. Recent published reports identify very closely linked flanking markers and refine the minimal region to which MEN2A is localized. The genomic long range restriction map and YAC contig span the entire refined MEN2A candidate region. This map and contig can now serve as a guide and a cloning source, respectively, for experiments designed to identify genes within this region, and an intensive search for potentially disease-causing mutations to identify genes important in the etiology of MEN 2.
CHAPTER I: INTRODUCTION

Medullary thyroid carcinoma occurs in the context of four genetic disorders: MEN 2A, MEN 2B, familial MTC and MEN 2A with cutaneous lichen amyloidosis.

Genes responsible for MEN 2A, MEN 2B and MTC map to the pericentromeric region of chromosome 10.

Recombination is repressed near the centromere of chromosome 10.

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Biologically plausible candidate genes within the MEN 2 region.

A positional cloning strategy for MEN2.

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## ABBREVIATIONS

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<tr>
<td>bp</td>
<td>base pairs</td>
</tr>
<tr>
<td>CEPH</td>
<td>Centre d'Etude du Polymorphisme Humain</td>
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<tr>
<td>CLA</td>
<td>cutaneous lichen amyloidosis</td>
</tr>
<tr>
<td>cM</td>
<td>centiMorgan</td>
</tr>
<tr>
<td>CMGT</td>
<td>chromosome-mediated gene transfer</td>
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<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>FIGE</td>
<td>field inversion gel electrophoresis</td>
</tr>
<tr>
<td>FISH</td>
<td>fluorescence <em>in situ</em> hybridization</td>
</tr>
<tr>
<td>FN RB</td>
<td>the gene encoding the B subunit of the fibronectin receptor</td>
</tr>
<tr>
<td>IPTG</td>
<td>isopropyl-B-D-thiogalactopyranoside</td>
</tr>
<tr>
<td>kb</td>
<td>kilobase pair</td>
</tr>
<tr>
<td>LOH</td>
<td>loss of heterozygosity</td>
</tr>
<tr>
<td>Mb</td>
<td>megabase pair</td>
</tr>
<tr>
<td>MEN 2</td>
<td>multiple endocrine neoplasmia type 2 (includes MEN 2A and MEN 2B)</td>
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<tr>
<td>MEN 2A</td>
<td>multiple endocrine neoplasmia type 2A</td>
</tr>
<tr>
<td>MEN2A</td>
<td>the locus responsible for MEN 2A</td>
</tr>
<tr>
<td>MEN 2A + CLA</td>
<td>MEN 2A with cutaneous lichen amyloidosis</td>
</tr>
<tr>
<td>MEN2A+CLA</td>
<td>the locus responsible for MEN 2A + CLA</td>
</tr>
<tr>
<td>MEN 2B</td>
<td>multiple endocrine neoplasmia type 2B</td>
</tr>
<tr>
<td>MEN2B</td>
<td>the locus responsible for MEN 2B</td>
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<td>MTC</td>
<td>medullary thyroid carcinoma</td>
</tr>
<tr>
<td>MTC 1</td>
<td>chromosome 10-linked, dominantly inherited medullary thyroid carcinoma</td>
</tr>
<tr>
<td>MTC1</td>
<td>the locus responsible for MTC 1</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PFGE</td>
<td>pulsed field gel electrophoresis</td>
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<td>RB</td>
<td>retinoblastoma</td>
</tr>
<tr>
<td>RBP3</td>
<td>the gene encoding the interstitial retinol binding protein</td>
</tr>
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<td>REM-PCR</td>
<td>human repeat element-mediated PCR</td>
</tr>
<tr>
<td>ROM</td>
<td>read-only memory</td>
</tr>
<tr>
<td>RFLP</td>
<td>restriction fragment length polymorphism</td>
</tr>
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<td>STS</td>
<td>sequence tagged site</td>
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<td>YAC</td>
<td>yeast artificial chromosome</td>
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I would first like to thank my graduate supervisor and friend, Dr. Paul Goodfellow, for making these years an exceptional learning and living experience. He has changed my attitude toward science and affected my outlook on life.

I want to thank my committee: Dr. Ross MacGillivray, Dr. Rob McMaster and Dr. Steve Wood for their helpful and encouraging comments both within and outside committee meetings.

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I am indebted to the many researchers and who contributed materials or expertise during the course of my studies: to Drs. Ken Kidd and Jay Lichter (Yale University) for a fruitful YAC collaboration; to Dr. Nancy Simpson (Queen's University), in whose laboratory the linkage analyses for D10S94 were done; to Dr. Charles Jackson (Henry Ford Hospital, Detroit) for providing the MEN 2B cell lines; to Steve Scherer for sharing his YAC techniques and to Dr. Lap-Chee Tsui (University of Toronto) for sharing Steve; to Trushna Desai of Dr. David Ward's laboratory (Yale University) for performing FISH analysis of the YAC clones; and to Bernard Chan of Q-Life Systems for the use of the prototype AutoBase FIGE apparatus. Drs. David Callen (Adelaide), Carol Jones (Denver) and Robert Norum (Detroit) contributed somatic cell hybrids used for regional mapping of repeat element-mediated PCR derived clones.

I am grateful to the Faculty of Medicine and Mr. R.M. Babicki, the Cancer Research Society of Canada, the Medical Research Council of Canada and the Genetic Diseases Network of the Networks of Centres of Excellence Program for financial support during my Ph.D. studies.

I thank my parents and family for their continued encouragement. Most of all, I want to thank Gary for another three and a half years of love and patience and toe-tickling.
FOREWORD

The following publications are based on work described in this thesis:


I wrote this paper, which describes the extension of the technique *Alu* element-mediated PCR to make use of human repeat elements (both *Alu* and LINE elements) to clone human-specific DNA markers. I developed *Alu* element-mediated PCR was developed while a research assistant in the laboratory of Dr. Paul Goodfellow. As a graduate student, I developed repeat element-mediated PCR and used it to clone 18 chromosome 10 DNA markers. Mr. D.E. Smailus assisted in mapping the DNA markers. Dr. H.-U.G. Weier contributed *in situ* hybridization data.


This paper describes the cloning and long range restriction mapping of a very CpG island-rich region at *D10S94*. I performed the long range restriction mapping experiments, interpreted data and wrote this paper. Mr. D.E. Smailus performed a cosmid walk from which probes for long range restriction mapping were obtained.


The mapping of the first single copy DNA marker (*D10S94*) very closely linked to *MEN2A* is described. I cloned this DNA marker by *Alu* element-mediated PCR, identified restriction fragment length polymorphisms and performed somatic cell hybrid mapping experiments that, combined with linkage mapping data generated by co-authors, allowed unambiguous mapping of *D10S94* relative to other chromosome 10 markers near the disease locus.


Dr. Goodfellow wrote these short reports, which describe the identification of polymorphisms at \textit{D10S94} near the MEN 2 gene(s) on chromosome 10. I cloned and mapped the probe and identified the polymorphisms described in the first report and developed the PCR primers described in the second report.

A.R. Brooks-Wilson, J.B. Lichter, D.C. Ward, K.K. Kidd and P.J. Goodfellow. A genomic long range physical map and yeast artificial chromosome contig linking six loci in 10q11.2 near the gene responsible for multiple endocrine neoplasia type 2A (\textit{MEN2A}). Submitted to \textit{Genomics}.

I performed all the long range restriction mapping experiments in both genomic DNA and YACs, and wrote this paper. Coauthors performed a major part of the YAC library screening to isolate YAC clones derived from loci near \textit{MEN2A}. Fluorescence \textit{in situ} analysis of the YAC clones was done in the laboratory of Dr. D.C. Ward.


I am writing this paper based upon the mutation-hunting experiments of chapter V, which were performed entirely by me under the direction of Dr. P. Goodfellow, using patient cell lines contributed by Dr. C.E. Jackson.

Angela R. Wilson

I certify the accuracy of these statements.

Dr. Paul J. Goodfellow,  
(Research supervisor and senior author)
CHAPTER I:

INTRODUCTION

The work described in this thesis forms an integral part of a laboratory-wide strategy to identify genes important in thyroid oncogenesis. Genes responsible for four rare dominantly inherited disorders involving medullary thyroid carcinoma (MTC) map to the pericentromeric region of chromosome 10: familial MTC, multiple endocrine neoplasia types 2A and 2B (MEN 2A and MEN 2B), and MEN 2A with cutaneous lichen amyloidosis. Nothing is known about the biochemical basis of these disorders, so no biochemical clues can be used in the pursuit of the corresponding disease genes. A genetic approach involving positional cloning is therefore being used to clone genes that, when inherited in mutant form, predispose to these disorders involving thyroid cancer.

Positional cloning, formerly referred to as reverse genetics, is a strategy in which knowledge of the chromosomal location of a gene is used in order to identify it. Mutations affecting a candidate gene are ultimately correlated with presence of disease (or gene carrier status) to identify the gene responsible for an inherited disorder. This strategy is now widely applied in the pursuit of human disease genes. It has been used successfully to isolate a number of genes of medical relevance, most notably the genes responsible for cystic fibrosis (Rommens et al., 1989; Riordan et al., 1989; Kerem et al., 1989), Duchenne and Becker muscular dystrophy (Koenig et al., 1987; Koenig et al., 1989), and myotonic dystrophy (Harley et al., 1992; Buxton et al., 1992; Aslanidis et al., 1992). Cloning a human gene is an interesting challenge in itself, rather than just
a physical task; it can provide valuable information about the genomic region in which a gene of interest is located, and information about the genetic nature of the disease.

Inherited cancers like MEN 2, retinoblastoma and familial adenomatous polyposis make up only one to two percent of all cancers (Ponder, 1990), but their importance in aiding the elucidation of the basic mechanisms of carcinogenesis greatly outweighs their relatively low incidence. Investigation of the genetic events underlying familial cancers with straightforward modes of inheritance provides a simplified way to approach a very complex disease: cancer. Identification of mutations in genes responsible for familial cancers can provides clues about the mechanisms of cancer and the genes involved in predisposing us to or protecting us from developing it.

Medullary thyroid carcinoma occurs in the context of four genetic disorders: MEN 2A, MEN 2B, familial MTC and MEN 2A with cutaneous lichen amyloidosis.

Medullary thyroid carcinoma makes up approximately 5% of all thyroid cancer (Bergholm et al., 1989), and occurs in both sporadic and familial forms. Most are sporadic cases; about 25% are familial (Block et al., 1980). MTC can occur alone or in combination with other endocrine tumors as a multiple endocrine neoplasia syndrome.

MEN 2A, also known as Sipple's syndrome (Sipple, 1961), is a dominantly inherited cancer syndrome characterized by medullary thyroid carcinoma, pheochromocytoma (a benign tumor of the adrenal medulla), and sometimes parathyroid hyperplasia. It has an incidence of approximately 1/50,000 (Carter et al., 1987; Narod et al., 1992). The rarer syndrome MEN 2B comprises MTC and pheochromocytoma as well as
mucosal and gastrointestinal ganglioneuromatosis and a Marfan-like appearance. Familial MTC is also observed in the absence of other disease features. In addition, both MEN 2A (Gagel et al., 1989a; Nunziata et al., 1989; Kousseff, 1992) and familial MTC (Ferrer et al., 1990) have been noted in combination with cutaneous lichen amyloidosis, a localized skin lesion in which amorphous amyloid material is deposited between skin layers (Gagel et al., 1989a). The nomenclature used here for these disorders is that recommended by the MEN 2 workshop organizing committee (Gagel et al., 1989b). These diseases are not genetically related to multiple endocrine neoplasia type 1 (MEN 1), in which patients develop adenomas or carcinomas of the pituitary, parathyroid and pancreas.

MTC is cancer of the calcitonin-secreting parafollicular or C cells of the thyroid; pheochromocytoma is derived from the chromaffin cells of the adrenal medulla. These cell types share a neural crest origin, as do the autonomic ganglion plexuses from which are derived the mucosal neuromas of MEN 2B patients. Parathyroid glands are thought not to be of neuroectodermal origin (Pearse, 1974).

The parathyroid hyperplasia seen in some MEN 2A families was at one time suggested to be the result of calcitonin excess from MTC. Neither sporadic MTC cases nor MEN 2B patients, however, show parathyroid hyperplasia. In addition, hypercalcemia can appear years after surgical eradication of thyroid cancer. Thus the parathyroid hyperplasia of some MEN 2A families appears to reflect the genetic predisposition of these families rather than paracrine secondary effects (Block et al., 1980). Tissues of both neuroectodermal and non-neuroectodermal origins are apparently involved in MEN 2A; the implications of this are not understood.
The age of onset of MTC in MEN 2A patients is variable, with a mean of approximately 35 years (Gagel et al., 1982). C cell hyperplasia precedes overt MTC (Jackson et al., 1973; Wolfe et al., 1973); chromaffin cell hyperplasia precedes pheochromocytoma (DeLellis et al., 1976). MTC occurs earlier in MEN 2B patients (at about 15 years of age) and the disease is usually more severe than in MEN 2A patients; mucosal neuromas appear early in life (Schimke, 1984). Age of onset of thyroid cancer is latest and the cancer least aggressive in MTC families (Noll et al., 1984). Penetrance of MEN 2A is high (Easton et al., 1989).

Hormone-based screening tests can detect the MEN 2 tumors before they are clinically apparent. High basal plasma calcitonin levels or, more sensitively, high calcitonin levels upon provocative testing, indicate C cell hyperplasia or MTC. Provocation with a combination of calcium gluconate and the calcitonin secretagogue pentagastrin is the most effective test of occult medullary thyroid disease described to date (Lairmore and Wells, 1991). Individuals destined to develop thyroid carcinoma can be cured by total thyroidectomy before appearance of overt MTC; the effectiveness of surgery can be checked by post-surgical provocation tests. The calcitonin provocation test, however, is not a specific test for MEN 2. C cell hyperplasia is found in 5-10% of the normal population (Howieson Gibson et al., 1981), including members of MEN 2 families who do not carry the disease allele (Wolfe et al., 1991; Landsvater et al., 1993). Unaffected members of MEN 2 families can be misdiagnosed as MEN 2 disease allele carriers on the basis of the provocation test. The use of DNA diagnostics in combination with the pentagastrin test will improve the accuracy of disease risk predictions. Pheochromocytoma and the adrenal medullary hyperplasia that precedes it can be detected through quantitation of catecholamines in urine (DeLellis et al., 1976).
Genes responsible for MEN 2A, MEN 2B and MTC map to the pericentromeric region of chromosome 10.

MEN 2A, MEN 2B and familial MTC show an autosomal dominant pattern of inheritance. MEN2A and MEN2B map to the pericentromeric region of chromosome 10 (Simpson et al., 1987; Mathew et al., 1987a, Norum et al., 1990; Lairmore et al., 1991). The gene responsible for familial MTC is linked to markers near the centromere of chromosome 10 in most but not all families, indicating genetic heterogeneity of this disease (Carson et al., 1991). Chromosome 10-linked familial MTC is now referred to as MTC 1 (summarized by Simpson and Cann, 1991). By definition, MTC 1 families exhibit only MTC with neither pheochromocytoma nor parathyroid hyperplasia; this differentiates them from MEN 2A families, which have been recently classified into three clinical subtypes, which appear to breed true (Figure 1). Early linkage analyses did not discriminate between affected families with different clinical features; it is now clear, however, that the gene(s) responsible for MEN 2B (Norum et al., 1990; Lairmore et al., 1991), MTC 1 (Lairmore et al., 1991; Noll et al., 1988), and all three subtypes of MEN 2A (Carson et al., 1990; Narod et al., 1989) map to the pericentromeric region of chromosome 10. The locus responsible for MEN 2A with cutaneous lichen amyloidosis (MEN 2A + CLA) also maps to this region of the chromosome (Robinson et al., 1991).

The relationship between the loci responsible for these diseases is not known; they may be closely linked loci or could represent different alleles of a single disease gene. It has also been suggested, based on phenotype mapping (Jackson et al., 1989), that they could define a contiguous gene syndrome (Schmickel, 1986), in which mutations affect adjacent genes governing the different clinical features of these diseases.
FIGURE 1: A classification system for MTC disorders of demonstrated chromosome 10 linkage (Adapted from Simpson, 1991).
A single family in which MEN 2A and MEN 2B cosegregate with chromosome 10 markers has been reported (Lacroix et al., 1991), implying an intimate relationship between the loci responsible for these diseases. Identification of mutations predisposing to MEN 2A, MEN 2B, MTC 1 and MEN 2A with cutaneous lichen amyloidosis will clarify the relationship between the gene(s) causing these cancers.

No cytogenetically visible chromosome alterations have been shown to be associated with chromosome 10 in MEN 2A, MEN 2B, MTC 1 or MEN 2A with cutaneous lichen amyloidosis patients. Knowledge of the localization of the disease genes is based solely upon linkage analyses in affected families.

*MEN2A* is flanked on the short arm of chromosome 10 by *FNRB* in 10p11.2 and on the long arm by *RBP3* in 10q11.2 (Wu et al., 1990). *MEN2A* (Wu et al., 1990; Carson et al., 1990), *MEN2B* (Lairmore et al., 1991) and *MTC1* (Narod et al., 1989; Lairmore et al., 1991) are all tightly linked to the chromosome 10 centromeric alphoid repeat locus, *D10Z1*. The localization of *MEN2B* has been further refined to the long arm of chromosome 10 (Norum et al., 1990). Linkage analysis predicts the most likely location of the gene directing MEN 2A with cutaneous lichen amyloidosis as between *FNRB* and *RBP3* (Robinson et al., 1991); *RBP3* flanks this disease locus distally on 10q. Figure 2 illustrates the "candidate regions" (the region to which each disease locus has been localized) to which *MEN2A, MEN2B, MTC1* and *MEN2A+CLA* have been mapped.
FIGURE 2: Candidate regions defined by DNA markers that flank the loci responsible for MEN 2A, MEN 2B, MTC 1 and MEN 2A with cutaneous lichen amyloidosis. A synthesis of data from the following sources is presented: Wu et al., 1990; Norum et al., 1990; Jackson et al., 1988; Lairmore et al., 1991; Narod et al., 1989; and Robinson et al., 1991.
Recombination is repressed near the centromere of chromosome 10.

There is a well-documented repression of recombination in the pericentromeric region of chromosome 10 in males relative to females (Wu et al., 1990; Lichter et al., 1992a). More recent studies have shown a repression of recombination relative to physical distance across the centromere in both males (Carson and Simpson, 1991; Lichter et al., 1991a) and females (Lichter et al., 1991a). This feature of the pericentromeric region, combined with limited family resources, has made difficult the use of meiotic mapping to refine the exact location of the MEN 2 disease loci.

It is difficult to estimate the size of the MEN2A candidate interval. The sex average genetic distance between FNRB and RBP3 is approximately 12 cM (Lichter et al., 1992a). From the approximate genome average of one megabase (Mb) of DNA per centiMorgan (cM) of genetic distance, one would expect this interval to be approximately 12 Mb. Because of the repression of recombination in this region, however, the true size could be much greater. In addition, chromosome 10 centromeric alpha satellite DNA is located within this interval. Alphoid repeat DNA is found at the centromere of every human chromosome. It is made up of 171 bp (base pair) monomers arranged into higher order repeat structures (reviewed by Willard and Waye, 1987). The size of the chromosome 10 alphoid DNA array is on average 2.1 Mb, although it varies by more than 1 Mb between different copies of chromosome 10 (Wevrick and Willard, 1989). It is not known whether the chromosome 10 alphoid repeats form a single block of repetitive DNA or whether patches of unique DNA could be located within the array; this question is of relevance in the search for disease genes that map very close to centromeres.
Progress in mapping the \textit{MEN2} region.

As of early 1993, additional markers have been mapped within the pericentromeric region of chromosome 10 and in some cases, markers more closely flanking \textit{MEN2A} (Miller \textit{et al.}, 1992a; Miller, 1992; Lairmore \textit{et al.}, 1993; Gardner \textit{et al.}, 1991 and 1993) or \textit{MEN2B} (Gardner \textit{et al.}, 1993) have been identified, allowing refinement of the regions to which the disease loci map. Subsequent chapters of this thesis will describe contributions to the cloning and mapping of DNA markers within the MEN 2 region. A synthesis of mapping data to date and a summary of the significance of these contributions, in the context of those of the MEN 2 mapping community, will be summarized in chapter VII. The order of the chapters of this thesis reflects chronologically the progress of this research; this type of presentation was chosen to facilitate the description of the rationale underlying each phase of the work.

Application of other gene mapping methods to \textit{MEN2}.

Haplotype analysis can be useful for localizing disease loci with low mutation rates. \textit{MEN2A} has a low mutation frequency; no new mutation cases of MEN 2A have, in fact, been reported (Narod \textit{et al.}, 1992). In contrast, 50\% of MEN 2B cases are new mutants (Norum \textit{et al.}, 1990). MEN 2A, with its low mutation frequency, would seem to be amenable to the use of haplotype analysis or linkage disequilibrium to localize the disease locus. Haplotype analysis is only effective, though, in situations where affected individuals can be assumed to be descended from a common founder. This is often the case when a disease clusters within an ethnic group or is found in a population in a certain geographic region. Cystic fibrosis, for example, has a major mutation that is particularly common in Caucasians; linkage disequilibrium was used effectively to locate the cystic
fibrosis gene (Kerem et al., 1989). The incidence of MEN 2A, however, has not been reported to vary markedly between countries.

While the use of haplotyping and/or linkage disequilibrium may not be optimally applicable to ethnically mixed collections of MEN 2A families, it may be valid for ethnically homogeneous collections of families where the assumption of identity by descent is not unreasonable. Narod and colleagues (1989, 1992) have reported the association, in six out of 24 French families analyzed, of an uncommon haplotype involving alleles at loci between and including \( FNRB, D10Z1, \) and \( RBP3, \) with both \( MEN2A \) and \( MTC1. \) Unfortunately, association of the disease allele with a haplotype that includes known flanking markers of the disease offers little information to help in localizing the MTC gene(s). Carson et al., (1990), did not observe a multilocus allelic association for their group of six North American families of varied ethnic origin. This could mean that there is a single mutation responsible for some familial MTC cases in France, but which is not necessarily shared by MTC families of non-French origin. Narod and colleagues (1992) also suggest, based on association of the disease alleles of both MEN 2A and MTC 1 families with the same haplotype, that these clinically distinct disorders could be caused by the same mutation, with penetrance of pheochromocytoma being influenced by other factors.

Cloning the gene(s) responsible for MEN 2 is of practical value to MEN 2 families.

DNA diagnostics using polymorphic markers linked to \( MEN2A, \) \( MEN2B, MTC1 \) and \( MEN2A+CLA, \) can complement existing hormone-based screening programs to provide more accurate presymptomatic diagnosis of these cancers. Genetic tests can spare non-carriers the routine but
reportedly unpleasant pentagastrin test. Cloning polymorphic markers closer to the disease loci will allow increasingly accurate test results for individuals in informative families. Identification of the gene(s) responsible for MEN 2A, MEN 2B, MTC 1 and MEN 2A + CLA will allow genetic tests of essentially 100% accuracy to be devised for many families.

Tumor suppressor genes and oncogenes.

Tumor suppressor genes are genes that, in their normal form, play a role in the regulation of cellular processes such as proliferation or differentiation. The loss or inactivation of a tumor suppressor is oncogenic. Oncogenes, in contrast, are genes whose gain-of-function mutant alleles play an active role in promoting inappropriate cell growth and proliferation. The wild type proto-oncogene may carry out a specific cellular role under restricted circumstances; the oncogenic form may carry out these functions constitutively, aberrantly, or at too high a level. The cloning and study of the normal cellular roles of both oncogenes and tumor suppressor genes is now a major focus of cancer research.

Tumor suppressor genes have been investigated by means of cell fusion experiments between normal and transformed cell lines, and observation of the transformed or non-transformed phenotype of the resultant hybrid lines. The fusion products, usually non-transformed, may revert to tumorigenicity upon loss of particular chromosomes or parts of chromosomes postulated to contain specific tumor suppressor loci (Reviewed by Sager, 1989). Another means by which tumor suppressor genes have been studied is through the identification of families whose members show a dominantly inherited predisposition to a particular type of tumor or tumors. Cataloguing of inherited human cancers (Mulvihill, 1977) suggests the existence of 200 or more cancer related genes.
The "two-hit" theory of carcinogenesis: Relevance to MEN 2.

Most cancers occur in both familial and sporadic forms (Knudson, 1986). Alfred Knudson, Jr., first formulated a model based upon observations concerning the age-specific incidence of cancer within families showing autosomal dominant inheritance of susceptibility to particular tumors, and of sporadic cases of the same tumors. He suggested, originally for retinoblastoma (Knudson, 1971) and later for other familial tumors (Knudson, 1976), that individuals within a family may inherit a mutant (inactive) allele of, for example, the RB locus. Somatic mutation at the remaining wild type RB allele within a cell of susceptible type (for retinoblastoma, a retinoblast) in such an individual results in the clonal expansion of this RB- cell into a tumor. The autosomal dominant inheritance pattern and the occurrence of multiple tumors and bilateral disease of affected individuals within RB families follows from a high probability of occurrence of the second (somatic) event. Sporadic cases, for which cancer is generally unilateral, result from somatic mutation of both copies of the RB locus within the same retinal cell.

Knudson's "two-hit" theory of carcinogenesis has been shown to apply to MEN 2A (Knudson and Strong, 1972). By analogy to RB, inheritance of a genetic defect is the first event and somatic mutation the second event thought to lead to tumor formation in MEN 2A. Age of onset data, and the multifocal clonal nature of inherited MTC (Baylin et al., 1978), are consistent with the two-hit model. This suggests that MEN2A encodes a tumor suppressor. It has been proposed, however, that the C cell hyperplasia that precedes MTC, and likewise that the chromaffin cell hyperplasia that precedes pheochromocytoma in MEN 2A, may be the physical manifestations of the first genetic event (Jackson et al., 1973; Wolfe et al., 1973; DeLellis et al., 1976): inheritance of a mutant
chromosome 10. Sporadic cases of MTC do not exhibit widespread C cell hyperplasia (Block et al., 1980). This implies that presence of a single mutant copy of the MEN2A gene produces a hyperplastic phenotype, and that MEN2A may be a dominantly acting oncogene. Alternatively, reduction to half the normal dosage of the MEN2A gene product may be sufficient to produce hyperplasia.

Conversion to overt MTC is thought to be the result of a second, somatic event. Loss of heterozygosity (LOH) of chromosome 13 markers from the RB region often heralds the second (somatic) mutation in RB tumors. Loss of heterozygosity is demonstrated through the study of constitutional and tumor DNAs from the same individual, using polymorphic DNA markers. Analogous LOH occurs at inherited susceptibility loci in other familial tumors (Wilms' tumor, neurofibromatosis type 2 and Von Hippel Lindau syndrome, for example) and in corresponding sporadic tumors. Such changes are considered to be indicative of involvement of a tumor suppressor gene. Consistent loss of chromosome 10 alleles has, however, not been found in the tumors of MEN 2A patients (reviewed by Kidd and Simpson, 1990) suggesting that the pattern of genetic events leading to MTC may be different from those producing retinoblastoma (Nelkin et al., 1989). The second event could occur at the normal copy of the chromosome 10 locus by mechanisms other than gross loss of chromosomal material (Nelkin et al., 1989). Alternatively, the second mutational event in an MEN 2 tumor could occur at a location other than the normal chromosome 10 allele. LOH of chromosome 1p alleles has been detected in both MTC and pheochromocytomas (Mathew et al., 1987b; Moley et al., 1992). This region also shows allelic loss in other tumors including neuroblastoma (Fong et al., 1989) and ductal breast carcinoma (Guernardi et al., 1989).
Allelic losses at 22q, 17p, 3p, 11p and 13q have also been noted (albeit less commonly than at 1p) in MEN 2 type tumors (Khosla \textit{et al.}, 1991; Mulligan \textit{et al.}, 1991a). Further studies are required to determine if these events contribute to tumor formation or to tumor progression. Loss of heterozygosity at a site other than a cancer's inherited predisposition locus has been noted for familial adenomatous polyposis (FAP): LOH of 17p and of 18q is frequent in colon cancers but reduction to homozygosity at the 5q FAP locus is generally not seen in familial cases (Vogelstein \textit{et al.}, 1988; Fearon \textit{et al.}, 1990).

Loss of heterozygosity has been noted to be a hallmark of many tumor suppressor genes; RB is the prototype for this mechanism of carcinogenesis. It is not necessary, however, that a tumor suppressor gene show LOH to be classed as such; FAP is an example of this. For MEN 2, examination of mutations at the chromosome 10 locus in constitutional and tumor DNAs will help to determine whether it is loss of activity of a tumor suppressor gene, or expression of an altered product of a proto-oncogene, which predisposes to this cancer syndrome. The genetic mechanisms involved may not be resolved until the \textit{MEN2A} gene has been cloned (Kidd and Simpson, 1990). Ultimately the most definitive means of categorizing a cancer gene as an oncogene or tumor suppressor gene may be according to whether the function of the wild type gene product is growth promoting (oncogene) or growth inhibitory (tumor suppressor gene). Such an experiment has been performed for RB with results consistent with its classification as a tumor suppressor (Huang \textit{et al.}, 1988).

\textbf{Biologically plausible candidate genes within the MEN 2 region.}

A candidate gene for \textit{MEN2}, mcs94-1 (the first \textit{MEN2} candidate at D10S94; McDonald \textit{et al.}, 1992), has been cloned at this locus in 10q11.2.
The cloning and mapping of sequences at \textit{D10S94} is the subject of a large part of this thesis. mcs94-1 has sequence similarity to nucleolin, an abundant nucleolar protein involved in rDNA transcription and ribosome assembly (Lapeyre \textit{et al.}, 1987). mcs94-1 shows 23\% protein sequence identity with nucleolin over a 219 deduced amino acid region that includes two potential RNA binding domains (McDonald \textit{et al.}, 1992).

Several genes originally identified independently of MEN 2 studies, have been mapped to the pericentromeric region of chromosome 10 and, on the basis of their localization, can be considered candidates for \textit{MEN2A}, \textit{MEN2B}, \textit{MTC1} and/or \textit{MEN2A+CLA}. \textit{RET}, which encodes a transmembrane tyrosine kinase (Takahashi and Cooper, 1987), is a particularly intriguing candidate. Tyrosine kinases were first identified as the oncogenic viral counterparts of the products of cellular proto-oncogenes. The normal cellular forms of many tyrosine kinases are thought to play a role in signal transduction. \textit{RET} has been localized to 10q11.2 by \textit{in situ} hybridization (Ishizaka \textit{et al.}, 1989) and by somatic cell hybrid mapping to a chromosomal interval near \textit{MEN2A} (Sozzi \textit{et al.}, 1991). To date no recombinants between \textit{RET} and \textit{MEN2A} have been described (Mulligan \textit{et al.}, 1991b; Gardner \textit{et al.}, 1993). Activated \textit{RET} genes in which the \textit{RET} tyrosine kinase-coding sequences are juxtaposed 3' to amino-terminal sequences derived from the H4 gene (Bongarzone \textit{et al.}, 1989; Grieco \textit{et al.}, 1990; Radice \textit{et al.}, 1991; Pierotti \textit{et al.}, 1992), have been found in several cases of papillary thyroid carcinoma, a cancer that arises from the follicular cells of the thyroid. \textit{RET} is expressed to a high level in both medullary thyroid carcinomas and pheochromocytomas (Santoro \textit{et al.}, 1990). An extensive search for disease related alterations has been made at \textit{RET} in the genomes of MEN 2 patients and in tumor DNA; to date no alterations have been reported (Mulligan \textit{et al.}, 1991b).
A number of T cell cDNAs encoding proteins containing zinc finger motifs of the Kruppel variety (Miller et al., 1985) have been mapped to chromosome 10 (Huebner et al., 1991). Eight such genes (ZNF11A, ZNF11B, ZNF22, ZNF25, ZNF37A, ZNF37B, ZNF33A and ZNF33B) map to chromosomal intervals that may contain the gene(s) responsible for MEN 2 (Rousseau-Merck et al., 1992; Mole et al., 1991) and can be considered candidates for genes involved in thyroid carcinogenesis. The possible involvement of zinc finger-containing genes is particularly interesting in light of the implication of a zinc finger gene (WT1, reviewed by Van Heyningen and Hastie, 1992) in the etiology of the childhood kidney cancer, Wilms' tumor. Interestingly, a cluster of putative zinc finger genes also maps to chromosome 11 near the gene responsible for MEN 1 (Saleh et al., 1992).

The type I cyclic GMP-dependent protein kinase gene (PRKG1B) has been mapped by in situ hybridization to 10p11.2-q11.2 (Orstavik et al., 1992). The choline acetyltransferase gene (CHAT) has been localized to 10q11.2, also by in situ analysis (Viegas-Pequignot et al., 1991). Identification of polymorphisms and linkage studies could demonstrate whether these genes are localized to the portion of 10q11.2 to which the MEN 2 gene(s) map. This will determine whether they should be considered, on the basis of localization, candidate disease genes and/or useful DNA markers within the MEN 2 candidate interval.

A positional cloning strategy for MEN2.

Positional cloning of different human disease genes has been aided by the existence of cytogenetically visible chromosome alterations such as deletions (Wilms' tumor, Francke et al., 1979), translocations (neurofibromatosis type 1; O'Connell et al., 1989), or fragile sites (fragile X syndrome, Sutherland, 1977). No cytogenetically apparent abnormalities
involving the chromosomal region to which the MEN 2 gene(s) map have been described in MEN 2 patients; cytogenetics to date has not provided clues useful in the cloning of the gene(s) responsible for MTC. In this way the search for the MEN 2 gene most resembles the search for the cystic fibrosis gene for which, similarly, no cytogenetic clues were available. The CF gene cloning, however, was aided by marked linkage disequilibrium, which helped to narrow the search for the gene.

Loss of heterozygosity of chromosome 13 markers was invaluable in narrowing the search for the retinoblastoma gene (Cavenee et al., 1983). No such allelic losses involving chromosome 10 have aided in the search for MEN2.

The candidate region to which MEN2 maps is potentially very large. A sensible approach to cloning this gene, then, combines the dual goals of refinement of the candidate region through cloning and mapping of new flanking markers demonstrated to recombine with disease, and characterization of candidate genes within the refined candidate region through examination of these genes for sequence alterations that could be causally associated with disease. In addition, examination of the candidate region, without prior identification of genes within it, could serve to identify potentially disease causing mutations; genes associated with the genetic alterations could then be sought.

This thesis describes a body of work that contributes to and is an integral part of a laboratory-wide strategy to clone the MEN 2 gene(s). It contributes to these goals in several definite ways.
THE SPECIFIC AIMS AND SCOPE OF THIS THESIS:

1. Development of human repeat element-mediated PCR (REM-PCR). REM-PCR is a method of generating human DNA markers from hybrid DNA sources. It is an extension of the technique Alu element-mediated PCR (Brooks-Wilson et al., 1990a). These methods allow the selective amplification and cloning of human DNA fragments from mixed DNA sources such as rodent/human somatic cell hybrid DNA, cosmids and YACs. In Alu element-mediated PCR, a single PCR primer directed to the 3' end of the human Alu element but not to rodent Alu-like elements, is used to amplify human DNA fragments located between Alu elements in opposite orientations and within a distance suitable for the PCR. In REM-PCR, a primer homologous to the 3' end of the human L1 element can be used singly or in combination with the human Alu primer to amplify human DNA fragments between L1 elements and fragments between Alu and L1 elements, in addition to those between two Alu elements.

2. Cloning of single copy DNA markers more closely linked to MEN2A than previously described markers. REM-PCR was developed for the purpose of cloning single copy DNA markers more closely linked to MEN2A than either FNRB or RBP3. A human/rodent somatic cell hybrid containing the pericentromeric region of chromosome 10, including FNRB, D10Z1 and RBP3 was used as a highly enriched cloning source. DNA from this hybrid was used as template for REM-PCR, to generate human-specific amplification products that were cloned, then mapped using Southern blots of REM-PCR products of panels of somatic cell hybrids allowing regional assignment on chromosome 10. Of 42 REM-PCR clones (pooled data from Brooks-Wilson et al., 1990a and Brooks-Wilson et
al., 1992a), two mapped to chromosomal intervals that could contain MEN2A. In the course of these studies, interesting observations about the distribution of Alu and L1 elements in the pericentromeric region of chromosome 10 were also made.

3. Contributions to linkage mapping of D10S94. One of the two REM-PCR clones mapping to the MEN2A candidate region was demonstrated to detect PvuII and RsaI polymorphisms. This clone, pC11/A1S-6-c23, defines D10S94. Somatic cell hybrid mapping results assigning pC11/A1S-6-c23 to an interval within chromosome band 10q11.2, combined with linkage mapping performed by Drs. Nancy Simpson (Queens University) and Paul Goodfellow, allowed mapping of D10S94 between D10Z1 and RBP3, within the MEN2A candidate interval. Linkage mapping also demonstrated that D10S94 was very tightly linked to MEN2A (Z_max=8.57 at θ=0.0 cM; Goodfellow et al., 1990a). D10S94 was the first single copy DNA marker demonstrated to be very tightly linked to MEN2A.

4. Expansion of the cloned region at D10S94. D10S94 was used as a starting point for expansion of the physically cloned segment within the MEN2A candidate region. Bidirectional cosmid walking (with Mr. Duane Smailus) and yeast artificial chromosome (YAC) cloning (in collaboration with Drs. Kenneth Kidd and Jay Lichter, Yale University) expanded D10S94 first to 180 kb and then to one Mb, respectively. A combination of genomic long range restriction mapping and physical mapping of YAC clones has allowed the establishment of physical linkage between D10S94 and other 10q11.2 DNA markers recently demonstrated to be tightly linked to MEN2A.
5. Identification of CpG islands at *D10S94*. CpG islands are landmarks for many mammalian genes. Long range restriction mapping was performed using rare cutting restriction enzymes that cleave mainly within CpG islands, in combination with pulsed field gel electrophoresis (PFGE) and DNA probes isolated during a cosmid walk from pC11/A1S-6-c23, to identify a dense cluster of six CpG islands within 180 kb at *D10S94*. Genes potentially associated with these CpG islands could be considered candidates for *MEN2A*, *MEN2B*, *MTC1* and/or *MEN2A+CLA* on the basis of their genomic location.

6. A search for potentially disease-causing alterations at *D10S94*. *D10S94* is a very CpG island-rich, and potentially very gene-rich region, and any gene in this area represents a candidate for *MEN2*. To implicate a gene in the etiology of thyroid cancer, mutations must be found in that gene in affected but not in unaffected individuals. Mutations can be sought first by identifying genes within the candidate region, then searching for mutations within those genes. Alternatively, one can look for mutations over a genomic area of interest without prior gene identification. Pulsed field gel electrophoresis was used to survey a 180-kb, CpG island rich region at *D10S94* for alterations detectable by this technique. This work establishes the utility of and quantitates the limitations of pulsed field gel electrophoresis as a tool to search for sequence alterations. No alterations were found at *D10S94* in the genomes of five *MEN 2A* or eight *MEN 2B* patients.
This work is a major part of a positional cloning strategy to identify the gene(s) responsible for MEN 2A, MEN 2B, MTC 1 and MEN 2A with cutaneous lichen amyloidosis. The cloning of a gene or genes responsible for these disorders would reveal the relationship between these diseases and would contribute to the understanding of the etiology of MTC. Although an MEN2 gene has not yet been cloned, this work brings us tangibly closer to the achievement of this goal.
CHAPTER II:

MATERIALS AND METHODS

Somatic Cell Hybrid Cell Lines

The somatic cell hybrids, their origin and chromosomal content are listed in table I. A group of seven hybrids (TRAXK2, TRAX10TG3, CRAB8, 64034p61c10, CHOK1-CZ28, CY5 and CY6) defines the eight chromosome 10 intervals depicted in figure 4 (chapter III). RAG (Klebe et al., 1970) and CHOK1 (Kao and Puck, 1967) were used as mouse and hamster controls, respectively, in human repeat element-mediated PCR experiments. The "centromere-fragment" (cen-frag) hybrids are from the "pp" series of X-irradiation reduced hybrids (Goodfellow et al., 1990b). A subset of these hybrids has been subjected to additional characterization (Miller et al., 1992b). Each cen-frag hybrid contains a fragment of human chromosome 10 including chromosome 10-specific alphoid repeat material, in a background of WG3H (hamster) chromosomes. A series of chromosome-mediated gene transfer (CMGT) hybrids has been made and characterized by Dr. Paul J. Goodfellow. These include the cloning source for REM-PCR, H2CL1, which contains a fragment(s) of chromosome 10 including the centromere, on a background of LMTK- (mouse) chromosomes (Brooks-Wilson et al., 1990a).

The chromosome 10 homologues of an MEN 2A family member were isolated in different somatic cell hybrids, for the purpose of comparing restriction maps and sequences derived from each. The lymphoblast cell line AC used as a source of human chromosomes was derived from a member of the "C" family (Duncan and Greenberg, 1986), for which chromosome 10 linkage of MEN2A had been demonstrated (Simpson et al.,
TABLE I: Somatic cell hybrids used in cloning and mapping chromosome 10 DNA markers.

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Rodent Parent</th>
<th>Chromosome 10 Content</th>
<th>Additional Human Material</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>762-8A</td>
<td>CHOK1 (hamster)</td>
<td>intact 10</td>
<td>Y</td>
<td>Fisher et al., 1987</td>
</tr>
<tr>
<td>64034p-61c10</td>
<td>CHOK1 (hamster)</td>
<td>10cen-qter</td>
<td>Y</td>
<td>Lathrop et al., 1988</td>
</tr>
<tr>
<td>853 (7631)</td>
<td>CHW1104 (hamster)</td>
<td>none</td>
<td>Y</td>
<td>Burk et al., 1985</td>
</tr>
<tr>
<td>3W4C15P2</td>
<td>IR (mouse)</td>
<td>intact 10</td>
<td>7, 11, 14, 15, 17, 21, X</td>
<td>Goodfellow et al., 1989</td>
</tr>
<tr>
<td>TRAXK2</td>
<td>RAG (mouse)</td>
<td>10q11.2-qter</td>
<td>11,15, 17, X*</td>
<td>Edwards et al., 1989; Mathew et al., 1990</td>
</tr>
<tr>
<td>TRAX10TG3</td>
<td>RAG (mouse)</td>
<td>10pter-q11.2</td>
<td>6, 15, 21, X*</td>
<td>Mathew et al., 1990</td>
</tr>
<tr>
<td>CRAB8</td>
<td>RAG (mouse)</td>
<td>10pter-p11</td>
<td>2, 9*, 14, 15, 21, X</td>
<td>Brooks-Wilson et al., 1992a</td>
</tr>
<tr>
<td>CHOK1-CZ 28</td>
<td>der10</td>
<td></td>
<td></td>
<td>Carson and Simpson, 1991</td>
</tr>
<tr>
<td></td>
<td>del(q11.2-q22.1)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CY6</td>
<td>10pter-q24.33</td>
<td></td>
<td></td>
<td>Callen et al., 1988</td>
</tr>
<tr>
<td>CY5</td>
<td>10pter-q26.3</td>
<td></td>
<td></td>
<td>Callen et al., 1988</td>
</tr>
</tbody>
</table>

*Portion of chromosome present as a translocation chromosome.
Human/hamster hybrids were generated and characterized by Dr. Paul Goodfellow. Polyethylene glycol fusion of AC lymphoblasts with proline auxotrophic CHOK1 Chinese hamster cells (Jones, 1975) was followed by hybrid selection by growth in proline deficient medium to produce hybrid clones retaining human chromosome 10. Clonal cell lines AC10A and AC8D were determined to retain different single chromosome 10 homologues by Southern blot analysis of RFLPs of known phase.

**Human Cell Culture**

Epstein Barr virus immortalized lymphoblast cell lines from eight MEN 2B new mutants and their parents were a gift of Dr. Charles E. Jackson. They were propagated in RPMI 1640 (1 x liquid, GIBCO) with 10% heat inactivated fetal calf serum (GIBCO) with 100 μg/ml gentamicin sulfate and 300 μg/ml supplemental L-glutamine.

**Growth of yeast strains**

Yeast artificial chromosome (YAC) clones in yeast host strain AB1380 (MATa ψ+ura3 trp1 ade2-1 can1-100 lys2-1 his5) were identified from the original Centre d'Etude du Polymorphisme Humain (CEPH) YAC library (Albertsen et al., 1990). They can be grown in rich medium (YPD: 1% yeast extract, 2% peptone, and 2% glucose) or selective medium (SD-URA-TRP: 0.67% yeast nitrogen base without amino acids and 2% glucose supplemented with amino acids [minus uracil and tryptophan], and 10 mg/L adenine sulfate). A subset of YACs showed a tendency to be lost when grown in non-selective medium (data not shown). For this reason, all yeast/YAC cultures from which DNA was to be prepared were grown under selection. A stock of AB1380 DNA for use as a negative control for Southern blots was prepared from a culture grown in YPD.
Repeat Element-Mediated PCR

The A1S primer (Brooks-Wilson et al., 1990a) corresponds to the 3' end of the human Alu element, with one G to A sequence modification to decrease potential primer self-complementarity. It corresponds exactly to the consensus sequence of greater than 40% of human Alu elements analyzed by Kariya et al., (1987). It also contains a Sall recognition site to facilitate cloning of PCR products. The A1B primer has the same 3', 18 nucleotide homology to the human Alu element as the A1S primer, but contains a BamHI cloning site. The L1 (LINE) element-specific primer L1S has 18 nucleotides of homology with the 3' end of the human L1 repeat element (Scott et al., 1987), and a Sall cloning site. The primers and their sequences are listed in table II. Cloning sites are underlined. Primers were purified by C-18 Sep-Pak (Waters).

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1S</td>
<td>5'-TCATGTGACGCGAGACTCCATCTCTAAAA-3'</td>
</tr>
<tr>
<td>A1B</td>
<td>5'-TCATGGATCCGCGAGACTCCATCTCTAAAA-3'</td>
</tr>
<tr>
<td>L1S</td>
<td>5'-TCATGTGACACGTTGTGCACATGTACC-3'</td>
</tr>
</tbody>
</table>

Polymerase chain reaction conditions were: 50 mM tris(hydroxymethyl)amino-methane, pH 8.0; 0.05% Tween-20; 0.05% NP-40, 1.8 mM MgCl₂; 200 μM each of dATP, dCTP, dGTP, and dTTP; 0.5 μM primer; 1.25 U of Taq polymerase; and 100 ng of template DNA.

Twenty-five cycles of a 1-min denaturation at 94°C, a 2-min annealing at 58°C, and a 3-min extension at 72°C, with an additional 10-s
extension increase per cycle and a final 72°C incubation for 10 min, were performed. A Perkin-Elmer Cetus thermal cycler was used.

Cloning of Human Repeat Element-Mediated PCR Synthesis Products

Human repeat element-mediated PCR products were generated from the chromosome-mediated gene transfer (CMGT) hybrid H2CL1. Alu element-mediated PCR was performed using the A1S primer, L1 element-mediated PCR was done using the L1S primer, and Alu/L1 combination PCR was carried out using the A1B and L1S primers together. The synthesized DNA was digested with Sall or with Sall and BamHI according to the restriction sites of the PCR primers used. Varying amounts of PCR fragments (5 to 20 ng) were ligated to 5 ng of pUC18 in 20-μl reactions at 25°C for 5 hours. Ligation products were transformed into Escherichia coli strain TG-1. Transformants were selected on LB agar plates containing 50 μg/ml ampicillin and spread with 50 μl 2% X-Gal in dimethylformamide and 20 μl 100 mM IPTG.

Gel Electrophoresis and Southern Blot Analysis of REM-PCR products

Repeat element-mediated PCR synthesis products (0.3 x reaction volume) were size fractionated on 1.0 or 1.5% agarose gels and detected by ethidium bromide staining. Repeat element-mediated PCR mapping panels were generated by Southern blotting an equivalent profile of PCR synthesis products onto Hybond-N (Amersham). Hybridization of "random primed" (Feinberg and Vogelstein, 1984) 32P-labelled DNA fragments isolated from repeat element-mediated PCR clones and post-hybridization washing were as recommended by the manufacturer. Hybridization and washing were performed at 65°C in most cases (except when preassociation of a probe with sheared human DNA was necessary to reduce background
hybridization). Autoradiography exposure times varied from 30 minutes to 5 days.

**Generation of probes for long range restriction mapping and YAC contig characterization**

Long range restriction mapping probes are listed in table III. DNA probes were tested using conventional genomic Southern blots to ensure that they gave rise to single hybridization bands prior to being used to probe PFGE blots. Probes used in long range mapping were restriction fragments or PCR products derived from plasmid clones. They were purified in low-melting-point agarose and radiolabelled by the random priming method (Feinberg and Vogelstein, 1984). Many of the probes were preassociated with a vast excess of sheared human genomic DNA prior to hybridization to Southern blots.

**Preassociation of probes with human DNA to minimize hybridization to human repeat elements.**

Incubation of probes with a vast excess of denatured and sheared human genomic DNA was used to compete out human repeat sequences present in some clones. Fifteen ng of \(^{32}\)P-labelled probe DNA was combined with 500 µg of sheared human placental DNA, boiled 5 mins, and preassociated in 1 ml of prewarmed hybridization mix at 65°C for 1 h. For "competed" probes, hybridization and post-hybridization washing were carried out at 70°C.
TABLE III: DNA fragments used as hybridization probes in long range restriction mapping and YAC contig characterization.

<table>
<thead>
<tr>
<th>Probe Name</th>
<th>Locus</th>
<th>Notes and references</th>
</tr>
</thead>
<tbody>
<tr>
<td>pC11/A1S-6-c65</td>
<td>D10S182</td>
<td>MspI fragment of Alu element-mediated PCR clone pC11/A1S-6-c65.</td>
</tr>
<tr>
<td>0.95 SstII</td>
<td>D10S94</td>
<td>An SstII subclone from cosmid pur-1-1 at D10S94 (P.J. Goodfellow and D. Smailus, unpubl.)</td>
</tr>
<tr>
<td>p0</td>
<td>D10S94</td>
<td>0.8 kb NotI/HindIII fragment isolated from a 1.3 kb EcoRI subclone from cosmid pur-1-1</td>
</tr>
<tr>
<td>p1</td>
<td>D10S94</td>
<td>0.35 kb EcoRI fragment from cosmid 1-1a, subcloned in pUC18</td>
</tr>
<tr>
<td>p2</td>
<td>D10S94</td>
<td>0.95 kb EcoRI/ SstII fragment from cosmid 1-1a, subcloned in pUC18</td>
</tr>
<tr>
<td>p3</td>
<td>D10S94</td>
<td>211 bp PCR product generated using cosmid 1-1a template DNA and primers P1 and P2 (Brooks-Wilson et al., 1992b)</td>
</tr>
<tr>
<td>p4</td>
<td>D10S94</td>
<td>0.43 kb EcoRI/BanII fragment isolated from a 6.5 kb EcoRI subclone from cosmid 2-4a-1</td>
</tr>
<tr>
<td>p5</td>
<td>D10S94</td>
<td>0.55 kb EcoRI/PstI fragment isolated from a 4.3 kb EcoRI subclone of cosmid 2-4a-1</td>
</tr>
<tr>
<td>p6</td>
<td>D10S94</td>
<td>2.7 kb PCR product generated using cosmid 6-11a-5 template DNA and primers homologous to the human L1 repeat element and to the T7 bacteriophage promoter in cosmid vector pWE15</td>
</tr>
<tr>
<td>p7</td>
<td>D10S94</td>
<td>0.6 kb EcoRI fragment of cosmid 1-6a, subcloned in pUC18</td>
</tr>
<tr>
<td>p8</td>
<td>D10S94</td>
<td>6.5-kb EcoRI fragment from a cosmid, 3-10a, which extends beyond cosmid 1-6a at D10S94 (P.J. Goodfellow and D. Smailus, unpubl.)</td>
</tr>
<tr>
<td>YP16</td>
<td></td>
<td>0.55-kb EcoRI/HindIII subclone from YAC 170G10 (P.J. Goodfellow, unpubl.)</td>
</tr>
<tr>
<td>pret9.1T3</td>
<td>RET</td>
<td>2.4-kb TaqI fragment excised from genomic clone pret9.1T3, gift of Dr. B. Ponder (Mulligan et al., 1991b)</td>
</tr>
<tr>
<td>pKW6ΔSacI</td>
<td>See Notes</td>
<td>Recognizes 3 genomic sites: <em>D10F38S1</em> (polymorphic <em>D10S97</em> in 10q11.2), <em>D10F38S2</em> (10p13) and <em>D10F38S3</em> (10q11.2, a site separable from <em>D10F38S1</em> by radiation hybrid mapping, &quot;<em>D10S97</em>-like&quot; [Miller et al., 1992b]) (Lichter et al., 1991b; Lichter et al., in press).</td>
</tr>
<tr>
<td>DM124</td>
<td>Recognizes two genomic sites, one at 10q11.2 and one on another chromosome (D.L. Miller and P.J. Goodfellow, unpubl.)</td>
<td></td>
</tr>
<tr>
<td>pMEN203DM1</td>
<td><em>D10S102</em></td>
<td>Gift of B. Robinson, subclone of cosmid MEN203.</td>
</tr>
<tr>
<td>DM44</td>
<td><em>D10S251</em></td>
<td>3-kb <em>EcoRI</em> subclone from λDM44 (Miller et al., 1992b)</td>
</tr>
<tr>
<td>DM55</td>
<td>ALOX5</td>
<td>0.7-kb <em>HindIII</em> fragment from genomic clone λDM55 (Miller et al., 1992b)</td>
</tr>
<tr>
<td>DM121</td>
<td>2.4-kb <em>HindIII</em> subclone from λDM121 (Miller et al., 1992b)</td>
<td></td>
</tr>
<tr>
<td>DM151</td>
<td><em>D10S252</em></td>
<td>1.35-kb <em>EcoRI</em> subclone from λDM151 (Miller et al., 1992b)</td>
</tr>
<tr>
<td>p1-3A</td>
<td><em>D10S253</em></td>
<td>2-kb <em>EcoRI/HindIII</em> fragment isolated from a 2.7-kb <em>Sall/SstII</em> subclone from λ1-3A (Miller et al., 1992b)</td>
</tr>
<tr>
<td>pα10RP8</td>
<td><em>D10Z1</em></td>
<td>Chromosome 10 alphoid repeats (Devilee et al., 1988)</td>
</tr>
</tbody>
</table>
Preparation and restriction digestion of high molecular weight human and yeast DNA in agarose blocks.

Agarose "blocks" containing $5 \times 10^5$ EBV transformed human lymphoblasts were prepared essentially as described by Herrmann et al. (1987). Preparation of yeast chromosomal DNA in agarose was as described by Scherer and Tsui (1991). Human or yeast DNA in agarose was digested with 20 U of restriction enzyme for 6 to 16 h. Blocks to be digested with a second enzyme were rinsed in TE prior to the second digestion.

Pulsed field gel electrophoresis

This section describes the PFGE methods used in chapter IV. DNA samples were electrophoresed in 0.8% agarose, in TBE (0.1 M Tris base, 0.1M boric acid, 2 mM EDTA) using an LKB pulsaphor apparatus with a hexagonal electrode array. Lambda (cI857 Sam7) concatamers and Saccharomyces cerevisiae strain YNN295 chromosomes (BIO-RAD) were employed as molecular size markers. Conditions were: 170V, 110°C, and 20-s pulses for 23.5h (gel A) or 60-s pulses for 38.5h (gel B). Gels were blotted to GeneScreen Plus (NEN). Blotting, hybridization and post-hybridization washes were according to the manufacturer's recommendations.

Field inversion gel electrophoresis

These FIGE methods apply to the experiments described in chapters V and VI. DNA samples were electrophoresed in 0.8% agarose in TBE (89 mM Tris base, 89 mM boric acid, 2.5 mM EDTA) using a prototype field inversion gel electrophoresis apparatus, AutoBase module AB 009 from Q-Life Systems Inc., Kingston, Ontario, Canada. The AutoBase unit uses pre-
programmed ROM cards to resolve DNA fragments of specific molecular weight ranges. Lambda concatamers (BRL Megabase II), *Saccharomyces cerevisiae* strain YNN295 chromosomes (BIO-RAD), yeast strain HY1 (which contains a 120-kb artificial chromosome on the background of host strain AB1380, gift of S. Scherer) and yeast strain YPH149 (which was derived from YNN295 and which contains 1010-kb and 90-kb derivative chromosomes (Vollrath *et al.*, 1988)) were employed as DNA size standards.

The multiple enzyme, *MluI*, and *NruI* human genomic FIGE gels of chapter VI were run using Q-Life Systems ROM card 5 (resolution in the 100 to 1100 kb range); the *NotI* human genomic FIGE gel and the YAC FIGE gel of chapter VI were run using Q-Life ROM card 3 (resolution in the 8 to 500 kb range). ROM programs used for experiments in chapter V were as indicated in the results section of that chapter. Human genomic FIGE gels were transferred to GeneScreen Plus (NEN); YAC FIGE gels were transferred to Hybond N (Amersham). Southern blotting, hybridization, and post-hybridization washes were performed according to the manufacturers' recommendations.

FIGE blots were hybridized with approximately 5 ng of *HindIII*-digested λ DNA radiolabelled by nick translation (using the BRL BioNick Labeling System) in order to better quantitate the migration of λ-based DNA size standards (λ *HindIII* fragments and λ concatamers).

**YAC expansion of D10S94 and RET**

*D10S94*- and RET- containing YAC clones were isolated from the CEPH YAC library (Albertsen *et al.*, 1990) using a PCR screening approach (Heard *et al.*, 1989) involving pooling of rows and columns of YAC clones arranged in groups of 96 well dishes, and testing of the pools by PCR.
One *D10S94* STS (sequence tagged site) derived from within probe p1 (primers Ms1 and Ms2, Brooks-Wilson *et al.*, 1992b) and one *RET* STS derived from pret9.1T3 (primers retF: 5' GCAATGAGATGCAACAGAGC 3', and retR: 5' TGTGGCAGTAAGATACCAAATC 3') were used. YAC 56G11 (gift of S. Mole and B. Ponder) is derived from *D10S102* and is also from the CEPH library. Yeast strains containing YACs were grown in selective medium lacking uracil and tryptophan. Small scale preparation of yeast/YAC DNA for Southern blot analysis was as described by Scherer and Tsui, 1991. Total yeast DNA from YAC-containing yeast strains was used for fluorescence *in situ* hybridization (FISH) analysis, performed by Ms. Trushna Desai in Dr. David Ward's laboratory at Yale University, to assess chimerism of the YACs. Experiments were performed as described for cosmid DNA (Lichter *et al.*, 1990). YAC DNA, labelled with biotin or digoxigenin, was hybridized to metaphase spreads of normal human lymphocytes and detected with avidin-FITC or antidigoxigenin-rhodamine, respectively, with diamidinophenylindole (DAPI) counterstaining.
CHAPTER III:

ISOLATION OF DNA MARKERS DERIVED FROM
THE MEN2 REGION OF CHROMOSOME 10
BY HUMAN REPEAT ELEMENT-MEDIATED PCR.

INTRODUCTION

The objective of the work described in this chapter was to clone single copy DNA markers more closely linked to MEN2A than known flanking markers. Somatic cell hybrids enriching for the pericentromeric region of human chromosome 10 were available for use as cloning sources for such experiments. One option in approaching the cloning of human DNA markers from this region would have been to construct a genomic DNA library from an enriched hybrid cloning source, then to screen for human clones from among a majority of rodent clones. To bypass this time consuming approach, Alu element-mediated PCR (Brooks-Wilson et al., 1990a) or Alu-PCR (Nelson et al., 1989) was developed. This method, developed during my time as a research assistant in Dr. Goodfellow's laboratory in 1989, was concurrently developed by another group.

Alu element-mediated PCR is a method of generating human-specific DNA fragments from somatic cell hybrid DNA. Genomic DNA from a human/rodent hybrid cell line is used as the template for a polymerase chain reaction (Saiki et al., 1988) in which a single human Alu-based oligonucleotide is used as the primer. Alu elements are abundant in human DNA. Rodent DNA contains sequences homologous to human Alu elements, but these repeat elements are less well conserved between species than they are within the human species (Jelinek and Schmid,
1982). Consequently, an Alu PCR primer can be used to preferentially amplify human sequences from human/rodent hybrid DNA template.

Alu PCR has become a valuable tool for the isolation of human sequences from mixed DNA sources (Nelson et al., 1989). Variations of this technique have been used to isolate series of human chromosome-specific clones from chromosome 5 (Cotter et al., 1990) and chromosome 19 (Aslanidis and de Yonge, 1990). Enrichment for Alu PCR isolates from chromosomal subregions can be achieved through the use of reduced human content hybrid cloning sources (Brooks-Wilson et al., 1990a), by using flow sorted material as a cloning source (Cotter et al., 1991), by visual comparison of Alu PCR fingerprints of hybrid cell lines containing different portions of a single human chromosome (Ledbetter et al., 1990; Patel et al., 1990), or by Alu PCR differential hybridization (Bernard et al., 1991).

Human repeat element-mediated PCR is an extension of Alu element-mediated PCR (Brooks-Wilson et al., 1990a). Primers homologous to the human Alu and L1 repeat elements have been used to amplify and clone a series of chromosome 10-specific inter-Alu, inter-L1, and inter-Alu/L1 DNA sequences. These primers permit the amplification of sequences between human Alu or L1 elements found in opposite orientations within several kb of each other. Because these primers are directed to the extreme 3' end of the repeat elements, amplification products do not contain extensive Alu or L1 sequences derived from the repeats directing PCR synthesis. The addition of restriction enzyme recognition sites to primers allows the direct cloning of human repeat element-mediated PCR products. Clones generated in this way can be mapped quickly and easily by hybridization to Southern blots of repeat element-mediated PCR products generated from somatic cell hybrid DNA templates.
Human repeat element-mediated PCR has been applied to the generation of DNA markers from the pericentromeric region of human chromosome 10, near the gene responsible for MEN 2A. MEN2A is flanked by FNRB in 10p11.2 (Wu et al., 1990) and by RBP3 in 10q11.2 (Liou et al., 1987; Lathrop et al., 1988), and is very tightly linked to the chromosome 10 centromeric alphoid repeats (D10Z1) (Simpson and Cann, 1990). FNRB and RBP3 map to the Giemsa-negative bands that flank the chromosome 10 centromeric heterochromatin. In situ hybridization studies using cloned repeats suggest that Giemsa-negative bands are rich in Alu elements, and that Giemsa-positive bands contain fewer Alu elements (Korenberg and Rykowski, 1988). Centromeric heterochromatic regions contain fewer Alu sequences than do other genomic regions (Moyzis et al., 1989). The MEN 2A region of chromosome 10 may therefore be a composite of Alu-rich and Alu-poor domains. For this reason, chromosomal region-specific cloning efforts were extended to include sequences between human L1 elements, and sequences between human Alu and L1 elements.

A chromosome-mediated gene transfer (CMGT) hybrid cloning source, H2CL1, was used to generate eighteen new chromosome 10 DNA markers: twelve Alu/Alu clones, two L1/L1 clones and four Alu/L1 clones. Two inter-Alu clones map to the pericentromeric region. A scarcity of suitably oriented Alu elements in the pericentromeric region of chromosome 10 has contributed to the low number of inter-Alu clones obtained from this region. Inter-Alu clone pCl1/A1S-6-c23 (D10S94) is tightly linked to MEN2A (Goodfellow et al., 1990a). This repeat element-mediated PCR clone provides an ideal starting point for experiments directed toward the identification of the MEN2A gene.
RESULTS

Repeat Element-Mediated PCR Fingerprints

Repeat element-mediated PCR amplification products generated using somatic cell hybrid DNA templates are visualized as a series of discrete bands following agarose gel fractionation and staining with ethidium bromide. The pattern of bands for any given hybrid template is a "fingerprint" reflecting the human DNA content of the hybrid (figure 3A). Mouse and hamster DNA templates do not produce visible bands with any of the human repeat derived primers used. Human genomic DNA template gives rise to a smear when the A1B (Alu) primer is used but produces a dense series of distinct bands when the L1S (L1) primer is used. This reflects differences in the number and/or organization of Alu and L1 elements in the human genome. When the A1B and L1S primers are used together, human genomic DNA template produces a smear with an average molecular weight lower than that generated by either primer alone, showing that the two primers together amplify a different spectrum of inter-repeat element sequences that is not merely the sum of the Alu and L1 synthesis products.

Cloning of Repeat Element-Mediated PCR Amplification Products

The CMGT hybrid H2CL1 (Brooks-Wilson et al., 1990a), which enriches for the pericentromeric region of chromosome 10, was used as the cloning source for all three types of repeat element-mediated PCR cloning: inter-Alu, inter-L1, and inter-Alu/L1 cloning. H2CL1 retains the chromosome 10 markers FNRB, D10Z1 and RBP3, and no other markers tested.
FIGURE 3: Repeat element-mediated PCR fingerprinting and subchromosomal localization of repeat element-mediated PCR clones. A: PCR products generated using the A1B, L1S or A1B+L1S primers together were separated on a 1.5% agarose gel and stained with ethidium bromide. B: Autoradiograph of the Southern blot of the gel in A. The filter was hybridized with equal counts of each of three $^{32}$P-labelled repeat element-mediated PCR clone inserts: (1) inter-L1 clone pC11/L1S-1-c20 (1.4 Kb), (2) inter-Alu clone pC11/A1S-6-c23 (0.9 Kb), (3) inter-Alu/L1 clone pC11/A1B-L1S-2-c8 (0.5 Kb). DNA size markers (M) are a mixture of HindIII-digested λ DNA and HaeIII-digested φX174 DNA. Template DNAs used in PCR were: 1, human genomic; 2, TRAX10TG3; 3, TRAXK2; 4, 3W4CL5P2 (chromosome 10 in a mouse background); 5, H2CL1; 6, RAG (mouse); 7, CHOK1 (hamster).
PCR amplification products were generated using H2CL1 template DNA and the A1S primer alone, the L1S primer alone, or the A1B and L1S primers together. The resulting products were cleaved with the appropriate restriction enzymes and cloned into SalI or SalI/BamHI digested pUC18.

Twelve different human inter-Alu clones (and one mouse-derived clone) were identified from among 75 colonies picked and analysed. The cloned human DNA fragments varied from 0.3 to 1.8 kb in length. Four of the twelve clones contained repeat sequences, as judged by hybridization with radiolabelled human genomic DNA. Clones identical to ones isolated from the previously described REG3CL4 Alu element-mediated PCR library (Brooks-Wilson et al., 1990a) are excluded from this total. Only two distinct inter-L1 clones were identified from among 200 colonies analysed: a 1.4 kb single copy fragment and a repeat-containing 1.3 kb fragment. Three mouse derived clones were also isolated during the inter-L1 cloning. The analysis of inter-Alu/L1 combination clones was complicated by the presence of inter-Alu and inter-L1 species in the A1B/L1S amplification product. An Alu primer with a BamHI site and an L1 primer with a SalI site were used with the expectation that the PCR products containing both cleavage sites (in oligonucleotide derived sequences) would be inter-Alu/L1 species. It seems that the presence of SalI or BamHI sites in inter-Alu or inter-L1 sequences, respectively; or incomplete double cleavage of the vector pUC18, led to the cloning of a mixture of inter-Alu, inter-L1, and true Alu/L1 combination clones. The criterion used to identify Alu/L1 clones was hybridization to PCR amplification product from somatic cell hybrid templates generated using the A1B and L1S primers together, but not to PCR products made using either of these primers alone. Four Alu/L1
clones were identified, with insert fragments from 0.3 to 2.6 kb; one clone contained repeat sequences.

Subchromosomal Localization of Repeat Element-Mediated PCR Clones

All mapping experiments were performed using Southern blots of repeat element-mediated PCR amplification products synthesized using various somatic cell hybrid and other DNA templates. An example is shown in figure 3B. DNA insert probes from a 0.9-kb inter-Alu clone (#2), a 1.4-kb inter-L1 clone (#1) and a 0.5-kb inter-Alu/L1 clone (#3) were hybridized simultaneously to a Southern blot of the "fingerprint" gel shown in figure 3A. The inter-Alu and inter-L1 clones hybridize to A1B products and L1S products, respectively, as well as to A1B+L1S synthesis products. The inter-Alu/L1 clone hybridizes only to A1B+L1S products.

To map markers, a panel of somatic cell hybrids containing chromosome 10 translocation or derivative chromosomes was used. These chromosomes, the breakpoints of which have been characterized cytogenetically, serve to divide chromosome 10 into eight intervals (figure 4). Markers were mapped to these intervals by scoring their presence or absence in these hybrids. Subsets of these hybrids are employed by other investigators, allowing comparison of hybrid defined map locations of DNA markers, between laboratories.

The panel of hybrids used for regional mapping confirms the chromosome 10 origin of clones and defines eight chromosomal intervals (1, 2, 3, 4a, 4b, 4c, 4d, and 4e), as shown in figure 4. Markers were assigned to these intervals with one ambiguity. Localization to interval 4a or 4c cannot be distinguished using this set of hybrids. The results of the subchromosomal mapping of repeat element-mediated PCR clones from H2CL1 are shown in table IV.
FIGURE 4: Intervals for regional localization of chromosome 10 markers. DNA markers present in the cloning source H2CL1 are shown to the left. The chromosome 10 content of the panel of translocation and derivative chromosome containing hybrids is indicated by vertical bars. The chromosome 10 intervals defined by this set of somatic cell hybrids: 1, 2, 3, 4a, 4b, 4c, 4d, and 4e are shown to the right. Interval 4 (Brooks-Wilson et al., 1990a) was subdivided when hybrids CHOK1-C228, CY6 and CY5 became available.
**TABLE IV: Initial Mapping of Repeat Element-Mediated PCR Clones**

<table>
<thead>
<tr>
<th>Chromosome 10 Interval*</th>
<th>Inter-\textit{Alu} Clones</th>
<th>Inter-L1 Clones</th>
<th>Inter-\textit{Alu}/L1 Clones</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>4a or 4c</td>
<td>4</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>4b</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>4d</td>
<td>5</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>4e</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>total</td>
<td>12</td>
<td>2</td>
<td>4</td>
</tr>
</tbody>
</table>

*The chromosome intervals are: 1, 10pter-p11.2; 2, 10p11.2-cen; 3, 10cen-q11.2; 4a, 10q11.2; 4b, 10q11.2-q22.1; 4c, 10q22.1-q24.3; 4d, 10q24.3-q26.3; and 4e, 10q26.3.
MEN2A is flanked by FNRB in interval 2 and by RBP3 in interval 4a. This places MEN2A in either interval 2, 3, or 4a. No repeat element-mediated PCR clones map to intervals 2 or 3. Six clones, however, (four inter-Alu, one inter-L1, and one inter-Alu/L1) map to either interval 4a or 4c. To identify those which originated from interval 4a, a subset of X-irradiation hybrids, which retain chromosome 10 alphoid repeat sequences (D10Z1) and little or no other human material, was used.

Identification of Clones Mapping to Interval 4a or 4c

Ten X-irradiation reduced complexity hybrids from the "pp" series (Goodfellow et al., 1990b) were used for further mapping of clones derived from interval 4a or 4c. All ten hybrids retain centromeric alphoid repeats (D10Z1) and no other markers tested. Markers mapping very close to the chromosome 10 centromere are likely to be present in a greater number of these "cen-frag" hybrids than markers mapping further away. Thus, it was expected that markers derived from interval 4a would be present in a greater number of these hybrids than those from interval 4c.

The six repeat element-mediated PCR clones from H2CL1 derived from interval 4a or 4c were mapped in the cen-frag hybrids (Table V). Inter-Alu clones pCl1/A1S-6-c23 (D10S94) and pCl1/A1S-6-c65 (D10S182) were present in three of the ten hybrids, pp7a, pp10c and pp11a. Inter-L1 clone pCl1/L1S-1-c20 was present in two hybrids, pp10c and pp12c. The other three clones were not present in any of the cen-frag hybrids. The clones expected to map closest to the centromere are therefore pCl1/A1S-6-c23, pCl1/A1S-6-c65 and pCl1/L1S-1-c20.

Genetic mapping of two of the Alu element-mediated PCR clones revealed that pCl1/A1S-6-c23 mapped between D10Z1 and RBP3 in interval 4a (Goodfellow et al., 1990a), while pCl1/A1S-6-c2 was linked to
TABLE V: Identification of Clones Mapping Near MEN2A by Analysis of Coretention with D10Z1 in X-Irradiation Hybrids Which Contain the Centromere of Chromosome 10 and no Other Human DNA Markers Tested.

<table>
<thead>
<tr>
<th>hybrid</th>
<th>pCl1/A1S-6-c2</th>
<th>pCl1/A1S-6-c23*</th>
<th>pCl1/A1S-6-c60</th>
<th>pCl1/A1S-6-c65*</th>
<th>pCl1/L1S-1-c20</th>
<th>pCl1/A1B/L1S-2-c8</th>
</tr>
</thead>
<tbody>
<tr>
<td>pp4c</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>pp6a</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>pp7a#</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>pp7b</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>pp10c</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>pp11a</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>pp12c</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>pp13c</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>pp14c</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>pp17b</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

+ present, - not present.

*pCl1/A1S-6-c23 defines the D10S94 locus; pCl1/A1S-6-c65 defines the D10S182 locus.

#pp7a did not detectably hybridize with a D10Z1 probe upon its initial characterization (Goodfellow et al., 1990b). Subsequent regrowth of this hybrid produced a line in which this locus is detectable (Miller et al., 1992b).
markers in interval 4c (P. Goodfellow, unpubl.; J. Lichter and K. Kidd, unpubl.). This supports the conjecture that clones derived from close to the centromere were more likely to be coretained with D10Z1 in the cen-frag hybrids. pCl1/A1S-6-c65 (D10S182) was found in the same three cen-frag hybrids as pCl1/A1S-6-c23, and so is predicted to map near D10S94 in interval 4a. pCl1/A1S-6-c60 and pCl1/A1B-L1S-2-c8 are predicted to map to interval 4c, like pCl1/A1S-6-c2. Linkage or an alternative physical mapping technique would be required to definitively map pCl1/L1S-1-c20.

Summary of the map locations of all REM-PCR clones.

Two series of chromosome 10 REM-PCR clones were generated. The first is the series of 15 Alu element-mediated PCR clones generated using CMGT hybrid REG3CL4 as a cloning source (Brooks-Wilson et al., 1990a). Of 15 human clones, 11 mapped to interval 1 and four mapped to interval 4. The somatic cell hybrids that allow subdivision of interval 4 into 4a, 4b, 4c, 4d and 4e were not available at the time of publication of the original mapping results, however, the four clones localized to interval 4 have since then been mapped to the subdivisions of interval 4. The other series is the 18 REM-PCR clones generated from CMGT hybrid H2CL1 (Brooks-Wilson et al., 1992a). Table VI is a summary of the map locations of all of these clones by name. It is included here because clones mapping to regions of chromosome 10 other than the regions where MEN2A may map, could be of use for other, unrelated studies.
TABLE VI: Summary of map locations of all series of REM-PCR clones.

<table>
<thead>
<tr>
<th>Chromosome interval</th>
<th>Clone name*</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>pC14/A1S-2-c1</td>
<td>0.5 kb unique</td>
</tr>
<tr>
<td></td>
<td>pC14/A1S-2-c2</td>
<td>1.8 kb repetitive</td>
</tr>
<tr>
<td></td>
<td>pC14/A1S-2-c6</td>
<td>0.9 kb unique</td>
</tr>
<tr>
<td></td>
<td>pC14/A1S-2-c8</td>
<td>1.1 kb repetitive</td>
</tr>
<tr>
<td></td>
<td>pC14/A1S-2-c15</td>
<td>1.5 kb repetitive</td>
</tr>
<tr>
<td></td>
<td>pC14/A1S-2-c16</td>
<td>1.2 kb unique</td>
</tr>
<tr>
<td></td>
<td>pC14/A1S-2-c19</td>
<td>0.6 kb unique</td>
</tr>
<tr>
<td></td>
<td>pC14/A1S-2-c23</td>
<td>1.3 kb unique</td>
</tr>
<tr>
<td></td>
<td>pC14/A1S-2-c28</td>
<td>0.8 kb unique</td>
</tr>
<tr>
<td></td>
<td>pC14/A1S-2-c40</td>
<td>1.2 kb unique</td>
</tr>
<tr>
<td></td>
<td>pC14/A1S-2-c43</td>
<td>0.5 kb unique</td>
</tr>
<tr>
<td></td>
<td>pC11/A1S-6-c27</td>
<td>0.9 kb unique</td>
</tr>
<tr>
<td></td>
<td>pC11/A1S-6-c29</td>
<td>0.8 kb unique</td>
</tr>
<tr>
<td></td>
<td>pC11/A1S-6-c38</td>
<td>1.2 kb unique</td>
</tr>
<tr>
<td></td>
<td>pC11/L1S-1-c12</td>
<td>1.4 kb repetitive</td>
</tr>
<tr>
<td>2</td>
<td>None</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>None</td>
<td></td>
</tr>
<tr>
<td>4a</td>
<td>pC11/A1S-6-c23</td>
<td>0.9 kb unique</td>
</tr>
<tr>
<td></td>
<td>pC11/A1S-6-c65</td>
<td>0.8 kb repetitive</td>
</tr>
<tr>
<td>4b</td>
<td>pC14/A1S-2-c9</td>
<td>0.7 kb unique</td>
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<tr>
<td></td>
<td>pC14/A1S-2-c12</td>
<td>1.0 kb unique</td>
</tr>
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<td>4c</td>
<td>pC11/A1S-6-c2</td>
<td>1.8 kb repetitive</td>
</tr>
<tr>
<td></td>
<td>pC11/A1S-6-c60</td>
<td>1.3 kb unique (predicted location)</td>
</tr>
<tr>
<td></td>
<td>pC11/A1B-L1S-2-c8</td>
<td>0.3 kb unique (predicted location)</td>
</tr>
<tr>
<td>4a or 4c</td>
<td>pC11/L1S-1-c20</td>
<td>1.3 kb unique</td>
</tr>
<tr>
<td>----------</td>
<td>----------------</td>
<td>--------------</td>
</tr>
<tr>
<td>4 d</td>
<td>pC14/A1S-2-c10</td>
<td>1.4 kb repetitive</td>
</tr>
<tr>
<td></td>
<td>pC14/A1S-2-c35</td>
<td>0.3 kb unique</td>
</tr>
<tr>
<td></td>
<td>pC11/A1S-6-c20</td>
<td>0.5 kb unique (10q25.3-q26.3)(^a)</td>
</tr>
<tr>
<td></td>
<td>pC11/A1S-6-c22</td>
<td>0.5 kb unique</td>
</tr>
<tr>
<td></td>
<td>pC11/A1S-6-c33</td>
<td>0.6 kb unique</td>
</tr>
<tr>
<td></td>
<td>pC11/A1S-6-c51</td>
<td>1.1 kb repetitive (10q24.3-q25.3)(^a)</td>
</tr>
<tr>
<td></td>
<td>pC11/A1S-6-c225</td>
<td>0.3 kb repetitive</td>
</tr>
<tr>
<td></td>
<td>pC11/A1B-L1S-2-c1</td>
<td>0.6 kb unique</td>
</tr>
<tr>
<td></td>
<td>pC11/A1B-L1S-2-c3</td>
<td>2.6 kb repetitive</td>
</tr>
<tr>
<td></td>
<td>pC11/A1B-L1S-2-c4</td>
<td>0.3 kb unique</td>
</tr>
</tbody>
</table>

4 e

None

*Clone names convey the cloning source, type of REM-PCR primer(s) used in their cloning, and individual identifiers. pC11 denotes a clone derived from H2CL1; pC14 denotes one from REG3CL4. Clones are named: /A1S, /L1S or /A1B-L1S to identify the primers used in their generation. The last numbers uniquely identify the clones by ligation and clone numbers.

\(^a\)These two clones were additionally mapped relative to the 10q25.3 breakpoint of a pair of chromosome 10 translocation chromosomes (Dr. P.J. Goodfellow and Helen McDonald, pers. comm.).
Detection of polymorphisms with pC11/A1S-6-c23

A screen for polymorphisms detectable with pC11/A1S-6-c23, the first marker found to map to a chromosomal interval (4a) near MEN2A, was conducted using a panel of genomic DNA samples derived from 47 unrelated individuals, digested with five restriction enzymes. *PvuII* and *RsaI* restriction fragment length polymorphisms were detected (Brooks-Wilson *et al.*, 1990b). The polymorphic locus detected by this probe was designated *D10S94*. pC11/A1S-6-c65 defines *D10S182*. A search for polymorphisms detectable by pC11/A1S-6-c65 was not conducted; expansion of *D10S94* by chromosome walking has shown that *D10S94* and *D10S182* are closely physically linked (see chapter VII).

Linkage studies of *D10S94* with other chromosome 10 markers near MEN2A (*FNRB, D10Z1, RBP3*) and with the disease locus itself, were conducted by Drs. Nancy Simpson (Queens University) and Dr. Paul Goodfellow (Goodfellow *et al.*, 1990a), using the *PvuII* and *RsaI* polymorphisms detected by pC11/A1S-6-c23. *D10S94* maps between *FNRB* and *RBP3*, approximately 5 cM from each flanking marker. It was not demonstrated to recombine with *D10Z1*, the centromeric repeat locus, or with MEN2A. *D10S94* was the first published single copy DNA marker not demonstrated to recombine with MEN2A. *D10S94* could not be assigned to either the short or the long arm of chromosome 10 through linkage analyses alone; a combination of the somatic cell hybrid mapping results, which place this locus in interval 4a within 10q11.2, and the linkage mapping data, however, allowed unambiguous ordering of loci: *FNRB, D10Z1, D10S94, RBP3*. *In situ* hybridization confirmed this localization (Dr. Kenneth K. Kidd, pers. comm.). MEN2A did not recombine with either *D10S94* or *D10Z1* in these studies; the disease locus therefore maps to any of three locations: proximal to *FNRB* on 10p, between *D10Z1* and *D10S94*,
or between \textit{D10S94} and \textit{RBP3}. The usefulness of \textit{D10S94} for diagnostic testing has been increased through the identification of \textit{MspI} and \textit{TaqI} polymorphisms. Primers have been developed for the PCR detection of the \textit{PvuII} and \textit{MspI} RFLPs (Brooks-Wilson \textit{et al.}, 1992b). To date, a single recombinant between \textit{D10Z1} and \textit{D10S94} has been reported (Norum \textit{et al.}, 1991).

**DISCUSSION**

Repeat element-mediated PCR cloning experiments were undertaken in an effort to generate DNA markers near \textit{MEN2A} in the pericentromeric region of chromosome 10. The cloning of 15 \textit{Alu} element-mediated PCR clones had previously been reported (Brooks-Wilson \textit{et al.}, 1990) from the CMGT hybrid REG3CL4 which, like H2CL1, contains the chromosome 10 centromeric marker \textit{D10Z1} as well as markers known to flank \textit{MEN2A}. None of those inter-\textit{Alu} clones mapped to intervals 2, 3, or 4a. The failure to generate markers from the region of interest could be due to a deletion or deletions in REG3CL4, or to an unfavourable distribution of \textit{Alu} elements in the pericentromeric region of this chromosome. An alternate cloning source, H2CL1, which also contains markers \textit{D10Z1}, \textit{FNRB} and \textit{RBP3}, has now been used. In addition, cloning efforts were expanded to include inter-L1 and inter-\textit{Alu}/L1 sequences, which could be more highly represented than clonable inter-\textit{Alu} sequences in this region of the human genome.

The distribution of inter-\textit{Alu} clones obtained from H2CL1 is comparable to that of REG3CL4. No inter-\textit{Alu} clones from intervals 2 or 3 were obtained from either cloning source. None of the 15 clones from REG3CL4 and only two of the 12 inter-\textit{Alu} clones from H2CL1 mapped to
interval 4a. pClI/A1S-6-c23 is not present in REG3CL4, however, indicating that REG3CL4 is deleted for some material from between D10Z1 and RBP3 in interval 4a (data not shown). These similar results obtained for the cloning of inter-Alu sequences from two different somatic cell hybrid cloning sources support the idea that a scarcity of Alu elements in the pericentromeric region of chromosome 10 contributed to the low success rate in generating inter-Alu clones from intervals 2, 3 and 4a.

Both hybrid cloning sources unexpectedly produced inter-Alu clones from the distal long arm of chromosome 10, reflecting the presence of additional chromosome 10 fragments that were not identified during the initial characterization of these CMGT hybrids. If this fragment(s) is richer in Alu elements than is the pericentromeric fragment, this could create a bias towards the cloning of DNA markers from distal 10q rather than from the region of interest.

A second line of evidence supports the conjecture that a scarcity of Alu elements in the pericentromeric region of chromosome 10 contributed to the low success rate in cloning inter-Alu elements from the region. Fluorescence in situ hybridization studies using Alu probes show little hybridization signal in this region of the genome (Korenberg and Rykowski, 1988). This is especially apparent when two-color fluorescence in situ hybridization with D10Z1 and Alu element probes is performed (Dr. Heinz-Ulrich G. Weier, pers. comm.). Alu-PCR products generated from total human DNA template and employed as a fluorescence in situ hybridization probe also show little signal in this region (Baldini and Ward, 1991).

Two inter-L1 isolates were cloned from H2CL1, one mapping in interval 1, the other in either 4a or 4c. The low number of independent clones obtained is consistent with the low complexity of L1 PCR products produced by H2CL1 template DNA (figure 3A, L1S lane 5). The four
Alu/L1 clones from H2CL1 all map to the distal long arm of chromosome 10, again indicating the presence of fragments derived from outside the region of interest, in the hybrid cloning source.

The success of human repeat element-mediated PCR is, like other chromosomal region-specific cloning strategies, highly dependent upon the cloning source employed. These results suggest that the success of this method is related not only to the degree of enrichment for the region of interest, but also to the nature and distribution of repeat elements in that region. These findings may be of practical importance to those planning the cloning of DNA markers from specific subchromosomal regions.

Human repeat element-mediated PCR has been used successfully to generate two DNA markers which map to a chromosome 10 interval that may contain MEN2A. One of these probes defines a polymorphic locus, D10S94, very tightly linked to the disease locus. D10S94 is therefore an ideal starting point for a chromosome walk and long range restriction mapping experiments directed to identifying CpG islands and genes in the MEN2A region.
CHAPTER IV:

A DETAILED PHYSICAL MAP CENTERED AT DIOS94 REVEALS
A CLUSTER OF CpG ISLANDS WITHIN THE MEN2 REGION

INTRODUCTION

CpG islands are short regions of DNA, usually one to two kb in length, that are found at the 5' ends of many mammalian genes (reviewed by Bird, 1986; Bird, 1987). The bulk of mammalian DNA shows a marked suppression of CpG dinucleotide relative to GpC dinucleotide. CpG islands are exceptional in that they are GC rich and do not show this scarcity of CpG dinucleotide. These factors make them about 10 times richer in CpG than most of mammalian genomic DNA. Mammalian DNA is methylated at the cytosine residue of most CpG dinucleotides. The scarcity of this dinucleotide in bulk DNA is thought to have arisen as a consequence of deamination of 5-methylcytosine residues to thymine residues over an evolutionary time scale. CpG islands may have been spared in this process because they are often unmethylated, possibly due to exclusion of the methylase by bound proteins, or because of functional conservation of their sequences. Methylation of a CpG island correlates with inactivity of its associated gene (reviewed by Bird, 1986).

CpG islands occur on average once every 100 kb in the human genome. They can be identified through the use of restriction enzymes for which the recognition sequences contain one or more CpG dinucleotides (Brown and Bird, 1986; Lindsay and Bird, 1987). These enzymes cleave rarely in the human genome and can be used in conjunction with pulsed
field gel electrophoresis to construct long range restriction maps. Points within a long range restriction map where "rare cutter" or "CpG enzyme" restriction sites cluster are considered to be CpG islands. The rare cutting restriction enzymes used in mapping at D10S94 were NotI (GCGGCCGC), SacII (CCGCGG), BssHII (GCGCGC) and SfiI (GGCCNNNNNGGCC, where N=A,T,G or C). Eighty-nine percent of NotI sites, and 74% of SacII or BssHII sites are expected to be found within CpG islands (Lindsay and Bird, 1987). SfiI cuts rarely by virtue of its long recognition sequence; it does not cleave preferentially within CpG islands.

CpG islands are associated with most "housekeeping" genes (genes expressed in most or all tissues) and some tissue specific genes. Construction of long range restriction maps to identify CpG islands that may mark the position of candidate genes for a disease locus has been and is a part of many positional cloning strategies such as those which successfully identified the genes responsible for neurofibromatosis type 1 (Fountain et al., 1989) and Wilms' tumor (Bonetta et al., 1990).

Pulsed field gel electrophoresis (PFGE) is a technique for separating large DNA molecules (currently up to about 6 Mb). It is capable of resolving DNA molecules of greater than about 20 kb that are too large to be sieved through the pores of an agarose gel (Schwartz and Cantor, 1984). If the conformation of a DNA molecule is changed, however, it can 'snake' through the pores of the gel in an end-on manner referred to as reptation (Smith and Cantor, 1986). Pulsed field gel electrophoresis uses an alternating electrical field to induce shape changes in DNA molecules, which will allow them to migrate through the pores of a conventional agarose gel. Upon a change in the direction of the electrical field, the molecules can be thought of as first reorienting, then beginning to move through the gel until bunching up of the molecules interferes with their
motion. Larger molecules take longer to reorient than do smaller ones. Pulse times can be chosen so as to allow separation of the desired size range of DNA molecules. Molecules too large to reorient in the pulse time will remain in an unresolved "compression zone".

Very high molecular weight DNA preparations are necessary for use in long range physical mapping. Isolated DNA is generally of inadequate size to use in these experiments. Instead, DNA is prepared from cells embedded in agarose. The desired number of cells is mixed with low melting point agarose and aliquots are cast in molds. After the "blocks" solidify, the cells within them are treated with proteinase K. Restriction digestions are carried out in the agarose, and whole, unmelted blocks are loaded onto gels for pulsed field gel electrophoresis. This method produces DNA of sufficiently high molecular weight for use in mapping very large DNA fragments.

Long range physical mapping using rare cutting restriction enzymes and pulsed field gel electrophoresis is a powerful method of defining the locations of CpG islands that may indicate the positions of genes. A search for CpG islands was undertaken at D10S94 as part of a positional cloning effort to identify the MEN 2 gene(s). D10S94 is very tightly linked to MEN2A. It is also located within the region of overlap of the candidate regions for MEN2A, MEN2B, MTC1 and MEN2A+CLA. Efforts to refine the MEN2A candidate interval are ongoing but time consuming, and are dependent upon the identification of new flanking markers defined by rare critical crossovers in the MEN2 region. Such studies have refined the MEN2A candidate region (Miller et al., 1992a; Miller, 1992; Lairmore et al., 1993), but D10S94 remained within the candidate area. D10S94 therefore provided an ideal starting point for long range restriction mapping and chromosome walking experiments directed to the
identification and cloning of candidate genes for these dominantly inherited cancers.

Pulsed field gel electrophoresis, rare cutting restriction enzymes and a series of DNA probes generated during a 160-kb cosmid walk at \(D10S94\) have been employed to produce a long range physical map spanning 570 kb at \(D10S94\). Six CpG islands were detected, five of them within a 145-kb \(N_{ot}I\) fragment spanned by the cosmid contig. This dense cluster of CpG islands suggests the presence of multiple genes at \(D10S94\), each of which may be considered a candidate for \(MEN2A, MEN2B, MTC1\) and/or \(MEN2A+CLA\).

RESULTS

A bidirectional chromosome walk at \(D10S94\)

The DNA probes used in long range restriction mapping were generated in the Goodfellow laboratory during a 160-kb bidirectional cosmid walk from pClI/A1S-6-c23, the \(Alu\) element-mediated PCR probe which originally defined \(D10S94\). Cosmid walking was performed mainly by Mr. Duane Smailus. A series of recombinant cosmids that make up the chromosome walk served as the source of cloned probes and allowed comparison of the restriction maps of genomic and cloned DNA at this locus. A map of the cosmid contig (represented by six cosmids that span the cloned region: 1-1a, 2-4a-1, 4-1a, 6-8a-1, 6-11a-5 and 1-6a) is shown in figure 5A. The starting point of the walk was pClI/A1S-6-c23 (figure 5A) which defines the polymorphic \(D10S94\) locus (Brooks-Wilson et al., 1990b). Cosmids were isolated from a commercially available human male lymphocyte DNA library (Stratagene) in cosmid vector pWE15. Cosmid 4-1a was included in analyses because it overlaps significantly with an
unstable recombinant, 2-4a-1. Attempts to obtain a cosmid extending to the right of 1-1a in the pWE15 library were unsuccessful. Cosmid pur-1-1 was isolated from a human male genomic library in cosmid vector pcos1EMBL (gift of Dr. A.-M. Frischaufl). The six step (not including 4-1a) cosmid contig spans approximately 160 kb. The walk was not oriented with respect to the centromere and telomere of the chromosome.

All cosmids were restriction mapped with the rare cutting enzymes used in long range mapping: NotI, SacII and SfiI. In addition, all the cosmids, with the exception of 2-4a-1 were mapped with BssHII (figure 5A). Additional rare cutting restriction sites were detected by sequence analysis of subclones derived from cosmid DNA (see below).

**A long range restriction map at D10S94**

Long range restriction mapping at D10S94 resulted in the identification of six CpG islands within a 180 kb region. The locations of DNA probes generated during the chromosome walk, which were employed in mapping (p0 to p7; see table III) are indicated in figure 5B.

DNA derived from lymphoblasts from an individual (AC) from an MEN 2A family was used for mapping with NotI, SacII and SfiI in single and double digestions. Pulsed field gel electrophoresis was performed using an LKB Pulsaphor apparatus with a hexagonal electrode array. A single Southern blot (blot A, figure 6A) was probed sequentially with each of the probes (p0 to p7) such that the resulting autoradiographs could be overlaid for direct comparison of the restriction fragments detected. The sizes of the observed restriction fragments are presented in table VII. Probes p1 to p6 detect a 145-kb NotI fragment, the ends of which fall within CpG islands #1 and #5 (figure 5). The NotI site in island #1 has
FIGURE 5: A Physical map at D10S94. A. The cosmid contig indicating restriction enzyme recognition sites detected in cloned DNA. The starting point of the chromosome walk, pC11/A1S-6-c23, is indicated by an asterisk. B. Rare restriction sites detected in lymphoblast DNA. p indicates partial cleavage. p0 to p7 are probes used in PFGE mapping. The cosmid and genomic restriction maps are drawn to the same scale; the dashed section to the left is not drawn to scale. CpG islands #1 to #6 are indicated by triangles. Restriction site abbreviations are: N = NotI, S = SacII, X = SfiI, B = BssHII.
FIGURE 6: Long range mapping at D10S94 by PFGE. A. Autoradiograph of PFGE blot A probed with p2. Lymphoblast DNA from an individual (AC) singly and doubly digested with the restriction enzymes indicated. A four day exposure is shown. Fragment sizes in kilobase pairs are to the left. B. Autoradiograph of PFGE blot B probed with p2. DNAs are from somatic cell hybrids in which the chromosome 10 homologues of a single individual have been segregated (AC10A and AC8D), lymphoblasts from the same person (AC), and CHOK1 hamster cells. Restriction digests are as indicated. A five day exposure is shown. Fragment sizes in kilobase pairs are to the left.
TABLE VII: Sizes of DNA fragments detected by probes from D10S94.

<table>
<thead>
<tr>
<th>PROBE:</th>
<th>p0</th>
<th>p1</th>
<th>p2</th>
<th>p3</th>
<th>p4</th>
<th>p5</th>
<th>p6</th>
<th>p7</th>
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<tbody>
<tr>
<td>ENZYME(S)</td>
<td>Size of fragment(s) in kilobase pairs*</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>NotI</td>
<td>20†</td>
<td>145</td>
<td>145</td>
<td>145</td>
<td>145</td>
<td>145</td>
<td>145</td>
<td>(29)</td>
</tr>
<tr>
<td>NotI/SacII</td>
<td>ND</td>
<td>8</td>
<td>30</td>
<td>30</td>
<td>58,</td>
<td>58</td>
<td>30</td>
<td>29</td>
</tr>
<tr>
<td>SacII</td>
<td>ND</td>
<td>9</td>
<td>30</td>
<td>30</td>
<td>58,</td>
<td>58</td>
<td>30</td>
<td>29</td>
</tr>
<tr>
<td>SacII/SfiI</td>
<td>ND</td>
<td>9</td>
<td>17</td>
<td>22</td>
<td>23</td>
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<td>SfiI</td>
<td>ND</td>
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<td>75</td>
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<td>37</td>
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<td>18</td>
</tr>
<tr>
<td>NotI/SfiI</td>
<td>ND</td>
<td>25</td>
<td>25</td>
<td>37</td>
<td>37</td>
<td>63</td>
<td>63</td>
<td>18</td>
</tr>
</tbody>
</table>

*Fragments detected by different probes and listed as the same size are identical as judged by overlaying of autoradiographs generated by sequential hybridization of probes to the same blot (blot A, figure 6).
†Size estimated from a different blot.
Bracketed fragment sizes indicate faint bands resulting from partial digestion.
been arbitrarily designated as 0 kb. The entire 145 kb NotI fragment has been cloned in cosmids.

Probe p7 detects three NotI fragments, a major band of 260 kb and fainter bands of 29 kb and 375 kb. Probes from within the 145-kb NotI interval do not detect any of these NotI restriction fragments. They are likely to result from partial restriction of NotI sites at map positions -175 kb, -405 kb and -520 kb. Probe p7 detects a single SacII fragment of 29 kb. This is the same size as the smallest of the NotI partial restriction products observed with p7. A CpG island (island #6) containing an unmethylated SacII site and a NotI site subject to partial cleavage is therefore located at -175 kb, outside the cloned region.

Probe p0 detects a 20-kb NotI fragment. The rightward end of this fragment may fall within another CpG island, which is not in the cloned region. Further mapping experiments will be required to determine whether or not CpG islands are located at map positions 20 kb, -405 kb and -520 kb.

Islands #2, #3 and #4 are detected in long range mapping experiments by digestible (unmethylated) SacII sites, and are within the 145 kb NotI interval. One SacII site that fails to digest fully lies between p4 and p6. It was detected by the presence of a faint 24-kb band in addition to the 58-kb major band observed upon hybridization with p4. Methylation of this SacII site was confirmed by conventional Southern blot analysis of EcoRI/SacII double digestion of AC DNA (not shown). The sum of the sizes of the different SacII fragments detected by probes p1 to p6 is approximately 145 kb. SfiI generally cuts outside CpG islands. SfiI single and SfiI/NotI and SfiI/SacII double digestions confirm the order and arrangement of NotI and SacII restriction fragments. Probe p6 spans an SfiI site at position -125 kb on the long range map. Most of the probe
length, however, falls to the right of the SfiI site and only the rightward, 63-kb SfiI band is observed upon p6 hybridization.

Mapping experiments were also performed using somatic cell hybrids (AC8D and AC10A) in which the different copies of chromosome 10 from individual AC were segregated, with the intention of examining separately the long range maps of the mutation bearing and normal chromosome 10 homologues of an MEN 2A patient. AC is a member of an MEN 2A family and had been deemed affected based on elevated serum calcitonin levels and the observation of C cell hyperplasia upon thyroidectomy. A report that C cell hyperplasia was not uncommon in the general population, however, and could be as high as 10% (Landsvater et al., 1993; Howieson Gibson et al., 1981), prompted a re-examination of AC's genetic disease status by means of haplotype analysis. AC was found not to carry the haplotype that segregates with disease in this MEN 2A family (P.J. Goodfellow, unpublished). Mapping in the segregated chromosome 10 homologues of AC does not fulfill the original objective of allowing comparison of an affected to an unaffected chromosome; it has the potential, however, to reveal polymorphisms and methylation differences at rare cutter restriction sites.

A representative hybridization to a Southern blot (blot B) including AC10A, AC8D, AC lymphoblast and CHOK1 hamster DNA is shown in figure 6B. No differences (between chromosome 10 homologues) in the size of fragments detected with any of the probes tested (p0, p1, p2, p3, p4, p5 and p6) were observed. Note that apparent band size differences between AC10A and AC8D (figure 6B) do not correspond to a doublet in AC lymphoblast DNA and so must be due to sample or PFGE artifacts. Hybridization of probe p7 to blot B failed to produce adequate signals for fragment size comparison. Hybridization of this probe to a PFGE blot of
NotI-digested DNA from 12 individuals produced bands of approximately 29 kb, 260 kb and 375 kb in all cases (not shown). The use of two blots in mapping also allows two independent comparisons of fragment size for the single enzyme digests.

The genomic and cosmid restriction maps are concordant. Where an unmethylated NotI, SacII or SfiI site was identified in genomic DNA, the same site was found to be present within the cosmid contig.

A combination of long range restriction mapping in genomic DNA and cosmid restriction mapping has been used to identify CpG islands. Each of the islands identified includes multiple CpG-containing restriction enzyme sites (SacII, NotI or BssHII), at least one of which is unmethylated in genomic DNA. Six CpG islands are present within a 180-kb region as shown in figure 5: five within the area spanned by the cosmid contig (CpG islands #1 to #5) and one at -175 kb (CpG island #6). In addition to the restriction sites indicated in figure 6A, DNA sequence analysis revealed NruI, HpaII and NarI sites at island #4 (Helen McDonald, pers. comm.).

DISCUSSION

A 570-kb physical map was generated at D10S94. DNA probes derived during a chromosome walk and restriction mapping using rare cutting restriction enzymes and pulsed field gel electrophoresis has revealed an exceptionally dense cluster of six CpG islands within 180 kb at D10S94. Five of these islands have been cloned as part of a cosmid contig. The cloned islands are marked by multiple CpG dinucleotide-containing restriction enzyme recognition sites within two kb. At least one of these sites (SacII or NotI) is unmethylated in lymphoblast DNA. The rare cutting restriction enzyme maps of genomic and cloned DNA are concordant; no
potential islands were detected in cosmid DNA that were not apparent in lymphoblast DNA. The restriction sites indicated on the long range map are unmethylated in at least two cell types (lymphoblast and human/rodent somatic cell hybrid cell lines); it is likely that they mark the position of transcribed genes. The extent of this apparently gene-rich region remains to be determined.

One gene, mcs94-1, associated with CpG island #2 has been characterized by McDonald and colleagues (1992). It encodes a putative polypeptide with amino acid similarity to nucleolin, a major nucleolar protein. A search for evolutionarily conserved sequences and transcription units is likely to reveal additional genes associated with CpG islands at D10S94. These experiments have been undertaken by other members of the Goodfellow laboratory.

D10S94 maps within the candidate regions for MEN2A, MEN2B, or MTC1. On the basis of location, any gene at D10S94 represents a candidate gene for any of these three diseases. Recombination is repressed relative to physical distance in the pericentromeric region of chromosome 10 (Lichter et al., 1991a), to which these loci have been localized. This makes it difficult not only to order loci on the chromosome but to estimate the distances between them. Physical mapping of the pericentromeric region will allow determination of the size of this region, which otherwise cannot be measured accurately. One means of identifying the MEN2A, MEN2B, and MTC1 genes is through the isolation, characterization and search for mutations in patients' DNA, of all genes within the genetically defined region.

Highly tissue-specific genes usually lack CpG islands (Bird, 1986). If the MEN2A, MEN2B, MTC1 and MEN2A+CLA genes are expressed in only a very limited set of tissues, they may lack these landmarks of genes.
Alternative gene identification strategies such as exon trapping (Duyk et al., 1990) or exon amplification (Buckler et al., 1991), or a search for conserved sequences (with or without regard for proximity to CpG islands) may be necessary. Hybridization-based strategies for entrapment of cDNAs (Lovett et al., 1991; Parimoo et al., 1991) could be useful in either case.

The characterization of this cluster of potential genes at D10S94 is of practical importance in the search for the gene(s) responsible for MEN 2A, MEN 2B and MTC1. It will also be of value in the construction of a physical map of the pericentromeric region of chromosome 10.
CHAPTER V:

A SURVEY OF A CpG ISLAND-RICH REGION AT D10S94 IN THE GENOMES OF MEN 2 PATIENTS FOR POTENTIALLY DISEASE-CAUSING ALTERATIONS DETECTABLE BY FIELD INVERSION GEL ELECTROPHORESIS

INTRODUCTION

The region near D10S94 is very rich in CpG islands, and potentially very rich in genes. Any gene at D10S94 can be considered a candidate for MEN2A, MEN2B, MTC1 and/or MEN2A+CLA on the basis of location alone. The physically mapped region at D10S94, however, is only a small part of the MEN2 candidate region. Identification of all transcribed sequences and comparison of the sequences of exons of candidate genes between affected and unaffected individuals is a very thorough but time consuming method of establishing or eliminating individual genes as candidates for the disease genes. Another approach is to survey portions of the candidate region in patients' genomes; then, if an alteration is found, isolate a gene or genes associated with that mutation.

One model put forward as a possible genetic basis for the MEN 2A and MEN 2B cancer syndromes is that MEN 2 is a contiguous gene syndrome in which different mutations affecting adjacent genes result in different phenotypes (Jackson et al., 1989). For example, MEN 2A could result from a deletion involving genes important in parathyroid hyperplasia, MTC and pheochromocytoma, whereas in MEN 2B a set of genes important in MTC, pheochromocytoma and mucosal neuromas could be affected. If such a hypothesis was correct, the mutations underlying MEN 2 would be expected to be more extensive than if the causative
mutations were as subtle as different point mutations in the same or different genes. Such mutations could be easier to detect than very localized sequence alterations like point mutations. The dense cluster of CpG islands at \textit{D10S94}, if the CpG islands do mark the positions of genes, seems to lend itself to the notion that the inherited cancer syndromes represent a contiguous gene syndrome. Regardless of whether or not MEN 2 is a contiguous gene syndrome, it is possible that a proportion of the MEN 2 mutations are sequence alterations that affect a relatively large region of DNA, rather than subtle defects. A broad survey for rearrangements could serve to identify some MEN 2 mutations.

Long range restriction mapping is capable of detecting rearrangements that are not visible cytogenetically. This technique has been used successfully to detect deletions in the genomes of Duchenne muscular dystrophy (den Dunnen \textit{et al.}, 1987) and neurofibromatosis type 1 patients (Viskochil \textit{et al.}, 1990). The larger the DNA fragment examined, the larger the alteration must be, to be detectable using pulsed field gel electrophoresis. Thus, smaller fragments are examined in effectively greater detail than are larger fragments.

To ask whether rearrangements involving \textit{D10S94} are involved in MEN 2A, MEN 2B, or MTC 1; this region was surveyed, in the genomes of affected individuals, for genetic alterations. The long range restriction maps of normal and affected individuals were compared. Each patient sample also has an internal control: presence of the normal, non-MEN 2 allele-bearing chromosome 10 homologue. Because \textit{D10S94} has a high density of CpG islands, \textit{NotI} and \textit{SacII} fragments in this region are relatively small. This has allowed a relatively detailed examination of this region of the genome in patient DNAs. It was not intended to be an exhaustive search for mutations, but a survey of \textit{D10S94} for alterations
detectable by this technique. Isolation of individual candidate genes associated with CpG islands at *D10S94* is an ongoing goal of other members of the Goodfellow laboratory; a gene, mcs94-1, associated with CpG island #2 has been described (McDonald *et al.*, 1992).

The experiments described in this chapter employ lymphoblast DNA from MEN 2 patients in a search for potentially disease-causing alterations. Because lymphocytes are not apparently involved in the clinical manifestation of MEN 2, the genomes of these cells can be considered to represent the inherited defect of the MEN 2 patient on a background of a 'wild type' copy of chromosome 10. It is the inherited mutation that governs the clinical presentation of disease. Examination of constitutional (lymphoblast) DNA, then, will reveal the relationship between the genes responsible for MEN 2A, MEN 2B and MTC 1.

There are several types of PFGE. Field inversion gel electrophoresis (FIGE) is a variation in which molecules are pulsed back and forth in one dimension, net pulse time being greater in the forward direction. Pulse time and other parameters are varied to achieve separation of different size ranges of DNA molecules. This type of PFGE has the advantage of generally producing straighter lanes than do other forms of PFGE. A prototype FIGE apparatus provided by Q-Life Systems, Inc., of Kingston, Ontario, was used to perform physical mapping at *D10S94* in the genomes of MEN 2A and MEN 2B patients and unaffected controls. No MEN 2A + CLA or MTC 1 patients are included in these analyses. Additional goals of this work were to determine both the quality of separation afforded by this FIGE system and the minimum difference in restriction fragment size detectable by this method.
The cloning and characterization of mutations underlying MEN 2A, MEN 2B and MTC 1 would clarify the relationship between the three diseases. It would also help us better understand how these cancers arise.

RESULTS:

Patients and families.

To date there is no evidence for genetic heterogeneity in MEN 2A or MEN 2B (Kidd and Simpson, 1990). MEN 2A patient DNAs examined in these experiments were derived from families whose defect shows chromosome 10 linkage. Cell lines from several MEN 2B new mutants and their parents were also used. These were a gift of Dr. Charles E. Jackson of the Henry Ford Hospital in Detroit. The MEN 2B new mutation resources are particularly valuable for several reasons: 1) if the extent of rearrangement was to correlate with the severity of disease (as in a contiguous gene syndrome), MEN 2B mutations may be more easily detected than those of MEN 2A; 2) the mucosal neuromas of MEN 2B patients appear early and are easily recognized, making diagnosis unequivocal; 3) alterations present in the new mutant but not in either parent are mutations rather than polymorphisms; and 4) these patients represent independent mutations. These considerations together make these MEN 2B new mutants a valuable resource in the search for the disease gene(s). It should be considered, however, that the possibility that such new disease cases could result from mutations in genes other than those predisposing to inherited MEN 2B, cannot be ruled out.

The lymphoblast cell lines available for FIGE analysis represent individuals from five MEN 2A families, and eight MEN 2B new mutants.
Table VIII summarizes the affected individuals and unaffected controls, disease and family.

Assessing the resolution achieved by a prototype FIGE apparatus for DNA of up to 200 kb.

The prototype FIGE apparatus AutoBase module AB 009 uses preprogrammed ROM cards to resolve DNA fragments of specific molecular weight ranges. Ten programs of general utility in PFGE analysis are available. In addition, Q-Life Systems will design specific programs for particular, special applications. The Goodfellow laboratory was a test site for the AutoBase module, and both the preprogrammed ROM cards and the specialty ROM card service were tested in the following sets of experiments.

ROM cards #1 and #2 are preprogrammed to resolve DNA fragments of 8 - 50 kb and 8 - 200 kb, respectively. To test the ability of these two programs to achieve separation of DNA fragments in these size ranges, a number of DNA size standards were subjected to FIGE using these programs.

Figure 7A and B are photographs of FIGE gels run using ROM cards #1 and #2, respectively. The DNA standards 4.9 kb ladder (BIO-RAD) and 1 kb ladder (for gel A only [BRL]) were chosen to illustrate the quality of resolution over a wide DNA size range. \(\lambda\) HindIII-digested DNA and linear \(\lambda\) DNA were also employed as size standards. Standards were size-separated either alone or mixed with CHOK1 (hamster) genomic DNA digested in an agarose block with SacII or SfiI (the digested DNA block was melted to facilitate mixing with the size standard). The mixing experiments were done to assess whether the quality of separation was affected by DNA quantity and complexity. The marker plus genomic DNA
TABLE VIII: MEN 2 patient and control DNAs examined by FIGE for potentially disease-causing alterations at *D10S94*.

<table>
<thead>
<tr>
<th>Individual</th>
<th>Disease</th>
<th>Family</th>
<th>Reference</th>
<th>Het. at <em>D10S94</em>&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>KW</td>
<td>MEN 2A-1 W</td>
<td>Keiser et al., 1973</td>
<td>Cousin, TaqI</td>
<td></td>
</tr>
<tr>
<td>SM</td>
<td>MEN 2A-2 R (or K)</td>
<td>Partington et al., 1981</td>
<td>Mother, PvuII</td>
<td></td>
</tr>
<tr>
<td>CP or KP</td>
<td>MEN 2A&lt;sup&gt;b&lt;/sup&gt;</td>
<td>P</td>
<td>P.J. Goodfellow and D. Gilchrist, pers. comm.</td>
<td>PvuII</td>
</tr>
<tr>
<td>YC</td>
<td>MEN 2A-1 B</td>
<td>Carson et al., 1990</td>
<td>TaqI</td>
<td></td>
</tr>
<tr>
<td>RC</td>
<td>MEN 2A-3 S</td>
<td>Jackson et al., 1973</td>
<td>RsaI</td>
<td></td>
</tr>
</tbody>
</table>

**MEN 2B new mutants**

<table>
<thead>
<tr>
<th>JT or CT&lt;sup&gt;c&lt;/sup&gt;</th>
<th>*</th>
<th>*</th>
<th>*&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>DM</td>
<td>*</td>
<td>*</td>
<td>PvuII, MspI</td>
</tr>
<tr>
<td>KS</td>
<td>*</td>
<td>*</td>
<td>MspI</td>
</tr>
<tr>
<td>CA</td>
<td>*</td>
<td>*</td>
<td>--</td>
</tr>
<tr>
<td>RW</td>
<td>*</td>
<td>*</td>
<td>--</td>
</tr>
<tr>
<td>AJ</td>
<td>*</td>
<td>*</td>
<td>--</td>
</tr>
<tr>
<td>MA</td>
<td>*</td>
<td>*</td>
<td>--</td>
</tr>
<tr>
<td>DeJ</td>
<td>*</td>
<td>*</td>
<td>PvuII, TaqI</td>
</tr>
</tbody>
</table>

**Unaffected controls**

<table>
<thead>
<tr>
<th>DaJ</th>
<th>father of DeJ</th>
<th>*</th>
<th>N/A</th>
</tr>
</thead>
<tbody>
<tr>
<td>AC</td>
<td>C</td>
<td>Birt et al., 1977; Duncan and Greenberg, 1986</td>
<td>N/A</td>
</tr>
<tr>
<td>HK</td>
<td>R (or K)</td>
<td>N/A</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup>Heterozygous for polymorphisms at *D10S94*, -- = The individual was not heterozygous at any sites tested. In two cases, homozygous individuals had an affected relative heterozygous for a *D10S94* polymorphism.

<sup>b</sup>It is unknown if members of this family develop parathyroid hyperplasia.

<sup>c</sup>JT and CT are the affected progeny of a new mutant, their deceased mother.

*Cell lines derived from MEN 2B new mutants and their parents were made available by Dr. Charles E. Jackson.

N/A = not applicable.
FIGURE 7: DNA size standards illustrate the level of resolution achieved by the AutoBase system in the 8 - 50 kb and 8 - 200 kb size ranges. (A): DNA size standards separated on a FIGE gel run using AutoBase ROM card #1 (for 8 - 50 kb). Lane 1, chromosomes of yeast strain YPH149 (the smallest chromosome is 90 kb); lane 2, linear λ DNA mixed with SacII-digested CHOK1 DNA; lane 3, λ DNA; lane 4, λ HindIII digest mixed with SacII-digested CHOK1 DNA; lane 5, λ HindIII digest; lane 6, 1-kb ladder mixed with SacII-digested CHOK1 DNA; lane 7, 1 kb ladder; lane 8, 4.9-kb ladder mixed with SacII-digested CHOK1 DNA; lane 9, 4.9-kb ladder. The λ DNA in lane 2 appears to migrate in the compression zone. The sizes of the 1-kb ladder bands visible on gel A are: 1.0, 1.6, 2.0, 3.1, 4.1, 5.1, 6.1, 7.1, 8.1, 9.2, 10.2, 11.2, and 12.2 kb. Sizes of the 4.9-kb ladder bands are multiples of 4.9 kb. (B): DNA size standards separated on a FIGE gel run using AutoBase ROM card #2 (for 8 - 200 kb). Lane 1, chromosomes of yeast strain YNN295 (the smallest yeast chromosome of this strain is 245 kb); lane 2, chromosomes of yeast strain HY1 (the smallest chromosome is a 120-kb YAC); lane 3, chromosomes of yeast strain YPH149 (the smallest chromosome is a fragmentation product of 90 kb); lane 4, λ concatamers (see text for explanation of extra bands between the monomeric 48.5 kb and dimeric 97.0 kb bands); lane 5, λ DNA; lane 6, λ DNA mixed with SfiI-digested CHOK1 DNA; lane 7, λ HindIII digest; lane 8, λ HindIII digest mixed with SfiI-digested CHOK1 DNA; lane 9, 4.9-kb ladder; lane 10, 4.9-kb ladder mixed with SfiI-digested CHOK1 DNA. CZ = The compression zone containing DNA fragments too large to be resolved using these FIGE programs. Positions and sizes of λ-based standards are indicated to the left of each gel. The smallest band of the 4.9-kb ladder is indicated to the right of each gel.
samples should mimic resolution of a DNA fragment within a restriction digest of genomic DNA. A number of yeast chromosomes were used on gel B as additional molecular weight markers.

The presence of hamster genomic DNA does not appear to alter the migration of the size standard fragments, although the linear λ DNA mixed with \textit{SacII}-digested CHOK1 (lane 1, figure 7A) appears to migrate in the compression zone (see below).

ROM card #1 is expected to provide good resolution of DNA fragments from 8 to 50 kb. The 49.0-kb band of the 4.9 kb ladder of gel A (figure 7), for example, is separated from the 44.1-kb band by 5 mm. Estimating conservatively that bands differing in migration by 2 mm or more would be discernible on an autoradiograph produced by hybridization of a Southern blot of such a FIGE gel, it follows that an alteration that changes band size by approximately 2 kb or more (2 mm x 4.9 kb/5 mm) would be detectable for a fragment in this size range. Two kb represents 4% of the size of a 46.6-kb band (calculating percentage using 46.6 kb because this is halfway between 44.1 and 49.0 kb). Analogous calculations for the low end of the separation range for card #1 show that a 9% difference in band size would be detectable for a fragment of 7.6 kb (the halfway point between the 7.1-kb and 8.1-kb bands of the 1-kb ladder). A difference of 5% for a 27-kb band is expected to be detectable.

ROM card #2 is intended to provide good resolution of DNA fragments from 8 to 200 kb. Calculating from the migration distances of the standard bands of gel B (figure 7), and again assuming that a band migration difference of 2 mm would be discernible, it is found that a 4% difference in size for a 7.4-kb or for a 121-kb band, and a 2.3% difference in size for a 218-kb band would be detectable. Thus, although it seems
that the standard bands are more widely separated at the low molecular weight ends of both gels, this does not directly illustrate the quality of separation achieved for different sizes of bands for these FIGE programs.

A 27.5-kb λ HindIII band is present on both gels. It results from the annealing of the cos ends of the 23.1- and 4.4-kb λ HindIII fragments. The 27.5-kb annealed fragment is resolved from the 23.1-kb fragment on both these FIGE gels, and is itself a useful size marker.

Several unexpected bands are present in the λ concatamer DNA standard (lane 4) of gel B. Three unexpected bands migrate between the 48.5-kb linear λ monomer and the 97.0-kb linear λ dimer. These extra bands seem to accumulate in this size standard preparation upon storage at 4°C. I suggest that these bands represent cos-annealed circular λ monomers, dimers, or multimers. Such molecules could migrate at different positions on the gel from their linear counterparts, analogous to the difference in migration between linear and relaxed circular plasmid molecules observed upon conventional agarose gel electrophoresis of some plasmid DNA preparations. Regardless of whether this suggestion is correct, it may be prudent to use λ concatamer standards in combination with other high molecular weight DNA standards, such as yeast chromosomal size standards, to aid in the identification of bands corresponding to the linear λ monomer, linear λ dimer, etc. Mixing with restriction digested genomic DNA may increase annealing of the λ cos ends. Comparison of lanes 4 and 5 of figure 7A reveals that less 23.1-kb band is present in the CHOK1-mixed sample in lane 4 than in the unmixed sample in lane 5. The linear λ that migrated within the compression zone of lane 2 of this gel may have done so because of having been circularized through cos annealing in the CHOK1-mixed sample.
Long range restriction mapping in patient DNA.

Individuals with MEN 2 are constitutionally heterozygous with respect to their MEN 2 mutations. DNA fragments derived from a normal copy of chromosome 10 serve as an important internal control for comparison with those from the mutation-bearing chromosome. Alterations in the MEN 2 chromosome homologue could result in a different band size than that produced by the normal chromosome. If the altered fragment were detectably different in size from that of the normal chromosome, then two bands would be observed: one derived from the normal chromosome and another derived from the mutation bearing chromosome.

The physical map previously generated at D10S94 by long range restriction mapping is reproduced in figure 8. The 145-kb NotI fragment and its flanking NotI fragments, as well as all SacII and SfiI fragments within or overlapping the 145-kb NotI fragment, were examined for detectable alterations. This provided redundancy in the examination of the CpG island-rich central NotI fragment.

Patient and control DNAs in agarose blocks were digested with NotI, SacII or SfiI, and then size-separated, along with chromosomal size standards, using ROM cards providing resolution in the appropriate molecular weight range. Southern blots of the FIGE "mutation hunting" gels were hybridized with radiolabeled probes derived from D10S94 and, in addition, with radiolabeled λ DNA. The hybridization of λ DNA to the blots allowed accurate determination of the mobility of λ concatamers and λ HindIII restriction fragments used as size standards on the FIGE gels. The resulting pattern of hybridization was then used to establish a standard curve of fragment size vs. distance migrated for each FIGE gel. Sizes of genomic bands were read from the standard curves.
FIGURE 8: A modified physical map at D10S94 that incorporates FIGE data. A: The cosmid contig indicating restriction enzyme recognition sites detected in cloned DNA. B: The original PFGE map, indicating restriction sites detected in lymphoblast DNA. p indicates partial cleavage. Restriction site abbreviations are: N = NotI, S = SacII, X = SfiI, B = BssHII. C: Modifications made to the map based on FIGE data. Horizontal lines and indicated sizes represent the SacII or SfiI bands detected in the FIGE analyses. Two SacII sites subject to incomplete digestion (Sp*) have been incorporated into the map. The site at position -35 is now indicated as fully methylated (Sm*). CpG island #3 has been moved 9 kb to the left. The estimated maximum non-detectable alteration for each SacII or SfiI band, for which these analyses could be done, is listed in brackets under the FIGE-determined fragment size.
Patient samples digested with *SacII* were resolved in the 8 to 50 kb range using ROM card #1, blotted, and hybridized with *D10S94* probes. The standard curves for the FIGE gels of patient DNAs digested with *SacII* (size-separated using ROM card #1) or *SfiI* (size-separated using ROM card #2) were very similar to those of standard gels A and B, respectively. The sizes of *SacII* restriction fragments detected by these probes are listed in table IX.

The relative sizes of the *SacII* bands detected by *D10S94* probes in these experiments are in agreement with those observed in the previous chapter. In addition, a number of bands not previously noted were observed. A modified physical map that incorporates the FIGE data is shown in figure 8. *p5* detects a 4-kb *SacII* band that reflects the presence of an incompletely cleaving *SacII* site on the left side (as shown in figure 8) of *p5*. Failure of the *SacII* site to the right of *p5* to be cleaved results in the production of a 24-kb band that can be detected by both *p5* and *p4*. When this site was cleaved, a 21-kb fragment detectable with *p4* was produced. This FIGE gel appears to afford greater resolution than gel A (figure 6) of chapter IV; *p4*, for example, detects 21- and 24-kb fragments (table IX) which are likely to correspond to the wide 24 kb-band noted previously.

Conventional genomic Southern blot and hybridization analysis and restriction mapping have helped to clarify the origins of the "new" *SacII* bands. *p5* detects a 3.9 kb *SacII* fragment on Southern blots of conventional agarose gels. Digestion of genomic DNAs with a methylation insensitive restriction endonuclease (*EcoRI*) in combination with the methylation sensitive restriction enzyme *SacII*, determined the extent of restriction digestion possible at these *SacII* sites (data not shown). The results of these experiments were consistent with the observation of a
TABLE IX: Sizes of DNA fragments detected by *D10S94* probes in MEN 2 patient and control DNAs resolved by FIGE.

<table>
<thead>
<tr>
<th>PROBE:</th>
<th>p0</th>
<th>p1</th>
<th>p2</th>
<th>p4</th>
<th>p5</th>
<th>p6</th>
<th>p7</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>ENZYME</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>NotI</em></td>
<td>230</td>
<td>ND</td>
<td>230</td>
<td>ND</td>
<td>ND</td>
<td>230</td>
<td>230</td>
</tr>
<tr>
<td></td>
<td>160</td>
<td></td>
<td>(160)</td>
<td></td>
<td>(160)</td>
<td></td>
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<tr>
<td></td>
<td>148</td>
<td></td>
<td>148</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>very small&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>SacII</em></td>
<td>ND</td>
<td>(90)</td>
<td>90</td>
<td>90</td>
<td>90</td>
<td>90</td>
<td></td>
</tr>
<tr>
<td></td>
<td>68</td>
<td>68</td>
<td>68</td>
<td>60</td>
<td>60</td>
<td>60</td>
<td>39&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>34</td>
<td>(24)</td>
<td>(24)</td>
<td></td>
<td></td>
<td></td>
<td>39&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>11</td>
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<td></td>
<td></td>
<td></td>
<td>39&lt;sup&gt;b&lt;/sup&gt;</td>
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<tr>
<td><em>SfiI</em></td>
<td>ND</td>
<td>ND</td>
<td>(260)</td>
<td>(260)</td>
<td>(260)</td>
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<tr>
<td></td>
<td>33</td>
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<td>33</td>
<td>(20)</td>
<td>(20)</td>
<td></td>
<td>17</td>
</tr>
</tbody>
</table>

*Fragments listed as the same size are identical as judged by overlaying of autoradiographs. Underlined sizes represent hybridization signal in the compression zone; the size of the hybridizing bands may be greater than or equal to the size listed. Bracketed fragment sizes indicate faint bands resulting from incomplete digestion. ND = not done.

<sup>a</sup>The sizes of these bands are too small to estimate using the special *NotI* FIGE blot, except that both are less than 48.5 kb.

<sup>b</sup>These fragments are non-identical as judged by band intensity differences and compression zone hybridization signals; see text.
4-kb FIGE band recognized by p5, and confirm the presence of partially digestible SacII site on the other (left) side of p5 (figure 8).

Similar experiments have demonstrated the existence of a SacII and a BssHII site at position -45 kb. These sites, however, were almost completely refractory to restriction digestion in genomic DNA. SacII and BssHII sites 9 kb to the left were partially digestible (data not shown). The position of CpG island #3 has been moved 9 kb to the left relative to the original map (Brooks-Wilson et al., 1992c) to reflect the position of the presumably incompletely methylated restriction sites. These changes have been made within the region spanned by cosmid 2-4a-1, which is unstable in culture. The positions of these restriction sites could not have been predicted by restriction mapping of cosmid DNA, due to the propensity of 2-4a-1 to rearrange.

p5 also detects a 39-kb fragment, which represents the left portion of a 60-kb partial digestion product (previously sized as 58 kb). The 21-kb fragment detected by p4 represents the remainder of the 60-kb fragment (60 kb - 39 kb = 21 kb).

p6 and p7 also detect fragments of 39 kb, although these fragments were previously sized as slightly smaller (30 kb and 29 kb, respectively). The ostensibly 39-kb fragments detected by p5, p6 and p7, however, are non-identical. They represent different restriction fragments, which migrated coincidentally in this experiment. Comparison of the relative intensity of bands between different lanes confirms that p5 and p6 do not recognize the same DNA fragment. The p7 SacII band cannot be the same as that detected by p6, because p6 produces hybridization signal in the compression zone and p7 does not. The p7 fragment is thus flanked by completely digestible SacII sites, whereas at least one flanking site of the p6 fragment is subject to partial digestion: the fragments detected by p6
and $p_7$ cannot be identical. This is consistent with the map described previously, in which $p_5$, $p_6$ and $p_7$ clearly hybridize to different SacII fragments.

It follows from the observation that compression zone hybridization signals were produced by four of the six probes hybridized to the SacII FIGE blot, that this set of digests was less complete than those described in the previous chapter. The 68-kb band detected by $p_4$ and $p_5$ presumably represents a SacII incomplete digestion product. It may result from cleavage at the SacII sites at -75 kb and -10 kb, but not at SacII sites between these map positions (figure 8).

Sample data for hybridization of the SacII FIGE blot with three probes: $p_6$, $p_2$ and $p_4$, are presented in figure 9. Note that in some lanes the resulting bands appear wider than in others. The SacII fragments detected by $p_6$, $p_2$ and $p_4$ are non-overlapping. Because the same samples produce wide bands for all three non-overlapping SacII fragments, the width of these bands is not due to incompletely resolved doublets potentially representing mutations, but is due to separation artifacts particular to individual samples. No potential mutations were detected by these SacII restriction mapping experiments. CP DNA in agarose blocks was prepared from a limited stock of cells; insufficient DNA was present to allow visualization of the SacII bands.

SfiI-digested patient samples were resolved in the 8 to 200 kb range by FIGE using ROM care #2. A Southern blot of the SfiI FIGE gel was hybridized with $D10S94$ probes. The sizes of DNA fragments detected (table IX) were approximately the same as those noted in the previous chapter, with one exception. $p_6$ detected a faint 20-kb band corresponding to the SfiI fragment at position -125 kb to -145 kb (figure 8), in addition to the larger 57 kb (previously sized as 63 kb) fragment. $p_6$ spans the SfiI
FIGURE 9: Hybridization of D10S94 probes to FIGE-separated SacII-digested DNA from MEN 2 patients. Portions of autoradiographs generated by hybridization of radiolabelled D10S94 probes p6, p2 and p4 to a Southern blot of a FIGE gel resolving SacII-digested patient and control DNAs are shown. The sizes of fragments detected by these probes and their positions of migration on the gel are shown to the left to illustrate the extent of separation of these fragments. Patient initials are shown above the lanes. The 24 kb band detected by p4 represents an incomplete digestion product; it is visible, though faintly, in all lanes of the original autoradiograph. CP DNA blocks were made with an insufficient number of cells to visualize these bands. Autoradiography was for 1 to 10 days.
site at -125 kb but most of the probe falls on the larger, right hand fragment. The more intense hybridization signals obtained in this experiment allowed both fragments to be visualized for the first time. In addition, a 97-kb band detected by both p4 and p5 and representing an SfiI fragment resulting from failure of the SfiI site at -65 kb to be cleaved, was also observed. KP (an affected relative of CP) was examined successfully in SfiI-digested DNA; agarose blocks made from a limited supply of frozen, nonviable cell were used for this experiment. No potential mutations or polymorphisms were noted in these SfiI analyses.

No alterations in patient DNAs were noted upon examination of SacII and SfiI restriction fragments at D10S94. It is therefore of interest to determine an approximate upper limit for the size of alteration that would not be detected in these experiments. The standard gels A and B (figure 7) have been used to estimate the size of alteration that could be detected, assuming that a fragment mobility difference of 2 mm is the minimum discernible difference. The DNA fragment size vs. mobility curves for the SacII and SfiI FIGE gels are very similar to those of standard gels A and B, respectively. Using pairs of DNA standards slightly larger and slightly smaller than each SacII or SfiI band at D10S94, calculations of the size of alteration necessary to produce a 2 mm difference in mobility can be done. Bands that were not visible in all patient lanes (generally those indicated as faint bands in table IX) were excluded from these calculations because they cannot be considered to have been examined in all patients. The estimated maximum non-detectable alteration for each SacII or SfiI band at D10S94 is listed in brackets under the FIGE-determined fragment size in figure 8.

The 145-kb NotI fragment at D10S94 contains or is flanked by six CpG islands, and was of particular interest in the examination of D10S94
for alterations potentially important in MEN 2. A special ROM card was made by Mr. Bernard Chan of Q-Life Systems (Kingston, Ontario) to optimally resolve DNA fragments close to 145 kb. It was agreed that Mr. Chan would design a program that would not have run time as a limitation; the resulting program took 105 hours. It was intended that this ROM card would provide size separation optimal for the detection of subtle size differences in the 145-kb range. Such a FIGE program would not necessarily provide good resolution of fragments outside the 145-kb range.

NotI-digested patient and control samples were subjected to FIGE using the special "145-kb" ROM card. A Southern blot of the special NotI FIGE gel was hybridized sequentially with p0, p2, p6, and p7 so than the resulting autoradiographs could be overlaid for direct comparison of bands. The sizes of DNA fragments detected are listed in table IX. A photograph of an autoradiograph produced by hybridization of p2 to a Southern blot of the special NotI FIGE gel is shown in figure 10.

p2 and p6 detect what appears to be a 148-kb NotI fragment, which corresponds to the 145-kb NotI band noted in chapter IV. A 160-kb band is also evident, though it is faint in most lanes. p0 hybridizes to the 160-kb band and a "very small" band, the size of which cannot be determined from this blot but which is consistent with the previous observation of a 20-kb p0 NotI band detected with probe p0 (table VII). The 160-kb fragment represents a partial digestion product in which the NotI site at position 0 kb at $D10S94$ (figure 8) fails to be cleaved, and the 145-kb and 20-kb NotI fragments recognized by p2 and p0, respectively, remain attached together and migrate as an approximately 60-kb fragment.

In the mapping experiments described in the previous chapter, p2 detected only a 145-kb band, not bands of 145- and 160-kb. One reason why the 160-kb band was not observed may be that the digestions
FIGURE 10: Searching for alterations in MEN 2 patient DNAs within a 145-kb NotI fragment at D10S94 by FIGE. An autoradiograph resulting from hybridization of radiolabeled probe p2 to a Southern blot of the special NotI FIGE gel is depicted. Patient initials are indicated above each lane. The NotI band recognized by p2 was sized relative to λ DNA standards in this experiment as 148 kb. Autoradiography was for 5 days. KP genomic DNA was not of sufficiently high molecular weight to produce fragments of this size. The 160-kb band represents an incomplete digestion product.
performed previously were more complete than these; a difference of this magnitude, however, would not have been recognized due to the width of the bands produced in that set of experiments (refer to figure 6).

DNA of individuals YC and KC appear to produce more of the 160 kb partial digestion product than do the other samples. Hybridization of a Southern blot of isolated genomic DNA from these and other individuals digested with both NotI and EcoRI and resolved in the 0.2 to 20 kb range by conventional agarose gel electrophoresis was designed to assess the tendency of the NotI site at position 0 kb to partial digestion (data not shown). Hybridization with p0 showed no difference in the ability of this NotI site to be cleaved in YC or KC DNA compared to DNA from their unaffected relatives. The darker 160-kb bands observed in DNA of YC and KC therefore seem to reflect only variation in digestion of these DNAs between experiments.

Hybridization of p7 to the special NotI blot produced a small band and a 223-kb band consistent with previous observations of 29 kb and 260 kb bands (recall that the special ROM card is not intended to provide good resolution of bands of these sizes). p0, p2, p6 and p7 all produce hybridization signals in the compression zone, indicating the presence of larger unresolved NotI incomplete digestion products.

A cell line (from which to make high molecular weight DNA embedded in agarose blocks) from individual KP was not available and previously isolated genomic DNA, subsequently embedded in agarose, was used for the NotI FIGE gel. The size of DNA molecules of this isolated (as opposed to prepared in agarose directly from cells) DNA sample was too small to allow detection of fragments in this FIGE experiment.

No aberrant NotI fragments were noted in these experiments. The size of the NotI fragment detected by p0 has been determined to be 14 kb
by restriction mapping in yeast artificial chromosome DNA (data not shown). The true size difference between the "145/148 kb" fragment and the 160 kb fragment is 14 kb. These bands are separated by 1.4 cm, a resolution of 1 mm/kb for fragments of close to 145 kb. Estimating from figure 10 that a doublet of bands (that might be expected in a situation in which an individual is heterozygous for a size-altering mutation or polymorphism) separated by 3 mm or more would be apparent (because the NotI bands are slightly wider than the SacII or SfiI bands for which a difference of 2 mm was assumed to be discernible), it follows that a sequence alteration resulting in a fragment size difference of 3 kb (a size difference of 2% for a 145-kb fragment) or more would be detected. It seems that no alterations of 3 kb or more occur within the 145 kb NotI fragment at D10S94 in this set of patients.

Genetic alterations changing the size of the 145-kb NotI band by 3-kb or more have not been found within this set of MEN 2A and MEN 2B patients. Alterations of 1.6 kb or more have been ruled out for all SacII intervals within the NotI fragment. Examination of the SacII fragments, however, excluded examination of small SacII fragments within CpG islands. For example, a 1.8-kb SacII fragment at CpG island #4 is not addressed in these experiments. Hypothetical deletion of this 1.8-kb fragment would not be detected using the SacII FIGE blot. Only alterations of 3 kb or more have been excluded over the entire 145-kb NotI fragment. The 39-kb SacII fragment detected by p7, however, has been examined at the 1.6-kb level. CpG island #5 contains only one SacII site; no small SacII fragments at this junction have been omitted from the survey for mutations. The 80-kb SfiI fragment detected by p2 has been examined at the 3.3-kb level, extending the surveyed area to map position +55 kb. The area from -180 kb to +55 kb (a 235 kb region) has been examined for
band alterations of 3.3 kb or more. Individual KP has been examined over
most, but not all of this area, at the 3.3 kb level.

These experiments would not detect rearrangements in which the
total D10S94 locus was deleted. In this situation, the normal copy of
chromosome 10 would contribute normal sized bands. Incomplete
digestion and differences in quantity of DNA between lanes could make it
difficult to detect a resulting decrease in band intensity. To rule out
deletions involving all of D10S94 in many of these patients, they have been genotyped for several D10S94 polymorphisms (Table VIII). Four
polymorphic restriction sites at D10S94 have been described (Brooks-
Wilson et al., 1990b; Brooks-Wilson et al., 1992b): PvuII, Rsal, MspI and
TaqI. The Rsal system is not highly informative and was not used here.
KC had, however, previously been shown to be heterozygous for Rsal alleles (Paul J. Goodfellow, pers. comm.). Patients were genotyped by
hybridization of probes detecting the polymorphic sites to Southern blots
(prepared by Ms. Heather Jenkins) of PvuII, MspI or TaqI digests of
patient genomic DNA. Not all patients were tested for all the
polymorphisms. Most patients were heterozygous at at least one site, or
had an affected relative who was heterozygous (KW and SM). Only four
patients: CA, RW, AJ and MA were apparent homozygotes at all sites
tested.
DISCUSSION:

Field inversion gel electrophoresis has several advantages over the other kinds of pulsed field gel electrophoresis, including straighter lanes, which make results much easier to interpret. The AutoBase module, used here in its prototype form, uses predetermined run profiles on ROM cards to resolve a variety of DNA fragment size ranges. It is much easier to use than a conventional apparatus that must be programmed for each run and it saves effort because it removes the necessity to optimize pulse time and other electrophoretic parameters. A disadvantage of the AutoBase module is that the run parameters are unknown to the user.

The detailed examination of the $D10S94$ region with a PFGE system affording superior resolution to the one previously used has helped to clarify fine points of the detailed physical map at this locus.

In general, genetic alterations that change the size of a long range restriction fragment by 5% or more of its size can be detected by PFGE (den Dunnen et al., 1987; Dunham et al., 1989; Viskochil et al., 1990). This observation is consistent with the results seen using the wide range separation profiles provided by AutoBase program cards #1 and #2 (where a 2 mm minimum difference is considered observable). The special program optimized for resolution of DNA fragments close to 145 kb exceeded this; it would allow a difference of 2% of the NotI fragment's size to be detected, even assuming a minimum discernible difference of 3 mm because the bands are slightly wider.

These FIGE experiments have ruled out the existence of an alteration that changes the size of the 145-kb NotI fragment at $D10S94$ by 3 kb or more as the cause for MEN 2 in 13 patients (excluding KP). Alterations of 3.3 kb or more have been ruled out for a larger area of 235 kb for these
Patients. KP did not have detectable alterations of any SfiI fragments examined. Complete deletion of the entire locus has not, however, been ruled out for four of the patients.

These experiments did not reveal rearrangements at D10S94. There are two possible explanations for this result. The first is that D10S94 is not the location of the disease gene(s), although this would still be a very interesting cluster of CpG islands. Another possibility is that MEN 2 mutations could be present at D10S94 but are too subtle (in these families) to be detected by the method employed.

These experiments have shown that D10S94 is not the site of large deletions or rearrangements in a large proportion of MEN 2 patients. If MEN 2 is a contiguous gene syndrome involving large overlapping deletions, then this 180-kb region examined at D10S94 is not likely to be where the set of contiguous genes responsible for the disease are located. Alternatively, if this region is the site of the MEN 2 gene(s), then a deletion syndrome is not likely to be the mechanism by which the diseases arise.

Although no alterations to NotI, SacII or SfiI bands at D10S94 were observed, subtle methylation differences between some individuals were noted. In one case, a possible methylation difference was demonstrated to be due to variation in restriction digestion between samples and experiments. Further studies would be necessary to implicate or to definitively rule out these restriction digestion differences potentially due to methylation differences between SacII sites in different individuals, as relevant to MEN 2.
CHAPTER VI:

A GENOMIC LONG RANGE PHYSICAL MAP AND YEAST ARTIFICIAL CHROMOSOME CONTIG LINKING SIX LOCI IN 10q11.2 NEAR THE GENE(S) RESPONSIBLE FOR MEN 2

INTRODUCTION

*MEN2A* maps between *FNRB* in 10p11.2 and *RBP3* in 10q11.2, and is tightly linked to the chromosome 10 centromeric repeat marker *D10Z1* (Wu et al., 1990). Recent studies have allowed refinement of the localization of *MEN2A*. *D10S34* (Lichter et al., 1992a, 1992b) and *D10S176* (Howe et al., 1992) in 10p11.2 have been demonstrated to flank this disease locus more closely than does *FNRB*. New DNA markers have been cloned and several of these demonstrated to flank *MEN2A* more closely than previous flanking markers. *D10S253* (p1-3A) and *D10S252* (DM151) (Miller et al., 1992b), and *D10S102* in 10q11.2 have recently been shown to flank *MEN2A* more closely than does *RBP3* (Miller et al., 1992a; Lairmore et al., 1993). Single crossovers have served to position *MEN2B* between the centromere (*D10Z1*) and *RBP3* (Norum et al., 1990; Lairmore et al., 1991). The candidate intervals to which *MEN2A* and *MEN2B* have been mapped overlap in proximal 10q11.2, a region that may contain any or all of *MEN2A, MEN2B* or *MTC1*.

The cloning and mapping efforts of several laboratories have served to identify a number of markers which map to 10q11.2, several of which have not been demonstrated to recombine with the disease loci. These include *D10S94* (Goodfellow et al., 1990a), *D10S97* (*D10F38S1*, Lichter et
al., 1991b; Lichter et al., in press), RET (Gardner et al., 1991) and the loci defined by DNA markers DM44 (D10S251) and DM55 (ALOX5)(Miller et al., 1992a, 1992b). Both genetic (Lichter et al., 1992a and 1992b) and radiation hybrid mapping (Miller et al., 1992b) have been used to establish the order of markers within 10q11.2. Additional loci and markers including DM121 (Miller et al., 1992b), D10S182 (pCl1/A1S-6-c65, Brooks-Wilson et al., 1992a), D10F38S3 (the "D10S97-like" locus mapped by Miller et al. (1992b)) and DM124 (D.L. Miller and P.J. Goodfellow, unpubl.) have been mapped within 10q11.2 by radiation hybrid mapping alone.

Other physical mapping methods such as genomic long range restriction mapping and yeast artificial chromosome (YAC) cloning and restriction mapping can be used not only to establish the order of DNA markers but also to determine physical distances between linked DNA markers.

Yeast artificial chromosomes (YACs [reviewed by Schlessinger, 1990]) are used to clone very large DNA fragments, often more than 1 Mb. The cloning and characterization of short sequences corresponding to yeast chromosomal centromeres, telomeres, and origins of replication (autonomously replicating sequences, or ARSs) has made possible the assembly of these chromosomal components, along with markers selectable in yeast and sequences necessary for propagation in bacteria, into a plasmid approximately 10 kb in size. These components are arranged such that YAC "arms", each containing a telomere (Tetrahymena telomeres are often used instead of yeast telomeres) and a selectable marker, and one of which contains a centromere and an ARS, flank a cloning site. Separation of the YAC arms and cloning of large DNA fragments into the cloning site results in a linear artificial chromosome, which segregates like a normal yeast chromosome and can be propagated stably in yeast cells. Unlike
vertebrates, yeast cells do not methylate the cytosine residues of CpG dinucleotides. Long range restriction maps of YAC DNA and of genomic DNA can be compared to identify rare cutting restriction sites (for methylation-sensitive restriction enzymes) which fail to be cleaved in genomic restriction digests because of methylation.

YACs have become a valuable tool in the physical mapping of DNA markers separated by hundreds and up to thousands of kb. Contigs of YACs can be assembled by means of determining the DNA markers they have in common. The PCR-based STS (sequence tagged site) mapping approach can provide information about marker order, but does not give physical distances between markers. Physical distances between markers must be determined by long range restriction mapping of the YAC clones. YACs are also a valuable source of cloned DNA from which to isolate genes of biological or medical interest from defined genomic regions.

The objective of assembling a YAC contig and performing long range restriction mapping using DNA markers within the MEN2 region was to establish physical linkage of and distances between markers tightly linked to the disease locus. Extension of such a map to include markers known to flank the disease loci would determine a physically defined region of known size, upon which disease gene cloning efforts could be concentrated.

YACs containing the $D10S94$ and $RET$ loci have been identified. A 1-Mb YAC contig containing $D10S94$ and $RET$ also encompasses $D10S182$, $D10F38S3$ and DNA marker DM124. The YAC contig did not contain $D10Z1$, $D10S102$, $D10S252$, $D10S253$, $D10S251$, $ALOX5$, or DM121.

Long range restriction mapping in genomic DNA confirmed physical linkage of $D10S102$, $D10S182$, $D10S94$ and $RET$ within 1 Mb. The probe recognizing $D10F38S3$, and DNA probe DM124 each hybridized to more than one site in the genome and as a consequence cannot be mapped.
unambiguously in genomic DNA. A long range restriction map of the YAC contig was therefore generated to establish physical distances between \textit{D10S182}, \textit{D10S94} and \textit{RET} and these markers.

Apart from expected differences attributable to methylation of human genomic DNA and not of the YACs, the long range restriction maps of are consistent with one another. This supports the general integrity of the YAC contig and provides confidence in the mapping of \textit{D10F38S3} and DM124, which is based solely on cloned DNA. This combined mapping approach predicts the following order: \textit{D10F38S3}, DM124, \textit{RET}, \textit{D10S94}, \textit{D10S182}, \textit{D10S102}.

Long range restriction mapping and YAC cloning and contig assembly have physically linked six loci or DNA markers within 1.4 Mb in the MEN 2 region of chromosome 10. The map includes \textit{D10S102}, which has recently been demonstrated to distally flank \textit{MEN2A} (Lairmore \textit{et al}., 1993). Both meiotic (Lichter \textit{et al}., 1992b) and radiation hybrid mapping (Miller \textit{et al}., 1992b) have served to position \textit{D10S102} telomeric to \textit{D10S94}. This allows orientation of the physically mapped region such that \textit{D10S102} is the most distal marker and such that \textit{D10F38S3}, DM124, \textit{RET}, \textit{D10S94} and \textit{D10S182} are within the \textit{MEN2A} candidate interval. Expansion of this physical map to include a marker or markers that flank the disease loci on the side opposite \textit{D10S102} will make possible the precise determination of the size of the MEN 2 region, complete cloning of this interval, and definition of a set of genes that can be considered candidates for \textit{MEN2A}, \textit{MEN2B} and/or \textit{MTC1}. 

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RESULTS

**YAC Cloning and Characterization**

Five yeast strains containing D10S94-derived YACs (183H7, 188H11, 351H2, 55A10 and 170G10) and three containing RET-derived YACs (344H4, 273D10 and 214A3) were identified by screening the Centre d'Etude du Polymorphisme Humain (CEPH) YAC library (Albertsen et al., 1990) using a PCR-based screening approach (Heard et al., 1989). In addition, a YAC (282C2) derived from ZNF22 in 10q11.2 and one (56G11) derived from D10S102 (gifts of Drs. Jay Lichter and Ken Kidd, and Drs. Sara Mole and Bruce Ponder, respectively) were included in the Southern blot analyses. Fluorescent *in situ* hybridization (FISH), performed by Ms. Trushna Desai of Dr. David Ward's laboratory (Yale University), was used to assess whether any of these YACs was chimeric. When used as a FISH probe, all of the YACs except 170G10 produced fluorescent signals only near 10cen; 170G10 hybridized to more than one chromosome, demonstrating chimerism of this YAC (data not shown). The size of the YACs and number of artificial chromosomes in each yeast isolate containing the YACs was determined by FIGE of yeast chromosomes prepared in agarose blocks, followed by Southern blotting and hybridization with radiolabeled sheared human genomic DNA (data not shown). YAC sizes range from 260 to 520 kb. 183H7 and 214A3 each contain two artificial chromosomes; the other eight yeast strains contain single artificial chromosomes. Results of YAC characterization: derivation, size and integrity are summarized in table X.
TABLE X: Characterization of yeast artificial chromosomes derived from
*D10S94, RET, ZNF22* and *D10S102*.

<table>
<thead>
<tr>
<th>YAC</th>
<th>Locus</th>
<th>Size (kb)</th>
<th>Integrity</th>
</tr>
</thead>
<tbody>
<tr>
<td>183H7</td>
<td><em>D10S94</em></td>
<td>450</td>
<td>non-chimeric by FISH, but rearranges</td>
</tr>
<tr>
<td></td>
<td></td>
<td>390</td>
<td></td>
</tr>
<tr>
<td>188H11</td>
<td><em>D10S94</em></td>
<td>290</td>
<td>non-chimeric by FISH</td>
</tr>
<tr>
<td>351H2</td>
<td><em>D10S94</em></td>
<td>300</td>
<td>non-chimeric by FISH</td>
</tr>
<tr>
<td>55A10</td>
<td><em>D10S94</em></td>
<td>370</td>
<td>non-chimeric by FISH, chimeric as judged by restriction mapping</td>
</tr>
<tr>
<td>344H4</td>
<td><em>RET</em></td>
<td>260</td>
<td>non-chimeric by FISH</td>
</tr>
<tr>
<td>170G10</td>
<td><em>D10S94</em></td>
<td>380</td>
<td>chimeric by FISH, deleted</td>
</tr>
<tr>
<td>273D10</td>
<td><em>RET</em></td>
<td>500</td>
<td>non-chimeric by FISH</td>
</tr>
<tr>
<td>214A3</td>
<td><em>RET</em></td>
<td>520</td>
<td>both rearranged</td>
</tr>
<tr>
<td></td>
<td></td>
<td>410</td>
<td>non-chimeric by FISH</td>
</tr>
<tr>
<td>56G11</td>
<td><em>D10S102</em></td>
<td>360</td>
<td>non-chimeric by FISH</td>
</tr>
<tr>
<td>282C2</td>
<td><em>ZNF22</em></td>
<td>510</td>
<td>non-chimeric by FISH</td>
</tr>
</tbody>
</table>

Locus refers to the locus derivation of the STS used to identify the YAC. Sizes were determined by FIGE, followed by Southern blotting and hybridization with radiolabeled sheared human genomic DNA. The integrity of each YAC was assessed by FISH (by T. Desai and Dr. D. Ward) to detect chimerism and by long range restriction mapping and Southern blot hybridization to reveal rearrangements.
Southern blot analysis of YACs and Contig Assembly

Preliminary characterization of YAC DNA by STS mapping suggested that one YAC, 170G10, contained sequences derived from both $D10S94$ and $RET$, thereby implying close physical linkage of these loci (data not shown). To confirm this and to determine whether the putative contig spanning $D10S94$ and $RET$ included other 10q11.2 loci, the ten YACs were tested with a battery of 20 markers derived from this region of the genome. YACs were scored for the presence or absence of each marker by hybridization of radiolabeled DNA probes to Southern blots of EcoR1-digested yeast/YAC DNA. Probes tested included pα10RP8 ($D10Z1$), pCl1/A1S-6-c65 ($D10S182$), probes p0, p1, p2, p3, p4, p5, p6, and p7 derived from a 160-kb cosmid contig (Brooks-Wilson et al., 1992c) at $D10S94$, pret9.1T3 ($RET$), pKW6ΔSacI (the $D10F38$ family), DM124, YP16, pMEN203DM1 ($D10S102$), DM44 ($D10S251$), DM55 ($ALOX5$), DM121, DM151 ($D10S252$) and p1-3A ($D10S253$). Hybridization results for probes present in one or more YACs are summarized in table XI. pα10RP8, DM44, and DM121 failed to hybridize to any of the ten YACs.

The existence of three groups of YACs, which do not have DNA markers in common with each other can be seen from examination of table XI. 56G11 contains only pMEN203DM1; ZNF22 YAC 282C2 contains the three markers p1-3A, DM55 and DM151; and the eight YACs identified by screening with $RET$ or $D10S94$ contain sequences derived from five 10q11.2 loci: $D10S182$, $D10S94$, $RET$, $D10F38S3$ and a 10q11.2 locus defined by marker DM124.

The mapping of loci detected by probe pKW6ΔSacI is complicated by the fact that this probe hybridizes to at least three loci. Hybridization of pKW6ΔSacI to a Southern blot of EcoRI-digested genomic DNA produces bands of different sizes, which define the genomic loci $D10F38S2$, 95
TABLE XI: DNA marker content of yeast artificial chromosomes derived from \textit{D10S94}, \textit{RET}, \textit{D10S102} and \textit{ZNF22}.

<table>
<thead>
<tr>
<th>YAC</th>
<th>Locus</th>
<th>DNA Markers</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>p D p Y p p p p p p p p c 2 D p D</td>
</tr>
<tr>
<td></td>
<td></td>
<td>K M r P 7 6 5 4 3 2 1 0 6 0 M 1 M</td>
</tr>
<tr>
<td></td>
<td></td>
<td>W 1 e 1 5 3 5 - 1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>6 2 t 6 D 5 3 5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4 M A 1</td>
</tr>
</tbody>
</table>

183H7 \textit{D10S94} - - - - - - + + + + + + + - - - -
188H11 \textit{D10S94} - - - - - - - - + + + + + + - - - -
351H2 \textit{D10S94} - - - - - - + + + + + + - - - -
55A10 \textit{D10S94} - - - - + + + + + + + + + + + + - - - -
344H4 \textit{RET} - - + + + + - - - - - - - - - - - - - - -
170G10 \textit{D10S94} - - + + - - - - + - + + + - - - -
273D10 \textit{RET} + + + + - - - - - - - - - - - - - - - - -
214A3 \textit{RET} + + + - - - - - - - - - - - - - - - - - - -
56G11 \textit{D10S102} + - - - - - - - - - - - - - - - - - - - - -
282C2 \textit{ZNF22} - + + +

Locus refers to the locus derivation of the STS used to identify the YAC. Presence or absence of DNA markers was determined by conventional Southern blot hybridization: + indicates presence, - indicates absence, a blank indicates not tested. Additional markers tested and not present in any of the YACs are listed in the text. Columns are ordered and boxes drawn to allow visual representation of the regions spanned by the YACs.
D10F38S1 (D10S97) and D10F38S3 ("D10S97-like") and which have been used to map these loci using radiation hybrids (Lichter et al., in press; Miller et al., 1992b). The smallest EcoRI band recognized by pKW6ΔSacI and diagnostic of D10F38S3 is present in YACs 214A3 and 273D10. An additional band of 6.5 kb is also present and appears to be of the same size as one of the monomorphic genomic sequences recognized by this probe.

These Southern blot hybridization analyses identified overlaps between the D10S94 and RET YACs. They also served to position the ends of those YACs (183H7, 188H11, 351H2, and 344H4) which terminate within a 160-kb CpG island-rich region at D10S94 (Brooks-Wilson et al., 1992c). In addition, they reveal a complex rearrangement in chimeric YAC 170G10. Probes p0 to p7 are numbered sequentially within the previously mapped D10S94 locus (Brooks-Wilson et al., 1992c). Presence of p2 and p4 and absence of p3 indicates a small deletion at D10S94 in 170G10, the YAC that originally implied close physical linkage of D10S94 and RET. The conclusion of close linkage, however, is strengthened by the observation that YAC 344H4 contains both D10S94-derived sequences (p5, p6 and p7) and RET, although it does not extend to the STS used to screen for YACs containing the D10S94 locus (data not shown). The columns of table XI are ordered and boxes drawn to allow visual representation of the YAC contig spanning D10S182, D10S94, RET, DM124 and D10F38S3.

A genomic long range restriction map spanning D10S182, D10S94 and RET.

Genomic long range restriction mapping confirmed the physical linkage of loci present in the YAC contig spanning D10S94, RET and D10S182. A detailed long range restriction map had been previously generated at D10S94 using probes p0 to p7 and restriction enzymes NotI, SacII and SfiI (Brooks-Wilson et al., 1992c). This region is CpG island-rich
and contains multiple SacII and SfiI sites within a 145-kb NotI fragment. To establish physical linkage of loci in this region over a greater distance than that previously mapped, restriction enzymes, which might cleave less often than SacII in this CpG island-rich region were used in these analyses. MluI and NruI were selected on the basis that their recognition sequences contain A and T residues in addition to CpG dinucleotides, a feature that might exclude them from many CpG islands (Bird, 1989). NotI was included in these analyses so that NotI sites at D10S94 could serve as reference points for aligning the new restriction map with the detailed map at D10S94.

The genomic long range restriction map was generated primarily with DNA from a single individual. EBV transformed lymphoblasts from an MEN 2B patient (CT) were used in an effort to identify map differences between the "wild type" and mutation bearing chromosome 10 homologues, which might be causally associated with disease. No such differences were noted. High molecular weight DNA prepared in low melting point agarose blocks was used for FIGE. A Southern blot of a FIGE gel generated using MEN 2B patient DNA digested with MluI, NruI, NotI and every two-enzyme combination of these, was sequentially hybridized with: pClI/A1S-6-c65 MspI-0.6, p1, p2, p7, p8, YP16, pret9.1T3 and pMEN203DM1. Autoradiographs were overlaid to facilitate direct comparison of fragments detected by different probes. Autoradiographs of this genomic FIGE blot following hybridization with p2 and with pret9.1T3 are shown in figure 11. The sizes of restriction fragments detected using this blot by each of the probes are summarized in table XII.

Additional long range restriction mapping was performed in the genomes of a series of normal controls and MEN 2 patients. Human genomic blots of FIGE-separated, MluI-, NruI- or NotI-digested DNAs were
hybridized sequentially with probes from \textit{RET}, \textit{D10S94}, \textit{D10S182} and \textit{D10S102}. In a number of DNA samples faint bands representing incomplete digestion products were observed in addition to the major bands detected previously. Differences in digestion conditions and easier visualization of faint bands on autoradiographs of blots made up of multiple lanes of similarly digested DNAs may account for the observation of partial digestion products in these experiments. The sizes of the complete and incomplete digestion products detected in single digests with \textit{MluI}, \textit{NruI} or \textit{NotI} are summarized in table XIII. The sizes of these bands have been normalized to those in table XII.

Figure 12A illustrates the long range restriction map incorporating all genomic mapping data. Restriction sites that are cleaved reproducibly in genomic DNA are unbracketed, sites subject to partial restriction digestion are in parentheses. The right end (as shown in figure 12) of the 150-kb \textit{NotI} fragment detected by p1 and p2 at \textit{D10S94} is set as 0 kb so that it can be aligned with a previously published map (note that this fragment was sized as 145 kb in chapter IV).

The relative position of \textit{D10S102} is deduced from \textit{NruI} incomplete digestion products. The \textit{D10S102} probe pMEN203DM1 falls on an 810-kb \textit{NruI} band that is also recognized by probes p2 and p5. Probe pMEN203DM1 does not fall on the 580 kb and 400 kb \textit{NruI} bands detected by these probes. pMEN203DM1 is therefore within the 230 kb interval at map position +470 to +700 kb (figure 12). This conclusion is further supported by the identification of STS \texttt{yWME28L} between \textit{D10S94} and \textit{D10S102} (Lairmore \textit{et al.}, 1993) in \textit{D10S94} YAC 183H7 (data not shown, see discussion).
FIGURE 11: Long range restriction mapping in genomic DNA using probes from 10q11.2. DNA from a single MEN 2B patient was digested with NotI (N), MluI (M), NruI (R), PvuI (P), or SfiI (X) singly or in combinations, and size-separated by FIGE. A single Southern blot of a FIGE gel was sequentially hybridized with eight probes; autoradiographs of the p2 and pRET9.1T3 hybridizations are shown. Sizes of three bands are indicated for illustration purposes: the 550-kb MluI band recognized by both p2 and pRET9.1T3, the 390-kb NotI/MluI pRET9.1T3 band and the 100-kb NruI/MluI p2 band. CZ= the compression zone, which includes unresolved fragments of approximately 1600 kb or greater. Selected band size data used in assembly of the genomic long range restriction map are listed in table 3. No discrete bands were evident in the NotI or NotI/PvuI lanes upon hybridization with pRET9.1T3.
TABLE XII: Sizes of Genomic Restriction Fragments Detected by Probes Derived From *D10S182*, *D10S94*, *RET* and *D10S102*.

<table>
<thead>
<tr>
<th>PROBE:</th>
<th>pret-9.1T3</th>
<th>YP16</th>
<th>p8</th>
<th>p7</th>
<th>p2</th>
<th>p1</th>
<th>c65-MspI-0.6</th>
<th>pMEN-203-DM1</th>
</tr>
</thead>
<tbody>
<tr>
<td>ENZYMES:</td>
<td></td>
<td>Size of fragment(s) in kilobase pairs</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MluI</td>
<td>550</td>
<td>550</td>
<td>550</td>
<td>550</td>
<td>550</td>
<td>40</td>
<td>NS</td>
<td>1360</td>
</tr>
<tr>
<td>MluI/NruI</td>
<td>470</td>
<td>470</td>
<td>470</td>
<td>470</td>
<td></td>
<td></td>
<td>NS</td>
<td>1360</td>
</tr>
<tr>
<td></td>
<td>300</td>
<td>180</td>
<td>180</td>
<td>180</td>
<td>100</td>
<td>40</td>
<td>NS</td>
<td>1360</td>
</tr>
<tr>
<td>NruI</td>
<td>550</td>
<td>550</td>
<td>550</td>
<td>550</td>
<td>1600</td>
<td>1600</td>
<td>NS</td>
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<tr>
<td></td>
<td>370</td>
<td>180</td>
<td>180</td>
<td>180</td>
<td>1530</td>
<td>1530</td>
<td>NS</td>
<td>1530</td>
</tr>
<tr>
<td>NruI/NotI</td>
<td>500</td>
<td>500</td>
<td>500</td>
<td>500</td>
<td>110</td>
<td>110</td>
<td>NS</td>
<td>NS</td>
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<tr>
<td></td>
<td>370</td>
<td>130</td>
<td>130</td>
<td>130</td>
<td>110</td>
<td>110</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>NotI</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>150</td>
<td>150</td>
<td>150#</td>
<td>1600</td>
</tr>
<tr>
<td>MluI/NotI</td>
<td>390</td>
<td>390</td>
<td>390</td>
<td>390</td>
<td>130</td>
<td>small$</td>
<td>NS</td>
<td>1360</td>
</tr>
</tbody>
</table>

DNA from a single MEN 2B patient was digested with rare-cutting restriction enzymes singly or in combinations and size separated by FIGE. A single Southern blot of a FIGE gel was sequentially hybridized with the eight probes listed. Fragments detected by different probes and listed as the same size are identical as judged by overlaying of autoradiographs. Underlined band sizes represent hybridization signals located within the compression zone. Their sizes may be greater than or equal to 1600 kb. NS = no signal. Poor hybridization signals were obtained with c65 MspI-0.6 and pMEN203DM1 and bands were not visible for all digests.

*No discrete NotI bands were observed upon hybridization with pret9.1T3, YP16, p8 or p7. This may be due to partial digestion at NotI sites near these probes and division of the hybridization signal between many faint bands, which may be difficult to visualize.

$The size of the small MluI/NotI fragment detected by p1 has been determined to be 8 kb by restriction mapping of cosmids derived from *D10S94* (data not shown).

#The 150-kb band recognized by c65 MspI-0.6 is not exactly the same as that recognized by p1 and p2, as determined by sequential hybridization of these probes to a Southern blot of a FIGE gel which resolves optimally at 150 kb (data not shown).
TABLE XIII: Sizes of Complete and Incomplete \textit{MluI}, \textit{NruI} and \textit{NotI} Restriction Digestion Products in Human Genomic DNA.

DNA samples from a series of normal controls and MEN 2 patients were digested with \textit{MluI}, \textit{NruI}, or \textit{NotI}. Samples digested with a single enzyme were size separated on the same FIGE gel. A Southern blot of each FIGE gel was sequentially hybridized with each of the probes listed. Fragments detected by different probes and listed as the same size are identical as judged by overlaying of autoradiographs. ND = not done. Note that the sizes of major bands (unbracketed) are consistent with those listed in table 3. They represent restriction fragments flanked by restriction sites that cut well and reproducibly in lymphoblast DNA. Fragment sizes in parentheses represent faint bands. Poor hybridization signals were obtained with c65 MspI-0.6 and pMEN203DM1. Underlined band sizes correspond to unresolved fragments in the compression zone (approximately 1600 kb for the \textit{MluI} and \textit{NruI} FIGE gels, and 560 kb for the \textit{NotI} FIGE gel). The actual sizes of the restriction fragments in the compression zone may be greater than or equal to the size indicated.
Long Range Restriction Mapping at D10S94 and RET in YAC DNA

Yeast/YAC chromosomal DNA prepared from seven of the eight D10S94 or RET YAC-containing yeast strains was digested with MluI, NruI, or NotI, or not digested, and resolved in the 8 to 500 kb range by FIGE. 170G10 was excluded from these analyses because it was known to have a complex rearrangement within D10S94. A Southern blot of the YAC FIGE gel was sequentially hybridized with p1, p2, p7, pret9.1T3, pKW6ΔSacI, DM124 and YAC right and left arm-specific probes (data not shown). Both the 450- and 390-kb artificial chromosomes of 183H7 contain D10S94-derived sequences; it is likely that one of these arose through rearrangement of the other. The 450 kb YAC produced darker autoradiographic signals from which the 183H7 restriction map was deduced (data not shown). The 520-kb artificial chromosome of 214A3 contains pret9.1T3, the 410-kb artificial chromosome contains sequences that hybridize to pKW6ΔSacI, and both contain DNA marker DM124. These artificial chromosomes appear to represent rearrangement products of an original YAC, which like 273D10, contained pret9.1T3, DM124 and D10F38S3. The long range restriction map of the right half of 55A10 was not consistent with the genomic map or with the maps of the overlapping portions of 183H7, 188H11 or 351H2 (figure 12). In FISH analysis, however, 55A10 produces a single fluorescent signal near 10cen. 55A10 may be rearranged in such a way that non-contiguous sequences both derived from near 10cen are juxtaposed, possibly by internal deletion of a larger YAC. MluI and NruI digests of cosmids derived from D10S94 (data not shown) aided in interpretation of the YAC long range restriction mapping data. The relationship of the YAC contig to the genomic long range map is illustrated in figure 12. 214A3 is not included because it has been demonstrated to be rearranged. The restriction mapping of YAC
clones that was performed did not necessarily detect every restriction fragment within each YAC. The YAC and genomic maps are consistent. Restriction sites present in genomic DNA and verified to be present in the YACs are not indicated on the YAC contig. The map generated in cloned DNA revealed the presence of several restriction sites (indicated by marked vertical lines intersecting the YACs in figure 12B) that are not apparent in human lymphoblast DNA, presumably because of methylation of the restriction sites.

The positions of RET, D10S94 and D10S102 deduced from long range restriction mapping in YACs are consistent with the genomic map. D10F38S3 and DM124 cannot be restriction mapped in genomic DNA because the probes hybridizing to these sequences recognize more than one locus. These two sequences have been mapped to the left end of the YAC contig (figure 12).
FIGURE 12: A genomic long range restriction map and YAC contig encompassing six loci in 10q11.2. (A) The genomic long range restriction map encompassing four loci: RET, D10S94, D10S182, and D10S102. (B) The YAC contig spanning five loci: D10F38S3, DM124, RET, D10S94 and D10S182. (C) Positions of probes used in mapping. The locations of RET and D10S94 specified are those of the STS used in YAC library screening. The scale (in kb) is oriented and positioned as for a published map of D10S94. M = MluI, R = NruI, N = NotI. Unbracketed restriction sites indicated on the genomic map represent sites that cleave well and reproducibly in lymphoblast DNA; sites in parentheses represent those which cleave only partially in lymphoblast DNA. Each YAC is indicated by a line proportional to its size. The genomic and YAC long range restriction maps concur. Sites present in genomic DNA and also verified to exist within the YAC contig are omitted for simplicity. Labelled vertical lines through the YACs represent restriction sites detectable only in cloned DNA. YAC 55A10 is derived entirely from near 10cen as judged by FISH; the right half of this YAC, however (indicated by a hatched bar), is not contiguous with the genomic map. Solid and hollow boxes represent the left and right arms of the YACs, respectively.
DISCUSSION

A combination of YAC contig assembly and genomic and YAC long range restriction mapping has been used to demonstrate physical linkage of six loci within 1.4 Mb at 10q11.2 within the MEN 2 region. The genomic map establishes the physical linkage of \textit{RET, D10S94, D10S182 and D10S102} within 1 Mb. A 1-Mb YAC contig encompasses \textit{RET, D10S94} and \textit{D10S182} as well as two loci that cannot be readily restriction mapped within genomic DNA: \textit{D10F38S3} and the 10q11.2 locus homologous to DM124. Physical mapping in YAC DNA allowed the positioning of the latter two loci relative to the genomic map. Genomic long range physical mapping is not subject to the problems of chimerism and rearrangement that plague YACs; a combined genomic and YAC map is therefore more reliable than a YAC map alone.

This work serves to illustrate the importance of careful characterization of YAC clones in establishing a contig of clones intended to reflect the arrangement of sequences found in the genome. A several-fold redundancy of YAC coverage of a region is also useful for the generation of a reliable map; it would not help, however, if an area were inherently unstable in yeast. FISH can reveal chimeric YACs (YAC 170G10 is an example); three of eight \textit{D10S94} or \textit{RET} YACs restriction mapped in detail, however, showed rearrangements not detectable by FISH. Comparison of the YAC restriction map to the genomic map was valuable in identifying an apparent rearrangement of YAC 55A10. YACs 214A3 and 183H7 each contained pairs of overlapping artificial chromosomes, which are likely to have arisen through rearrangement during propagation in yeast. These observations emphasize the importance of genomic long range restriction
mapping or of assembling a highly redundant YAC contig for generating a reliable map of a genomic region of interest.

Meiotic (Lichter et al., 1992a) and radiation hybrid mapping (Miller et al., 1992b) have served to position D10S102 distal to both RET and D10S94. In addition, in a critical crossover between MEN2A and D10S102, D10S94 was informative and segregated with markers proximal to D10S102 (Lairmore et al., 1993). This information makes possible orientation of the six-locus physical map within 10q11.2: 10cen, D10F38S3, DM124, RET, D10S94, D10S182, D10S102, 10qter. This order is inconsistent with that previously predicted by radiation hybrid mapping: 10cen, D10S182/D10S94, RET/D10F38S3, D10S102, 10qter (Miller et al., 1992b). The only difference between the two maps is the order of D10S182/D10S94 and RET/D10F38S3. Lairmore and colleagues (1993) have used YAC cloning and mapping to give order and relative distances between RET, D10S94 and D10S102. Their results concur with those described here. In addition, the STS yWME28L (Lairmore et al., 1993), which is located between D10S94 and D10S102, is present only in YAC 183H7, consistent with 183H7 extending furthest towards D10S102. It therefore seems likely that the order and arrangement of loci shown in figure 12 correctly reflect the true arrangement of these loci in the human genome. Differences between the long range restriction map and the radiation hybrid map, which was constructed assuming the least number of chromosome breaks, could be explained by an unrecognized deletion or rearrangement in one or more of the somatic cell hybrids.

Two other YACs did not contain D10S94 or RET markers. YAC 56G11 is derived from D10S102 and is expected to be located at the distal end of the YAC contig, or perhaps just distal to it. The DNA maker content analysis of these YACs has not addressed whether 56G11 overlaps with the
distal end of the RET/D10S94 YAC contig. YAC 282C2, derived from ZNF22, contains two markers recently demonstrated to flank MEN2A: p1-3A and DM151 (Miller et al., 1992a and 1992b), as well as marker DM55 of the ALOX5 locus (Goodfellow and Miller, 1992). A single critical crossover has served to position p1-3A (D10S253) and DM151 (D10S252) distal to D10S102 (Miller, 1992). YAC 282C2 and the ZNF22 locus can therefore be predicted to be located, with p1-3A, DM151 and ALOX5, distal to the intensively mapped region illustrated in figure 12. Close linkage of these loci is consistent with the observation that YACs 183H7 (D10S94), 273D10 (RET), 56G11 (D10S102) and 282C2 (ZNF22) produced superimposed fluorescent signals near 10cen when studied by simultaneous four-color FISH (T. Desai and Dr. D. Ward, pers. comm.). ALOX5 encodes the arachidonate 5-lipoxygenase gene. YAC 282C2 does not contain D10S102 probe pMEN203DM1, and is located distal to this locus. These facts together place ALOX5 and ZNF22 outside the refined MEN2A critical region and make it unlikely that these genes are involved in the etiology of MEN 2A.

A combination genomic and YAC mapping approach has been used to physically link six loci in 10q11.2 within the MEN 2 region. Most of the physically mapped region lies within the MEN2A interval; it includes its distal border, D10S102. Expansion of the physical map to include a marker or markers that flank the disease loci on the side opposite D10S102 will make possible the precise determination of the size of the MEN 2 region, complete cloning of this interval, and definition of a set of genes that can be considered candidates for MEN2A, MEN2B and/or MTC1.
MEN 2A, MEN 2B, MTC 1 and MEN 2A with cutaneous lichen amyloidosis are dominantly inherited cancer syndromes, which have in common medullary thyroid carcinoma. The work described in this thesis forms part of a laboratory-wide positional cloning strategy to identify the gene(s) responsible for these cancers. Cloning of these genes will be an important step toward understanding their role in endocrine oncogenesis.

When this work was undertaken in 1990, MEN2A had been mapped to pericentromeric chromosome 10 between FNRB on the short arm of chromosome 10 and RBP3 on the long arm (Wu et al., 1990). The first goal, to clone DNA markers more closely linked to MEN2A than either FNRB or RBP3, was fulfilled with the cloning of pC11/A1S-6-c23 and pC11/A1S-6-c65, DNA markers defining the D10S94 and D10S182 loci, respectively. These DNA markers were cloned by human repeat element-mediated PCR, a method for cloning human DNA fragments from hybrid DNA sources.

In repeat element-mediated PCR, PCR primers directed to human repeat elements result in human-specific DNA synthesis; template DNA derived from a reduced complexity somatic cell hybrid containing the human chromosomal region of interest provides region specificity. A series of repeat element-mediated PCR clones was generated using a reduced complexity somatic cell hybrid containing the chromosome 10 centromere and markers known to flank MEN2A. A total of 27 inter-Alu, two inter-L1 and four inter-Alu/L1 repeat element-mediated PCR clones were mapped to intervals of chromosome 10 by hybridization to Southern blots of repeat element-mediated PCR products amplified from somatic cell hybrid DNA templates. Two inter-Alu clones mapped near the centromere. A scarcity
of Alu elements in the pericentromeric region of chromosome 10 is proposed to have contributed to the low number of clones obtained from this region. One inter-Alu clone, pCl1/A1S-6-c23, defines the D10S94 locus, which is tightly linked to MEN2A and to D10Z1 (Goodfellow et al., 1990a).

D10S94 mapped within the region of overlap of the genetic intervals to which the disease loci have been mapped; genes located at D10S94 are therefore candidates for MEN2A, MEN2B, MTC1 and/or MEN2A+CLA on the basis of localization. Long range restriction mapping was undertaken at this locus, using pulsed field gel electrophoresis and restriction enzymes that cleave within CpG islands, molecular landmarks for the positions of many vertebrate genes. A 570-kb long range restriction map was generated by pulsed field gel electrophoresis using probes developed during a 160-kb bidirectional cosmid walk at D10S94. A dense cluster of six CpG islands was found within a 180-kb region; five CpG islands fall within a 145-kb NotI restriction fragment, which is contained in its entirety in the cosmid contig. The SacII, SfiI and NotI restriction maps for lymphoblast and cloned DNA are concordant. These CpG islands may represent the 5' ends of candidate genes for MEN2A, MEN2B and/or MTC1. One gene designated mcs94-1, which is associated with CpG island #2 of this cluster, has been isolated and characterized by Ms. Helen McDonald (McDonald et al., 1992).

The presence of six CpG islands at D10S94 indicated the possibility of several genes at this locus. A search for potentially disease-causing alterations in the genomes of MEN 2A and MEN 2B patients was undertaken concurrently with a search for transcribed sequences performed by other members of the Goodfellow laboratory. Field inversion gel electrophoresis was employed as a method of surveying the
CpG-rich region for alterations detectable by this method. Quantitation of the size of alteration detectable by this method was performed. No genetic alterations were noted in DNA of five MEN 2A and eight MEN 2B patients. Experimental conditions were such that alterations changing the size of restriction fragments by 3.3 kb or more were excluded over a 235-kb region that includes at least six CpG islands. Alterations of less than 1.6 kb were excluded over most of the region spanned by a 145-kb NotI fragment.

Refinement of the MEN2A candidate region through the description of critical crossovers that establish new, more closely flanking DNA markers, is an ongoing goal of both the Goodfellow laboratory and other MEN 2 researchers. It depends, as well, on the prior cloning of DNA markers derived from near the disease gene.

Genetic mapping studies have refined the MEN2A candidate interval; several 10q11.2 loci have been described that are very tightly linked to or have not been demonstrated to recombine with MEN2A. Determination of the physical relationships among these closely linked markers is a step toward the complete mapping of the MEN 2 region and would lead to an estimate of the number of genes therein. Genomic long range restriction mapping was used in combination with yeast artificial chromosome (YAC) cloning and restriction mapping to establish physical linkage, order and distances between the following 10q11.2 markers: \( D10S102, D10S182, D10S94, RET, D10F38S3 \) and DNA marker DM124. \( RET, D10S94, D10S182 \) and \( D10S102 \) were long range restriction mapped in human genomic DNA. \( RET, D10S94 \) and \( D10S182 \) are encompassed by a 1-Mb YAC contig, which also demonstrates physical linkage of \( D10F38S3 \) and the 10q11.2 sequences recognized by DM124 to these loci. These data together give an order and orientation of: 10cen, \( D10F38S3 \), DM124, \( RET, D10S94, D10S182, \)
D10S102, 10qter and place these loci within 1.4 Mb at 10q11.2, in a region that may contain MEN2A, MEN2B and/or MTC1.

These efforts are a significant contribution to the mapping and characterization of the MEN 2 region of chromosome 10. Figure 13 is my synthesis of a map of the pericentromeric region of chromosome 10 incorporating both results reported in the literature and this thesis work.

As described in the introduction, MEN2A is located between FNRB in 10p11.2 and RBP3 in 10q11.2. D10S34 (Mathew et al., 1991; Gardner et al., 1991; Lairmore et al., 1991; Lichter et al., 1992a; Lichter et al., 1992b) and D10S176 (Howe et al., 1992), have been positioned centromeric to FNRB within 10p11.2, more closely flanking the disease locus than does FNRB. Three 10q11.2 loci: D10S102 (Lairmore et al., 1993), D10S252 and D10S253 (Miller et al., 1992a) flank MEN2A more closely than does RBP3; D10S102 is the most proximal of these and is the closest flanking marker. MEN2B has been localized between D10Z1 and RBP3 (Norum et al., 1990). MTC1 and MEN2A+CLA are tightly linked to pericentromeric chromosome 10 markers; RBP3 flanks MEN2A+CLA distally on 10q.

The region of overlap between the candidate intervals to which the disease genes have been localized extends from D10Z1 to D10S102 and encompasses D10F38S3, DM124, RET, D10S94 and D10S182. If the diseases are allelic, then the disease gene would be expected to be located within this interval.

The order of loci depicted in figure 13 is a synthesis from several types of mapping. Orders established through genomic long range restriction mapping and YAC contig assembly have been considered to be more definitive than marker orders deduced by radiation hybrid mapping, which employs somatic cell hybrids that are subject to rearrangement.
The order of markers shown is in agreement with that reached by meiotic mapping (Gardner et al., 1991; Lichter et al., 1992a; Lichter et al., 1992b).

Two very recent reports (published in March 1993) have served to radically refine the MEN2A and MEN2B candidate regions. Gardner et al. (1993) describe critical crossovers in MEN 2 families which map MEN2A between D10S141 and D10S94 in 10q11.2. D10S141 (Tokino et al., 1992a), which maps proximal to D10S94, is defined by one of a series of cosmid clones mapped to the pericentromeric region (Mole et al., 1993a). An accompanying paper (Mole et al., 1993b) describes a YAC contig and long range restriction map of YAC clones which spans D10S141, RET and D10S94. The new MEN2A flanking markers, D10S141 and D10S94, are 480 kb apart; RET is between them. Comparison of the restriction map of Mole and colleagues to that in chapter VI reveals that D10S141 is located between map positions -510 kb and -420 kb (figure 12). The polymorphic sites at D10S94 are located at position -30 kb. The distance between positions -510 and -30 kb is 480 kb, in agreement with the size of the MEN2A candidate interval as determined by Mole et al. (1993b). Most of the CpG island-rich region at D10S94 falls within the newly refined MEN2A interval; only islands #1 and #2 are outside this region. The mcs94-1 gene is located at position -9 to -25 kb. It appears to lie outside the new MEN2A interval; it is, however, within a refined MEN2B candidate region, which extends from D10S141 to RBP3. RET remains a candidate for MEN2.

A number of genes that were formerly MEN2 candidates on the basis of their localization, lie outside the newly refined MEN2A region. ZNF22 (zinc finger gene 22; Rousseau-Merck et al., 1992) and ALOX5 (arachidonate 5-lipoxygenase; Goodfellow and Miller, 1992) were already ruled out as candidates on the basis that they map distal to MEN2A flanking marker D10S102. Four genes containing zinc finger motifs
FIGURE 13: Relative map positions of markers near MEN2. Chromosomal intervals 2, 3, and 4a are indicated to the left. Positions of DNA markers are shown in the center. Distances are not to scale. MEN2A is flanked by D10S141 and D10S94; MEN2B is flanked by D10S141 and RBP3. Position of the genomic map is indicated by a hatched bar; YAC contigs are represented by dotted bars. B = Brooks-Wilson et al., submitted; M = Mole et al., 1993b; L = Lairmore et al., 1993; T = Tokino et al., 1992b. Lengths of bars are not to scale. Mapped genes are indicated to the right.
(ZNF11A, ZNF25, ZNF37A and ZNF33A) map centromeric to D10S141 and are unlikely to be involved in MEN 2. ZNF33B is not within the D10S141 to D10S94 interval (Gardner et al., 1993). Two zinc finger genes (ZNF11B and ZNF37B) map within chromosomal interval 4a but have not been mapped relative to other markers in this interval, which is now predicted to contain both MEN2A and MEN2B. RET is a candidate for any or all of MEN2A, MEN2B, MTC1 and MEN2A+CLA.

The locations of YAC contigs reported by four groups, and the genomic long range restriction map described in chapter VI, are depicted in figure 13. The genomic map extends from D10S141 (deduced by map comparison) to D10S102. The YAC-based map of Mole et al. (1993b) overlaps extensively with that described in chapter VI, spanning D10S141, RET and D10S94. Lairmore and colleagues (1993) have cloned sequences from D10S141 to D10S102. The distal end of their contig overlaps with a 950-kb cloned region at D10S102 (Tokino et al., 1992b). The physical map described in this thesis is the only genomic long range restriction map of the entire MEN2A interval.

The examination of constitutional (for example, lymphoblast) DNA can bring to light the inherited mutations of MEN 2A, MEN 2B and MTC 1. These diseases breed true within families. It is therefore the inherited mutation, which determines disease phenotype. Comparison of inherited mutations will, then, provide information about the relationship between the genes responsible for these diseases. Several possibilities exist: the diseases may be allelic, they may be caused by separate mutations in different genes, or they may be due to a contiguous gene syndrome in which single mutations affect different sets of genes and give rise to different disease phenotypes. This last scenario, while still possible, now seems less likely if the disease genes are at D10S94. At present these
possibilities cannot be resolved; it will require the identification of
disease-causing mutations to elucidate the relationship between the genes
responsible for MEN 2A, MEN 2B, MTC 1 and MEN 2A with cutaneous lichen
amyloidosis.

MEN 2A has a very low mutation frequency; 50% of MEN 2B cases, in
contrast, are the result of new mutations. It will be interesting to find out
whether this is due to MEN 2A mutations being very specific (for example,
substitution of a particular nucleotide) and those of MEN 2B being more
general (for example, any mutation leading to loss of function).
Characterization of MEN 2 mutations will reveal if this is the case.

*RET*, an intriguing candidate for *MEN2*, is located within the refined
*MEN2A* candidate region. It was originally identified as a portion of a
fusion gene isolated from papillary thyroid carcinoma DNA by focus
formation upon transfection into NIH3T3 cells. *RET* thus has a
demonstrated ability, when suitably activated, to act as a dominant
oncogene. High levels of several normal sized *RET* transcripts have been
noted in MTC and pheochromocytomas (Santoro *et al.*, 1990). No genetic
rearrangements, however, were noted upon hybridization of a *RET* probe,
pret9.1T3, to Southern blots of FIGE gels of patient DNA during long range
restriction mapping (data not shown). In addition, an extensive search for
disease related alterations has been made at *RET* in the genomes of MEN 2
patients and in tumor DNA (Mulligan *et al.*, 1991b); to date no alterations
have been reported. From this result it seems likely that, if *RET* activation
plays a role in MEN 2, that activation occurs by means of relatively subtle
alterations in the majority of patients.

Examination of constitutional DNA does not provide insight into the
nature of the somatic events in tumorigenesis. Examination of tumor DNA
will be necessary to discover somatic genomic alterations and ultimately to
understand the mechanism of carcinogenesis underlying these hereditary endocrine cancers. Numerous DNA markers have now been mapped to the pericentromeric region of chromosome 10, near the MEN 2 gene(s). This will facilitate the examination of tumors derived from MEN 2 patients for local loss of heterozygosity in this region. Sets of polymorphic markers previously used in such experiments spanned chromosome 10 but were not concentrated in the pericentromeric region. This will determine definitively whether a localized LOH is part of the mechanism of carcinogenesis of MEN 2, or whether this type of event does not commonly contribute to the etiology of this cancer.

RET is within 480 kb of MEN2A. The failure to detect alterations at RET in tumor DNA by PFGE (Mulligan et al., 1992b) does not lend support to the notion that allelic loss at MEN2A plays a role in tumorigenesis; these studies, however, do not specifically assess heterozygosity at RET polymorphisms. Deletion of the entire RET locus and surrounding region from the normal chromosome 10 homologue may not have been detectable by the methods used. If a dominantly acting oncogene is the basis for MEN 2, then loss of heterozygosity at the disease locus would not be expected to occur in MEN 2 tumors.

There are many possible roles of the normal MEN 2 gene product(s). It may play a role in the differentiation of neuroendocrine cells, regulation of the cell cycle, or signal transduction. Cloning of the MEN 2 gene(s) will tell us whether the limited number of tumor types observed in this syndrome is related to the expression pattern of the gene or whether, like retinoblastoma, the gene is widely expressed but its loss induces formation of tumors only in certain tissues.

The work described in this thesis has contributed to progress toward the cloning of the MEN 2 gene(s). The cloning and characterization of the
polymorphic \textit{D10S94} locus is of use in the presymptomatic diagnosis of at risk members of MEN 2 families. Probes derived from and PCR primers which direct amplification of polymorphic sequences at this locus are currently in use in diagnostic laboratories.

\textbf{Prospects for future study.}

The genomic long range restriction map and YAC contig, which span six loci in 10q11.2, are a prelude to the construction of a physical map of the pericentromeric region of chromosome 10, and to an understanding of the organization of this region of the human genome.

The \textit{MEN2A} candidate interval has been refined to 480 kb (Gardner \textit{et al.}, 1993; Mole \textit{et al.}, 1993b), a size of region that is amenable to relatively detailed analyses. The entire \textit{MEN2A} candidate region has been cloned in YACs and has, as well, been long range restriction mapped in genomic DNA (Brooks-Wilson \textit{et al.}, submitted). These cloning and mapping efforts provide the basis for an intensive examination of this region, directed toward the identification of the \textit{MEN2A} gene. A search for evolutionarily conserved and transcribed sequences, either in proximity to CpG islands or not, is likely to identify more genes within this region. Examination of each candidate gene at the level of sequence, in the genomes of affected individuals, may be necessary to identify the gene(s) responsible for MEN 2.

The cloning of and study of the normal cellular product of the MEN 2 gene(s) will help to elucidate the role that mutations of this gene or genes play in endocrine oncogenesis. Identification of the gene(s) will allow experiments to be designed specifically to determine the normal cellular role of the gene product, and its relevance to processes such as cell cycle regulation, signal transduction, or cellular differentiation. Exploration of a
role in cellular differentiation would also contribute to the understanding of the normal development of the endocrine system. Comparison of the differences and similarities of the MEN2 gene, gene product and function, to those of other oncogenes or tumor suppressor genes, will help to address the tissue specificities of the action of such genes; these studies will contribute to our general understanding of human cancers.
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