Localization of the Niemann-Pick Type II Gene

by

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DEDICATION

This thesis is dedicated to my best friend, my sister Norma J. Hustins, who helped me grow up into the person I am today and for being one of the most courageous people I know. My admiration and respect for her can not be expressed in words alone. Thanks for everything 'sis'!

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ABSTRACT

Niemann-Pick Type II (NP Type II) disease is an autosomal recessive childhood neurological disorder. The biochemical defect is an impairment in transport of intracellular cholesterol. This disease has been classified into two subtypes, C and D. NPC is found to comprise at least two complementation groups, whereas NPD has been identified only in descendants of an Acadian population from Yarmouth County, NS. NPD and the major complementation group of NPC both map to chromosome 18q11-12, therefore they are likely allelic variants (Carstea et al., 1993. PNAS. 90:2002-2004; Greer et al., 1997. Am. J. Hum. Genet. 61:139-142.) The objective of this study was to localize the NP Type II gene utilizing three approaches; two of these were directed toward positional cloning, while the third was a functional approach. The first approach of classic linkage analysis, attempted to reduce the NP Type II critical region by identifying new NPD families with potentially recombinant individuals, that may have emigrated from Yarmouth County, NS. The second approach involved retrofitting candidate YACs with the mammalian selectable marker, neomycin, in preparation for complementation studies with NP Type II fibroblasts and NP Type II mutant CHO cells. The final approach was based on the fact that progesterone induces a NP-like phenotype in normal fibroblasts. Normal and NPC cells were photoaffinity labeled with [^{3}H]-progesterone as an attempt to identify a candidate NP Type II protein that was present in normal but absent in mutant cells. Four new NP Type II families were identified, only one of which was an NPD family from Massachusetts. The NP Type II gene was previously localized between microsatellite markers D18S44 and D18S1108 (Greer et al., Am. J. Hum. Genet., in prep), however a recombination in one of the newly identified NPC families moved the distal limit of the critical region to D18S1101. This reduces the critical region to a 1cM interval between D18S44 and D18S1101. The NP Type II gene has been narrowed to a set of three overlapping YACs, 877F12, 906C12 and 911D5, that spans the previously defined proximal (D18S44) and new distal (D18S1101) limits. Retrofitting of YACs 877F12 and 906C12 with the mammalian selectable marker, neomycin, has been completed and they can now be used to further define the location of the NP Type II gene by attempting to rescue normal LDL-stimulated ACAT activity in cultured NP Type II fibroblasts and NP Type II mutant CHO cells. Photoaffinity labeling of normal and NPC cells with [3H]-progesterone identified a 60kDa cytosolic protein that was present in normal but not NPC cell lines

LIST OF ABBREVIATIONS

ACAT	acyl-CoA:cholesterol acyltransferase
APS	ammonium persulfate
BAC	bacterial artificial chromosomes
BSA	bovine serum albumin
CHO	chinese hamster ovary cells
cM	centimorgan
DMEM	Dulbecco's Modified Eagle Medium
DMSO	dimethylsulfoxide
DTT	diethylthreitol
EBV	epstein barr virus
EDTA	ethylenediamine tetraacetic acid
ER	endoplasmic reticulum
EtBr	ethidium bromide
FBS	fetal bovine serum
G418	geneticin
[^{3}H]-PRG	[^{3}H]-progesterone
HLA	human leukocyte antigen system
HMG-CoA reducatase	hydroxymethylglutaryl CoA reductase
LDL	low density lipoprotein
LPDS	delipidated serum

MDR	multiple drug resistance
neo^r	neomycin resistance
NPA	Niemann-Pick Type A
NPB	Niemann-Pick Type B
NPC	Niemann-Pick Type C
NPD	Niemann-Pick Type D
NP Type II	Niemann-Pick Type II
p60	60 kDa protein
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
PEG	polyethylene glycol
PMSF	phenylmethylsulfonyl fluoride
RT	room temperature
SC	synthetic complete media
SDS	sodium dodecyl sulfate
SSCP	single strand conformation polymorphism
STS	sequence tagged sites
TBS	tris buffered saline
TE	Tris-EDTA
TEMED	N,N,N',N'-tetramethylethylenediamine
VNTR	variable number of tandem repeats
YAC	yeast artificial chromosome
25-HC	25-hydroxycholesterol

ACKNOWLEDGMENTS

1.0 INTRODUCTION

Niemann-Pick disease is an autosomal recessive neurodegenerative disorder that usually occurs during childhood. It was first identified in 1914 by Drs. Albert Niemann and Ludwig Pick. It was characterized as causing an increase in tissue phospholipids, particularly sphingomyelin, with variability in the age of onset, clinical expression and lipid storage. In 1958 a review of 18 patients was published by Crocker and Farber which describes a more detailed account of the clinical and pathological manifestations of the disease. It was recognized that there was a great deal of phenotypic heterogeneity among individuals with Niemann-Pick disease, and therefore the disease was classified into 4 subgroups based on biochemical and clinical data (Crocker and Farber, 1958 and Elleder and Jirasek, 1983). Type A (NPA) was the 'classical' form which involved central nervous system degeneration with death occurring in early infancy; type B (NPB) had heavy visceral involvement without neurodegeneration; type C (NPC) had a moderate amount of neurodegeneration and individuals usually only live until early adolescence. and type D (NPD) which seems to be a milder variant of NPC. Interestingly, of the 18 patients originally described by Crocker and Farber, 4 of them, which he classified as type D, had ancestry tracing to a French Acadian population residing in Yarmouth County, Nova Scotia.

Analysis of various lipid contents in Niemann-Pick patients revealed that individuals with NPC and NPD have more cholesterol than sphingomyelin stored in their tissues, as compared to NPA and NPB patients who have high levels of sphingomyelin

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and normal levels of cholesterol (Crocker, 1961). Additional studies by Brady et al (1966) and Schneider and Kennedy (1967) showed greatly reduced or absent levels of sphingomyelinase in NPA and NPB patients and relatively normal levels in those patients with NPC and NPD, providing increasing evidence that accumulation of some other lipid(s) are at least partly responsible for the characteristic phenotype of NPC and NPD patients. These studies indicated that there were at least 2 distinct types of Niemann-Pick disease, and hence NPA and NPB were subsequently referred to as Niemann-Pick Type I disease (NP Type I) and NPC and NPD were known as Niemann-Pick Type II (NP Type II) disease (Spence and Callahan, 1989). The remainder of this thesis will focus on NP Type II disease.

1.1 CLINICAL MANIFESTATIONS OF NP TYPE II DISEASE

There is a great deal of phenotypic heterogeneity among individuals with NP Type II disease (Vanier et al., 1991a, Vanier et al., 1991b and Natowicz et al., 1995). Vanier et al. (1988) developed a classification system for NP Type II patients based on the age of onset and neurological symptomatology. There is a severe infantile form in which the individual presents with hypotonia and delay of developmental motor skills evident by age 1-1.5 years with loss of acquired motor skills, intention tremor, spasticity and pyramidal tract involvement and death between 3-5 years. Also described were a late-infantile form which includes ataxic gait, clumsiness and the age of onset between 2-4 years, and a juvenile form in which cerebellar involvement, variable mental regression and poor school performances are present between 4-15 years with ataxia, dysarthria, cataplectic attacks, epileptic manifestations and vertical supranuclear ophthalmoplegia

occurring later in life. The final classification was an adult form of the disease which has no well-defined pattern but several patients were identified with psychiatric involvement. NPD seems to resemble the milder juvenile form of NPC. Hepatosplenomegaly is usually seen in most cases of NP Type II disease as a result of the excessive accumulation of lipids in the tissues. The onset of vertical supranuclear ophthalmoplegia is the neurological hallmark of the disease (Pentchev et al, 1995 and Brady et al., 1989). To date there is no effective treatment available for individuals with NP Type II disease.

1.2 PATHOLOGICAL FEATURES OF NP TYPE II DISEASE

The characteristic pathologic feature in NP Type II disease is the presence of visceral foamy cells and neuronal storage. Foamy cells and sea-blue histocytes are found prominently in the spleen, tonsils, lymph nodes, liver and lung. One of the distinguishing features in the brain is cytoplasmic ballooning of the cortical, basal ganglia, thalamus, substantia nigra and locus ceruleus neurons, however the degree of ballooning varies with each case. In severe cases, Purkinje and granular cells are lost and replaced with dense fibrillary gliosis, and many of those that survive have perikarya distended with storage materials. Neuronal storage is seen in the anterior and posterior horn neurons of the spinal cord and in the myenteric plexus, but is absent in the small pyramidal neurons in layers II, III and IV (Pentchev et al., 1995).

1.3 REGULATION OF INTRACELLULAR CHOLESTEROL METABOLISM BY LDL

Early studies of Niemann-Pick disease suggested that accumulation of lipids, specifically sphingomyelin, occurred in the tissues. This was later found to be the primary biochemical defect in NP Type I disease; however further studies indicated that possibly cholesterol storage may play a role in NP Type II disease. The exogenous low density lipoprotein (LDL)-cholesterol pathway is the preferred way for most cells to derive cholesterol for membrane function, and is subject to strict intracellular cholesterol regulation. LDL particles are delivered to the cell membrane where they bind to LDL receptors and become internalized via endocytosis. The LDL particle then disassociates from the LDL receptor which is recycled and returned to the cell membrane (to wait for another LDL particle to arrive). The internalized LDL particle is then delivered to the lysosome, where its LDL phospholipid coat and cholesterol esters are degraded and the LDL protein is hydrolyzed into amino acids. Unesterified cholesterol leaves the lysosome and is used by the cell to synthesize cellular membranes, bile acids and steroid hormones (Brown and Goldstein, 1986). Cholesterol in excess of cellular requirements is re-esterified by the endoplasmic reticulum (ER) enzyme acyl-CoA:cholesterol acyltransferase (ACAT), resulting in formation of cytoplasmic cholesteryl ester droplets for storage. The cell displays several responses to LDL cholesterol including down regulation of cholesterol synthesis by suppression of hydroxymethylglutaryl-CoA reductase (HMG-CoA reductase), suppression of the synthesis of LDL receptors and as previously mentioned stimulation of ACAT (Goldstein and Brown, 1977 and Brown and Goldstein, 1986). All of these regulatory mechanisms work together to maintain stable,

normal levels of unesterified cholesterol despite variations in its supply and demand (Figure 1).

1.4 BIOCHEMICAL LESION INVOLVED IN NPC DISEASE

Insight into the biochemical defect in NP Type II disease was assisted by the discovery of a mutant BALB/c mouse with accumulation of sphingomyelin, glucocerebrosidase and cholesterol in lysosomes (Pentchev et al., 1980, Pentchev et al., 1984). A similar inbred C57BL/KsJ mouse strain was reported in Japan that carried a genetic lesion with a clinical and biochemical phenotype similar to the BALB/c mouse mutant (Miyawaki et al., 1982 and , Sakiyama et al., 1982). Since both mouse strains present with an autosomal recessive lesion and clinical and biochemical phenotypes similar to that of NP Type II disease, the murine disorder was therefore suggested to represent an animal counterpart of this human lipid storage disorder. A predominating feature of the murine storage disorder was the excessive accumulation of unesterified cholesterol in tissues. Hepatic levels of unesterified cholesterol were observed as high as 15 times the normal level (Pentchev et al., 1980). Due to the phenotypic similarities between this murine disorder and NP Type II disease, an investigation involving the utilization of cholesterol in the mutant mice and NPC fibroblasts was undertaken in hopes of gaining a better understanding of the underlying basis of this inborn error of metabolism.
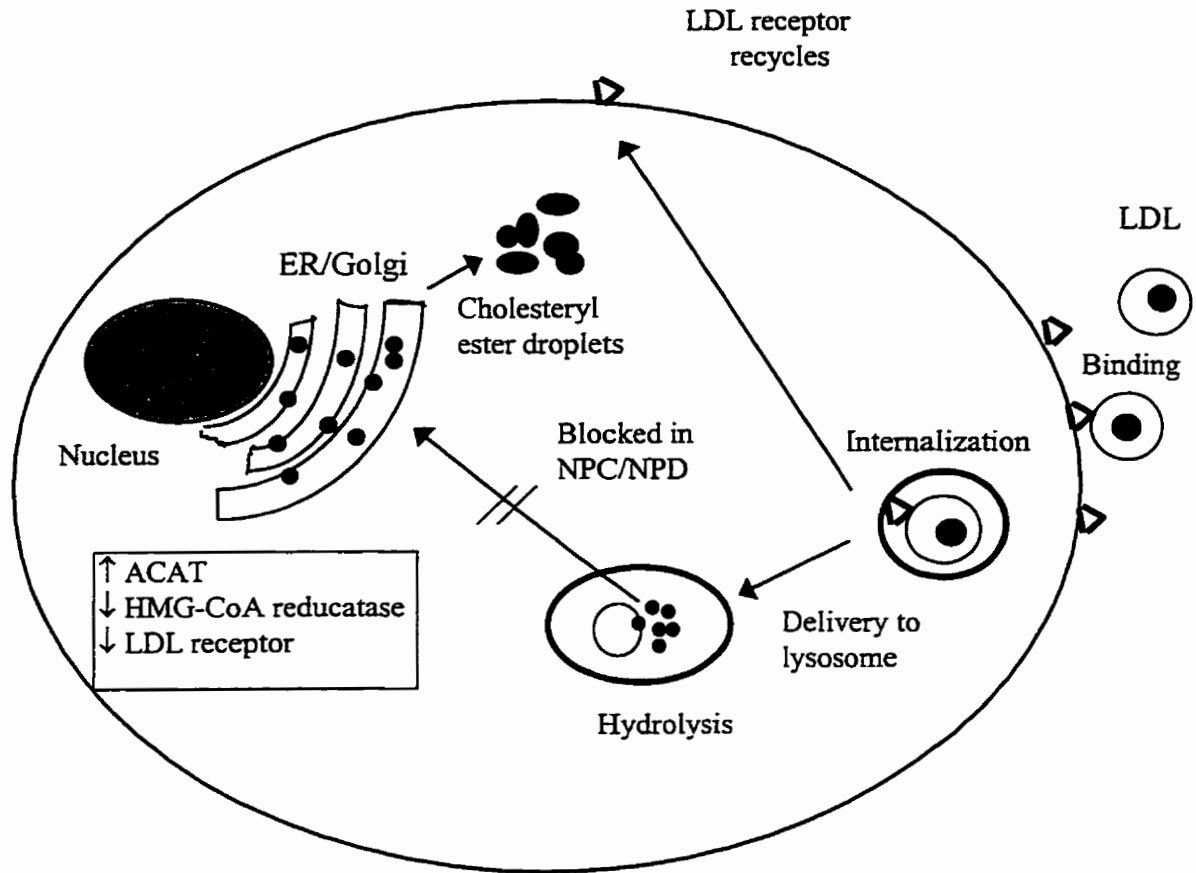
Figure 1: LDL-cholesterol pathway in mammalian cells

An LDL particle will recognize and bind to an LDL receptor, become internalized and subsequently be delivered to the lysosome, while the LDL receptor is recycled back to the membrane. The LDL is hydrolyzed to release free cholesterol. After leaving the lysosome, excess cholesterol travels to the ER to stimulate the enzyme ACAT, which re-esterifies the cholesterol in cytoplasmic cholesteryl ester droplets for storage. An increase in ACAT activity, a decrease in LDL receptor recycling and a decrease in HMG-CoA reductase are seen in response to LDL-cholesterol. In NPC/NPD cells, trafficking of free cholesterol from the lysosome to the ER and Golgi is blocked. This figure was modified from Brown and Goldstein (1986).

Murine in vitro experiments demonstrated a metabolic block in the esterification of exogenously derived cholesterol. In normal mouse fibroblasts, an increase in the net level of LDL-derived cholesterol and thus an increase in the rate of synthesis of cholesteryl esters was seen. In comparison, the uptake of exogenous LDL-derived cholesterol was not accompanied by a comparable rise in cholesteryl esters in the homozygous affected mouse fibroblasts (Pentchev et al., 1984). A study with 31 separate Niemann-Pick cell lines, 13 NPA/NPB and 20 NPC, showed that intracellular cholesterol esterification is severely compromised in cultured fibroblasts of patients with NPC only (Pentchev et al., 1985). These results indicate that the mutant BALB/c mouse is a good model for studying NPC and that they are both closely linked to an error in the ability to esterify exogenous cholesterol (Pentchev et al., 1985 and Pentchev et al., 1986). NPC heterozygote fibroblasts were found to be able to synthesize only intermediate levels of cholesteryl esters. Furthermore, in addition to a defect in cholesterol esterification, NPC cells accumulated substantially more total cholesterol when incubated with LDL or FBS than the normal fibroblasts (Kruth et al., 1986). These results relevantly link the defect in cholesterol storage and esterification to the primary mutation in NPC disease. Additional studies have confirmed that impairment in intracellular cholesterol esterification is a key feature of NPC disease (Liscum and Faust, 1987, Vanier et al., 1988).

The regulatory functions normally elicited in response to LDL-cholesterol are not seen in NP Type II cells. LDL does not down-regulate cholesterol synthesis by suppression of HMG-CoA reductase, LDL does not down-regulate LDL receptor activity and there is no stimulation and subsequent increase in ACAT activity in response to LDL-

cholesterol (Pentchev et al., 1985, Liscum and Faust, 1987). However, LDL binding, internalization and lysosomal hydrolysis of cholesterol esters is normal. Normal cholesterol regulation is observed in NP Type II cells in response to nonlipoprotein compounds such as oxysterols (i.e. 25-hydroxycholesterol (25-HC)) and mevalonate. 25-HC was shown to be capable of suppressing cholesterol synthesis and LDL receptor activity as in normal cells and mevalonate-derived sterol regulation of cholesterol synthesis was also normal. This suggests that there are different mechanisms for endogenous (mevalonate-derived) versus exogenous (LDL-derived) sterol regulation of cholesterol (Liscum and Faust, 1987). Transport of *de novo* synthesized cholesterol to the plasma membrane was also found to be normal in NPC cells suggesting again that cholesterol transport appears to be affected only when trafficking is through the lysosomes (Liscum and Faust, 1989 and Pentchev et al., 1994).

1.5 BIOCHEMICAL LESION INVOLVED IN NPD DISEASE

Due to the fact that NPC and NPD are clinically quite similar, studies were initiated to determine if LDL-derived cholesterol esterification was defective in NPD fibroblasts. Byers et al. (1989) found stimulation of ACAT by LDL was also defective in NPD cells. Accumulation of unesterified LDL-derived cholesterol was found in the lysosomes of NPD fibroblasts and subsequent reduction of cholesterol esterification was seen (Sidhu et al., 1992). These studies also revealed that NPC and NPD seem to exhibit separate and distinct biochemical defects. Not only do patients with NPD appear to have a milder clinical phenotype than those with the more severe forms of NPC, but they also seem to have a milder biochemical phenotype. NPD fibroblasts appear to have a lower

amount of cholesterol accumulation in their lysosomes and demonstrate a higher LDL-induced stimulation of ACAT activity and cholesterol esterification (Byers et al., 1989 and Sidhu et al., 1992). Further studies by this group revealed that the same biochemical defect seen in both NPC and NPD fibroblasts was also present in NPC and NPD Epstein-Barr virus-transformed lymphoblasts (Byers et al., 1994).

1.6 DIAGNOSIS OF NP TYPE II DISEASE

Clinical symptoms and the presence of foam cells are a good indication of NP Type II disease; however reduced cholesterol esterification and lysosomal cholesterol storage provides a more accurate diagnosis. Two biochemical assays are routinely used in conjunction as a method of detection of NP Type II disease in fibroblasts. Distribution of cholesterol within the cell can be detected by staining with the fluorescent probe filipin, an antibiotic that forms specific complexes with unesterified cholesterol. NP Type II cells show brightly fluorescing lysosomes due to the unesterified cholesterol stored there, as compared to normal cells which reveal only small, dispersed fluorescing areas throughout the cell (Severs et al., 1983). The second biochemical assay involves the measurement of ACAT activity, which esterifies excess internalized cholesterol for storage. When NP Type II fibroblasts or lymphoblasts are cultured in the presence of LDL, very low levels of ACAT activity are seen, in comparison to normal cells which exhibit a dramatic increase in ACAT activity in response to the LDL-cholesterol (Byers et al., 1994). Interestingly, heterozygotes show intermediate levels of ACAT activity in response to LDL-cholesterol (Kruth et al., 1986). Even though ACAT activity in heterozygotes differs significantly from either affected or normal individuals, this assay can not be

routinely used as a diagnostic carrier test due to the considerable overlap between carrier and normals as well as carrier and affected individuals.

1.7 NP TYPE II MODELS

In addition to the BALB/c mouse model, there are various other NP Type II models that are being researched in laboratories around the world in hopes of providing insight into NP Type II disease and cholesterol transport. Chinese hamster ovary (CHO) mutant cell lines were first described by Cadigan et al. (1990) who reported a CHO mutant defective in intracellular LDL-cholesterol trafficking. In 1992, Dahl et al. isolated several other CHO mutants that displayed a biochemical phenotype similar to NPC fibroblasts. Complementation analysis with six of the mutants reported by Dahl et al. (1992) and the cholesterol transport mutant developed by Cadigan et al. (1990) revealed the existence of two complementation groups. Six of the seven mutants were found to express a biochemical phenotype closely resembling that of NPC. The seventh mutant seemed to display a variant NPC phenotype similar to that seen in an NPC heterozygote (Dahl et al., 1994). The development of this NPC mutant CHO cell type provides a valuable resource for gaining information into both the biochemical and genetic basis of this disorder. The use of the CHO mutants will facilitate genetic manipulation as compared to their human and murine counterparts as they are the most widely used cell line for somatic cell genetics of cholesterol metabolism (Cadigan et al., 1990 and Dahl et al., 1994). These CHO mutants are also ideal for further complementation studies involving NPC fibroblasts, exogenous DNA and YACs. Spheroplast fusion of YACs

containing human DNA from the critical NPC/NPD gene region to NPC mutant CHO cells could aid in the refinement of the position of the NP Type II gene.

A number of hydrophobic amines that accumulate in the lysosome have also been shown to be able to induce lipidosis in a variety of cell types (Rodriguez-Lafrasse et al., 1990). The exact nature and pattern of accumulation varies depending on cell/tissue type and the hydrophobic amine used. Two hydrophobic amines imipramine and 3-β-(2-diethylaminoethoxy)androst-5-en-17-one hydrochloride (U18666A), are commonly used to induce a NP Type II-like phenotype in normal human fibroblasts by their ability to block movement of cholesterol from lysosomes to the plasma membrane, ER or other cellular compartments. When normal fibroblasts are incubated in the presence of LDL with either of these compounds, unesterified cholesterol accumulates within the lysosome as seen by measurement of ACAT activity and filipin staining, and down regulation of the LDL receptor and HMG-CoA reductase is reduced (Rodrigiez-Lafrasse et al., 1989, Roff et al., 1991 and Underwood et al., 1996). Similar results were also reported in CHO cells incubated with U18666A (Liscum and Faust, 1989). The biochemical phenotype is almost identical to that seen in NP Type II cells and therefore provides another model for learning more about cholesterol metabolism and NP Type II disease.

In addition to hydrophobic amines, the steroid progesterone has also been shown to induce a NP Type II-like phenotype in normal, human fibroblasts and macrophages (Butler et al., 1992 and Mazzone et al., 1995). Progesterone is known to inhibit esterification of LDL-derived cholesterol by preventing the delivery of LDL-cholesterol to ACAT (Brown and Goldstein, 1980). Progesterone also inhibits the transport of LDL-

cholesterol from the lysosomes to the plasma membrane (Butler et al., 1992) and the transport of LDL-cholesterol from the plasma membrane to the ER (Lange et al, 1994). Metherall et al. (1996a) demonstrated that progesterone inhibited cholesterol biosynthesis in CHO cells which resulted in the accumulation of sterol precursors. The exact mechanism of how and why progesterone can inhibit LDL-cholesterol esterification is not fully understood, however it is believed to be associated with progesterone's ability to also inhibit the activity of the multiple drug resistance (MDR) P-glycoprotein (Metherall et al., 1996b). MDR P-glycoproteins are believed to have a role in facilitating the transport of normal cellular lipids across membranes and have recently been demonstrated to be required for the esterification of LDL-derived cholesterol suggesting that they are involved in a general cellular process that is required for esterification (Metherall, et al., 1996b and Debry et al., 1997).

Early studies showed that a variety of steroid ketones are photoactive and will covalently bind to specific proteins in the presence of ultraviolet light. The α,β-unsaturated ketone structure in progesterone has been shown to be inherently photoactive and attachment of axido or axidobenzoyl groups is not required for its covalent binding to protein under UV light (Martyr and Benisek, 1973). Photoaffinity labelling of binding sites of these ketones is a novel and potentially useful approach for identifying a specific protein in a complex mixture of proteins that contains the site of interest. Photoaffinity labelling of progesterone has been used in several cases to identify a progesterone binding protein. Progesterone and its synthetic counterpart, R5020 have been found to photoaffinity label P-glycoprotein in MDR human leukemic lymphoblasts, as well as an

adrenocortical nuclear progesterone-binding protein and progesterone receptors in human breast cancer cells (Qian and Beck, 1990, Demura et al., 1991, Horwitz and Alexander, 1983 and Horwitz et al., 1985). Since progesterone has been shown to induce normal cells to exhibit a phenotype similar to NP Type II cells, it may be possible that the progesterone is binding to the protein defective or absent in NP Type II cells and therefore causing the resulting phenotype. A novel approach of photoaffinity labelling normal and NP Type II cells with [^{3}H]-progesterone ([H^3]-PRG) may provide a mechanism for identifying candidate NP Type II proteins.

1.8 GENETIC AND DEMOGRAPHIC ASPECTS OF NPD DISEASE

In 1978, Winsor and Welch published a study investigating the genetic and demographic aspects of NPD disease. This study looked at several NP Type II families from the Yarmouth County region and tried to establish a link to a common ancestor. It was found that parents of all the affected children could be traced to one particular couple, Joseph Muise, born in Nova Scotia in 1679 and Marie Amirault, born in Nova Scotia in 1674 (Figure2). This finding provided clear evidence that NPD is a genetic isolate of Yarmouth County, NS as compared to its NPC counterpart which is of panethnic origin. All of the NPD families were found to live within an approximate 10 mile radius within Yarmouth County. Recent genealogical studies by Greer et al. (1997c) have since updated the original NPD pedigree published by Winsor and Welch (1978). These findings suggest that the NPD mutation could have more recent origins than previously proposed. It is believed that one of the 12 or so returning couples to Yarmouth County

Figure 2: The original Winsor and Welch NPD pedigree

This pedigree links all 15 NPD patients to a possible founding couple, Joseph Muise and Marie Amirault born c. 1680. It is thought that at least one of these founders may have carried the NPD mutation and passed it down through the generations. This figure was reproduced from Winsor and Welch (1978).

Nova Scotian Niemann–Pick Disease
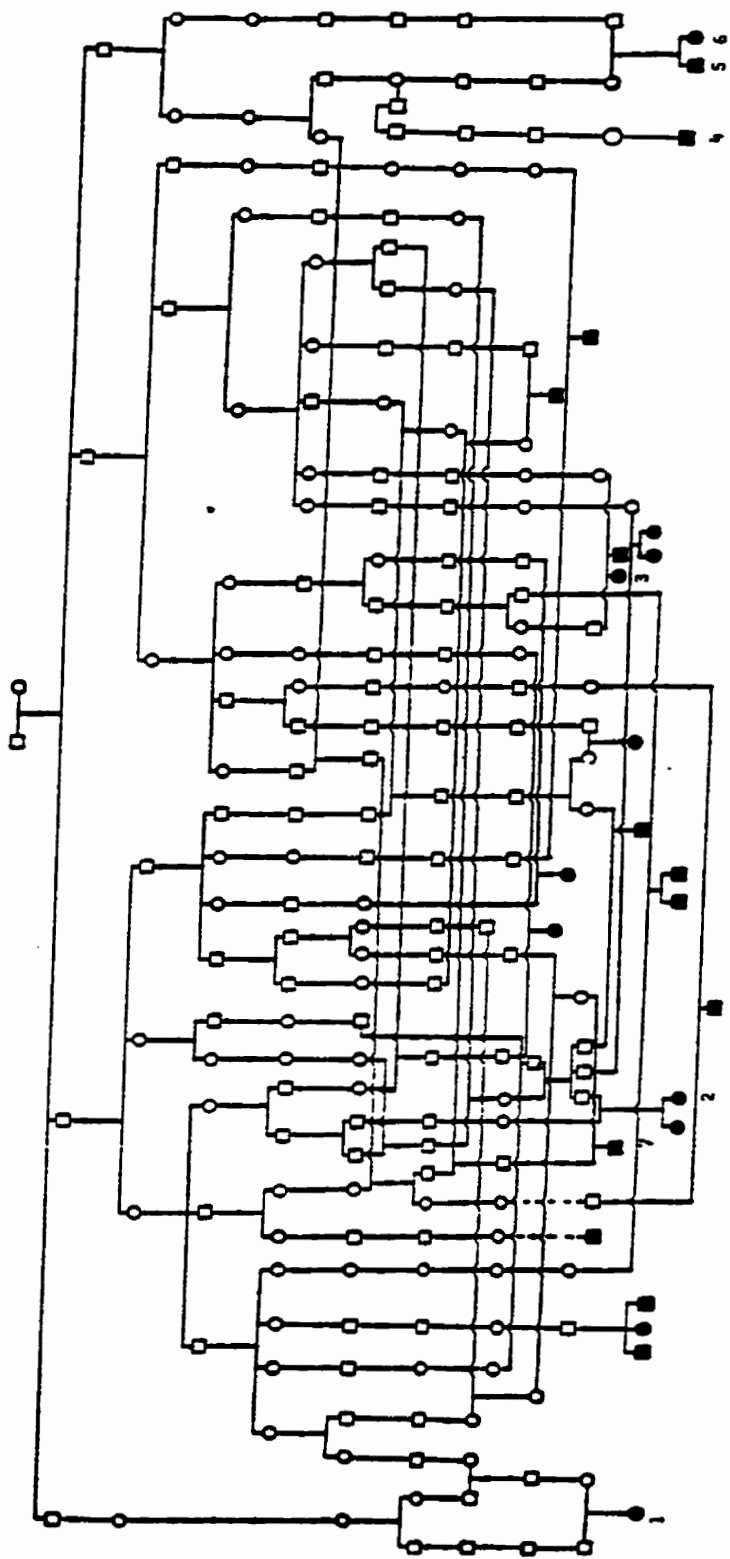
Figure 2

after the Acadian expulsion may have carried the NPD mutation and passed it through the family (Figure 3). One particular couple (#5 and 6) was found to be the most recent common ancestral pair to which 34 of the 38 NPD parents were found to descend from this couple and hence, were arbitrarily marked as the NPD ancestral pair (Greer et al., 1997b).

1.9 PREVALENCE OF NPD DISEASE IN YARMOUTH COUNTY, NS

Winsor and Welch (1978) also assessed the prevalence of the NPD gene in the Yarmouth County community. Yarmouth County was found to have the highest incidence of NP Type II disease in the world with an estimated frequency of affected children of 1% and a heterozygote carrier frequency between 10-26%. The high prevalence of this gene can be explained by a founder effect where one of a relatively small group of original 'founders' of the community carries a rare or mutant allele which is then subsequently passed down from generation to generation, becoming fixed in the closed population. Since all NPD families are thought to be descendants of a common ancestor, then theoretically all NPD patients should have the same mutation, thus producing a very homogenous population as compared to the phenotypically indistinguishable, NPC heterogenous population. This extensive NPD kindred along with the founder effect make this family extremely useful in localizing and identifying the NPD gene

Figure 3: The updated Winsor and Welch pedigree

Recent genealogical searches have indicated that another founding couple, born after Joseph Muise and Marie Amirault, may be the founding couple. One particular couple (#5 and 6), born c. 1770, was the most recent common ancestral pair to which 34 of the 38 NPD parents were found to desend from, This figure was reproduced from Greer et al., (1997b).
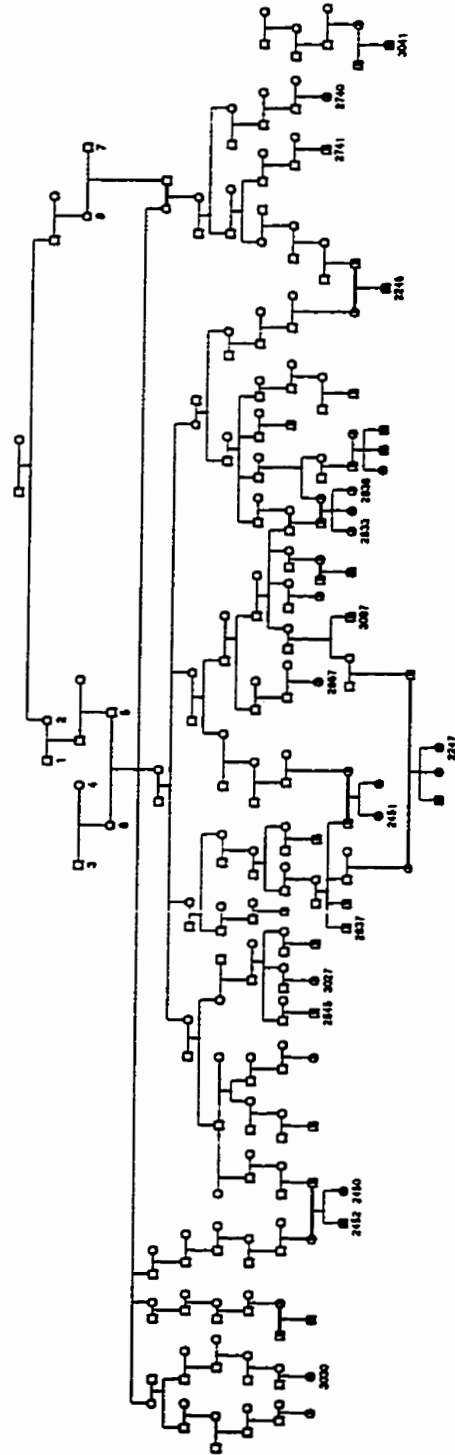
Figure 3

1.10 LOCALIZATION OF THE NPC GENE

An ongoing, extensive search for the genetic defect responsible for NPC and NPD is being pursued. Early studies involved the use of a cell line derived from the sphingomyelinosis mouse (spm/spm) which exhibited abnormal intracellular accumulation of exogenous cholesterol. Portions of intact human chromosomes, specifically chromosomes 9, 11 and 18 were transferred by microinjection into the mutant cell lines in hopes that one would restore normal activity. Indeed, transfer of mouse chromosome 18 to the *spm*-3T3 cell line displayed complete restoration of cholesterol esterification as seen by filipin staining. This provided the first piece of evidence that the gene responsible for NP Type II disease was located on chromosome 18. One of the cell lines had deletions on 18p11.3 and 18q21.3 and did not complement when transferred to NPC cells suggesting that the gene may be located in one of these regions (Kurimasa et al., 1993). Regions of mouse chromosome 18 are syntenic with human chromosomes 5 and 18, therefore subsequent experiments transferred human chromosome 5 and 18 DNA to human NPC fibroblasts. It was found that transfer of human chromosome 18 to NPC cells restored normal cholesterol esterification activity suggesting that the gene was located somewhere on this chromosome. The strategy of linkage analysis utilizing polymorphic microsatellite markers specific for human chromosomes 5 and 18 was examined by Carstea et al. (1993). There was no evidence of localization of the NPC gene to chromosome 5, however certain chromosome 18 markers did appear linked to the centromeric region of chromosome 18, specifically 18q11-12. Additional studies by Carstea et al. (1994) showed localization of the NPC gene to a 5 cM interval between

microsatellite markers D18S44 and D18S66 (Figure 4). Complementation studies by Steinberg et al. (1994) and Vanier et al. (1996) showed the existence of two NPC complementation groups. The major complementation group, which consisted of 27/32 unrelated patients who spanned the entire phenotypic spectrum, was shown to map to 18q11-12. The remaining 5/32 patients, who presented with a phenotype indistinguishable from the major NPC complementation group did not map to 18q11-12. Complementation studies with the C57BL/KsJ mouse model revealed that it belonged to the major NPC complementation group as well (Akaboshi, 1997). The chromosomal assignment of the minor complementation group is still unknown. These studies provide evidence that mutations in at least two different genes are responsible for NPC, which may offer a partial explanation for the broad range of phenotypic heterogeneity seen in this disease except for the heterogeneity seen within the major complementation group. It has been suggested that the two gene products may function together or sequentially in a common metabolic pathway affecting intracellular cholesterol transport (Vanier et al., 1996).

1.11 LOCALIZATION OF THE NPD GENE

Recently, studies in our lab have shown that the gene responsible for NPD maps to the same pericentromeric region of chromosome 18 as the major complementation group of NPC (Greer et al., 1997a). Cell fusion experiments that assessed complementation between NPC and NPD fibroblasts did not appear to restore normal cholesterol.
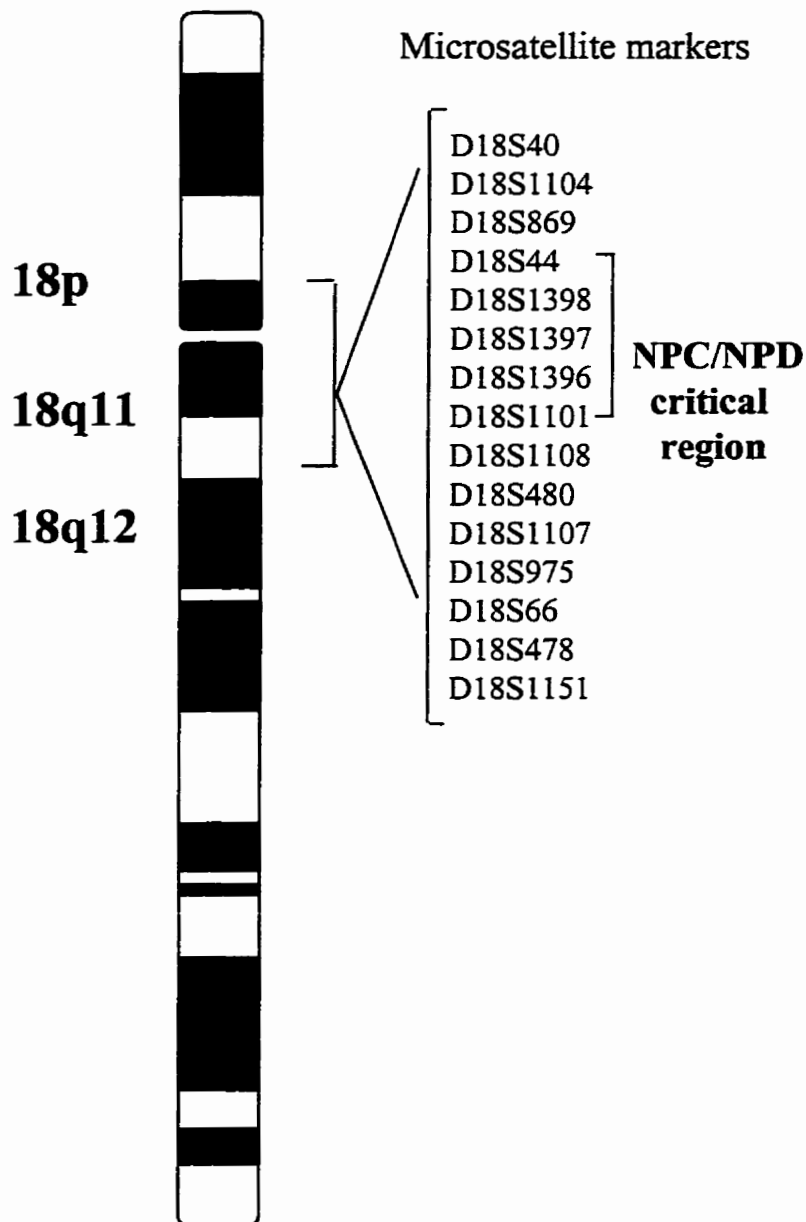
Figure 4: Chromosome 18q11-12

Ordered polymorphic microsatellite makers from a 15 cM interval on chromosome 18q11-12. These markers were used for linkage analysis in the NP Type II families

esterification activity (Sidhu et al., 1993). These results together with the linkage data indicate that NPD is an allelic variant of the major complementation group of NPC. A strategy of linkage disequilibrium mapping, which takes advantage of the founder effect in our NPD population, was utilized to further refine the position of the NPD gene. In this approach one assumes that specific combinations of alleles at closely linked loci occur more frequently with the affected allele than would be expected by chance (Thompson et al., 1991). This approach has defined the critical region of the NPD gene to a 2cM interval between microsatellite markers D18S1398 and D18S1108 (Greer et al., 1997b).

1.12 POSITIONAL CLONING OF HUMAN DISEASE GENES

Postitional cloning is the molecular cloning of a gene on the basis of knowledge of its map position without prior knowledge of its gene products. There are various components of positional cloning that can be utilized to localize and isolate genes.

1.12.1 Genetic mapping

Linkage analysis takes advantage of the fact that alleles close together or linked on the same chromosome are likely to segregate together as an intact unit during meiosis. This is useful for constructing genetic or linkage maps which reflect the relative order and distance of genetic markers within an interval of DNA. A few requirements are necessary in order to carry out linkage analysis. Firstly, availability of families, either many small, nuclear families or several large extended families is essential; secondly, markers in the

chromosomal region of interest are needed. In order for genetic markers to be useful they must be polymorphic and informative. The variability of these markers allows their segregation pattern to be followed through the family. They become informative when the parents are heterozygous, thus enabling one to see which chromosome allele was passed on to the offspring. If a marker is tightly linked to a gene, they are almost always transmitted together, however, if they are unlinked, they will segregate randomly (Thompson et al., 1991). The greater the distance between a marker and a gene, the greater the frequency of a meiotic recombination occurring between them. Recombination involves the exchange of chromosomal material between two homologous chromosomes resulting in a new hybrid chromosome. Recombination events can be extremely useful in mapping a particular gene. They can aid in the ordering of makers with respect to one another, and also with respect to a disease locus. They can also limit the critical region of a gene by excluding a portion of the chromosome above or below the recombinant breakpoint. This is due to the fact that some previously linked markers may no longer cosegregate (Green et al., 1995).

1.12.2 Genetic markers

The majority of genetic markers used today are DNA sequence differences including RFLPs, VNTRs and microsatellites. RFLPs represent a DNA sequence variation producing fragments of different sizes following digestion with a restriction endonuclease. VNTRs, or variable number of tandem repeats, are 11-60 bp tandemly repeating segments of DNA. The most widely used VNTRs are microsatellite markers due to their great degree of variability and hence informativeness, and frequent

occurrence in the genome. They consist of either di-, tri-, or tetra-nucleotide repeats that can be easily detected by PCR amplification and resolved on a denaturing polyacrylamide gel. CA repeats are the most common microsatellites occurring approximately every 30-60 kb (Green et al., 1996 and Weber, 1990).

1.12.3 Physical mapping

Once a gene has been localized to a certain area, it is often necessary or useful to begin to physically map the area. Physical maps of DNA consist of ordered landmarks at known distances from one another (Green et al., 1996). There are various types of landmarks that can be used to construct physical maps of DNA. One of the most common techniques is the development of a restriction map which provides a fingerprint of the DNA segment based on cutting sites of several restriction endonucleases. In particular, long range restriction maps that use rare-cutting restriction enzymes and are resolved on pulse field gel electrophoresis are useful when trying to position and isolate a gene. These maps will not only provide physical distances between restriction sites and help order loci close to one another, but they often identify CpG islands which are frequently associated with genes (Bird, 1986). The development of sequence-tagged sites (STS) has begun to dominate the world of physical mapping. STSs are short stretches of approximately 60-1000 bp of unique DNA sequence that can be readily detected by PCR (Green and Green, 1991). Due to the ever increasing numbers of STSs being reported and the ease with which they can be identified, they are extremely useful for generating relatively detailed physical maps of a critical region. The most detailed type of physical maps available are DNA sequence maps which give the exact order of nucleotides along a

stretch of DNA. Comparing sequence maps between normal and mutant genes will detect the individual mutation(s) responsible for the resulting phenotype of the particular disease of interest.

1.12.4 Yeast artificial chromosomes

Yeast artificial chromosomes (YACs) are an extremely powerful tool for human genome analysis as they can be used to map and study large genomic regions and genes (Gobin et al., 1991). YACs contain all the necessary sequence elements that are needed to propagate a chromosome in yeast: an origin of replication, a centromere, and telomeres as well as the yeast selectable markers, TRP1 and URA3 (Figure 5). They are extremely useful because as little as 100 kb to over 1000 kb of foreign DNA can be cloned into them, giving them an advantage over plasmids, cosmids and BACs which can only contain segments of DNA from a few kb to up to 100 kb. The sheer size of YACs allow them to provide overlapping DNAs across large genomic regions, facilitating the recovery of very large intact genes.

1.12.5 YAC complementation studies

When a complete YAC contig over a critical gene region is obtained, and when the disease is associated with an identifiable phenotype, one way to exclude one or more of the YACs is through complementation studies. This can be done by transferring intact YACs into mammalian cells which express the mutant phenotype of interest. With this approach, all of the intact genes present in the YAC should be expressed in the mammalian cell. Therefore, the functional, normal genes present on the YAC should

Figure 5: Yeast artificial chromosome (YAC)

A representative figure of a YAC with its centromere, replication origin and telomeres, all of which are needed o propagate a chromosome in yeast. Joint digestion with SmaI and BamHI creates two linear fragments with telomeres on each end for stability and flush ends on the opposite side where foreign DNA can be inserted. Each fragment, designated left and right arms, carries the yeast selectable markers, URA3 and TRP1 respectively. Figure modified from Lewin (1994).
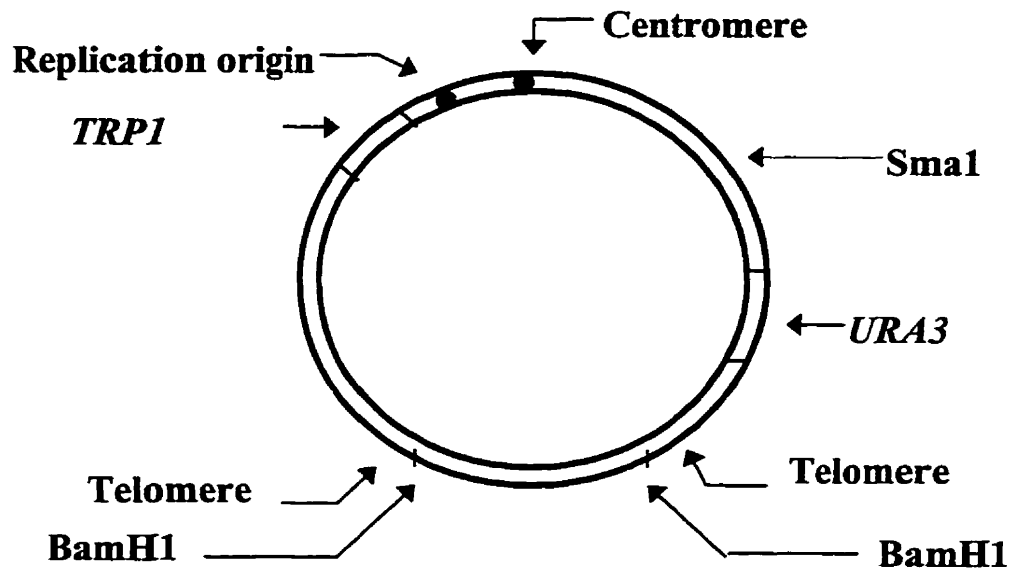
Cut with BamHI
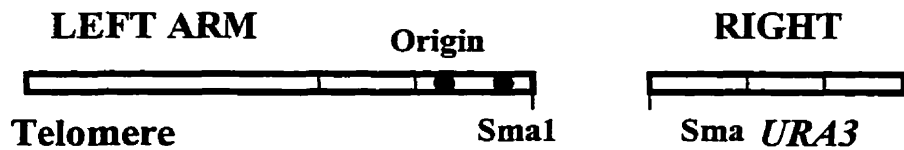
Ligate with foreign DNA
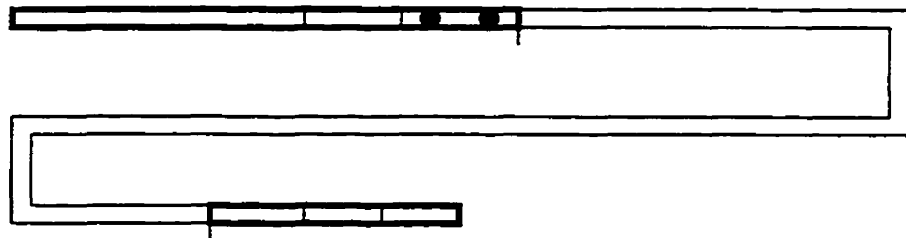
Transform into yeast cells

Figure 5

complement and restore normal activity in the recipient cell. If a particular YAC is able to restore a normal phenotype to a cell line of interest (i.e. restoration of cholesterol esterification activity in NP Type II fibroblasts), it can be concluded that the gene responsible for the disease must lie on that particular YAC. Electroporation, microinjection, lipofection and spheroplast fusion can all be used to transfer YACs into mammalian cells with demonstrable expression of the transferred genes. Each method has its advantages and disadvantages related to the amount of handling which may cause breakage of YACs and their DNA, the amount of yeast DNA introduced alone with the YAC, and the efficiency of the procedure. Spheroplast fusion of YACs, up to several megabases in size, to mammalian cells appears to be a widely used and successful approach (Davies et al., 1992, Demmer and Chaplin, 1993, Huxle et al., 1991, Jakobovitis et a., 1993, Pachnis et alk., 1990 and Mogayzel et al., 1997).

1.14 OBJECTIVES

The objective of this thesis was to gain information that would aid in the localization of the NP Type II gene. Three approaches were utilized to localize the defective gene; two of these were directed towards positional cloning, while the third was a functional approach. The first approach of classic linkage analysis attempted to reduce the NPD critical region by identifying new NPD families with potentially recombinant individuals, that may have emigrated from Yarmouth County to other regions of Canada and the United States. Genealogical information about the families was used to try to tie them into the extensive Yarmouth County kindred. The second approach was directed toward limiting the NP Type II critical region through complementation studies, in which

individual YACs from a contig that spans the NPD critical region (Greer et al., 1997b) would be fused to either NPC/NPD fibroblasts or NP Type II mutant CHO cells in an attempt to restore normal phenotype. Complementation could limit the gene to a single YAC. The final approach involves a model system in which progesterone is used to induce a NP Type II-like phenotype in normal fibroblasts. It is hypothesized that progesterone binds and inactivates the protein defective or absent in NP Type II cells. Therefore, [^{3}H]-progesterone binding through a photoaffinity labelling technique was used as a novel approach to identify a candidate NP Type II protein that was present in normal cells, but absent or altered in mutant cells.

Isolation and characterization of the gene would allow for carrier detection for NP Type II families, contribute to the understanding of the biochemical defect responsible for the disease, and open the door for the development of an an effective treatment.

2.0 METHODS AND MATERIALS

2.1 IDENTIFICATION OF NP TYPE II FAMILIES AND AQUISITION OF SAMPLES

Niemann-Pick C and D families from Canada and United States were identified through The National Niemann-Pick Foundation who provided a mailing list for those families registered in this foundation. Each family was contacted with a letter describing Niemann-Pick Type II Disease, our research project and our goal of identifying NP Type II families with possible Yarmouth County, Nova Scotia ancestry (Appendix I). Included in the letter was a list of common Yarmouth County French Acadian surnames that are known to be associated with the Niemann-Pick Type D families (Appendix II) A letter of consent for each participant was also included (Appendix III). Families with possible Nova Scotian ancestry who were interested in participating in the study contacted us directly. Arrangements were made with local hospitals or clinics for 2 vacutainer-EDTA tubes and 2 Heparin-EDTA tubes of blood to be taken and shipped to the Niemann-Pick Research lab at the QEII Hospital, VG site, immediately after being collected (Appendix IV). These were processed within 48 hours. Permission was granted for the use of human subjects by the ethics committee of the IWK-Grace Health Centre, Halifax, NS. Two additional families from New Brunswick were brought to our attention by the department of Medical Genetics at the IWK-Grace Health Centre. EBV transformed lymphoblast cell lines from these two families were obtained from the Niemann-Pick cell bank in this laboratory.

Genealogical information on each responding family was collected through personal communication with contact individuals and was used to see if their ancestry could be traced to Yarmouth County, NS. Additional resources from the Public Archives of Nova Scotia and the University of Moncton, Centre of Acadian Studies, were used to try and confirm genealogical ties.

2.2 DNA EXTRACTION

2.2.1 Extraction of DNA from Peripheral Blood

Four × 5 ml blood samples were collected in EDTA vacutainer tubes, maintained at room temperature and extracted within 48 hours. To isolate white blood cells for DNA extraction, blood was fractionated into layers of serum, white blood cells (buffy coat) and red blood cells by centrifugation for five minutes at 2000 rpm at room temperature (RT). The top layer of serum was removed and discarded and the buffy coats were collected and combined in a fresh 50ml tube. Five volumes of warm (37°C) NH_4Cl:Tris solution (0.155 M NH_4Cl:0.170 M Tris, pH 7.65) was added. The tubes were then incubated at 37°C for five to ten minutes, and then centrifuged for five minutes at 2000 rpm at RT. The supernatant was decanted, and pellet was washed twice with 10 ml of warm (37°C) saline, and centrifuged again for five minutes, 2000 rpm at RT. The final pellet was resuspended in 5ml saline-EDTA (5 M NaCl:0.02 M EDTA), 500µl 10% SDS, 50µl 20 mg/ml Proteinase K and incubated overnight at 56°C. The following day, tubes were placed at 4°C for a minimum of one hour or until a white precipitate formed. Two ml of 5M NaCl was added and tubes were inverted several times to mix and then centrifuged for 15

minutes at 2500 rpm at RT. The supernatant was transferred to a new 50ml tube and two volumes of 95% EtOH were added. The solution was mixed by gently inverting several times until long strands of DNA precipitated out. DNA was then collected with a hooked pasteur pipette and washed first with 70% EtOH and then in 95% EtOH. DNA was then blotted dry on a kimwipe and added to 200-800μl of low TE (10mM Tris-Cl, pH 8.0, 1mM EDTA) buffer in a 1.5ml screw cap tube. A small sterile glass bead was added to each tube to aid in the dissolving of the DNA. Tubes were then placed in 37°C incubator rotating overnight or until dissolved. Tubes were then stored at 4°C indefinitely.

2.2.2 Extraction of DNA from Peripheral Blood Lymphoblasts and Fibroblasts

Fibroblasts were grown in 2-4 T-150 flasks until 95% confluent in Dulbecco's Modified Eagle Media (DMEM) (Gibco/BRL) + 10% FBS. EBV transformed lymphoblasts were also grown in DMEM + 10% FBS in 2-4 T-75 flasks until the culture approached confluency. Both cell types were centrifuged at 1500 rpm for five minutes at RT and washed twice in 10ml of sterile saline. To the final pellet, 5 ml of NaCl-EDTA was added, 500μl 10% SDS and 50μl 20 mg/ml Proteinase K and incubated at 56°C overnight. Precipitation of DNA was according to previously described protocol.

2.3 QUANTIFICATION OF DNA

A 1:5 dilution of DNA:water was made for each DNA sample. 2μl of each diluted sample was then added to 2ml of the fluorescent dye (1mg/ml Hoechst, 1X TNE). The fluorometer was set with a water blank and the each dilution was measured in ng/μl.

2.4 ISOLATION AND STORAGE OF WHITE BLOOD CELLS FROM WHOLE BLOOD FOR FUTURE TRANSFORMATION TO CELL LINES

A 10ml blood sample was taken in Heparin-EDTA vacutainor tube and processed within 48 hours. The tubes were centrifuged at 2000 rpm for 5 minutes at room temperature. The top serum layer was removed and the white blood cell buffy coat was collected and mixed with 4.5ml of sterile saline. The saline-wbc mixture was then layered at a 45° angle on top of 3 ml of sterile ficoll (Pharmacia Biotech) in a 15ml tube. Tubes were then centrifuged for 25 minutes at 2000 rpm for 5 minutes at room temperature. The white blood cell band was collected and transferred to a fresh 15ml tube and washed twice with 5ml sterile saline and centrifuged 5 minutes, 2000 rpm at room temperature. The cell pellet was resuspended in 1.5ml of freezing solution (20% FBS, 10% DMSO in RPMI + pen/strep) and was divided into two cryovials. Cells were placed in -70°C freezer O/N and then transferred to liquid nitrogen indefinitely.

2.5 MICROSATELLITE GENOTYPING ANALYSIS

Fifteen polymorphic microsatellite markers spanning a 15 cM region of 18q11-12 were used to construct genetic haplotypes for each individual. Of the 15 markers, only 10

were consistently used for all 4 families, these included D18S40, D18S869, D18S44, D18S1101, D18S1108, D18S480, D18S975, D18S66, D18S478, and D18S1151. The remaining 5 markers, D18S1104, D18S1396, D18S1397, D18S1398 and D18S1107 were only used for some of the families.

2.5.1 PCR Reaction

Each 25μl reaction included 1X of either Buffer A (25mM Tris-Cl, pH 9.3, 50mM KCl, 2mM MgCl$_2$, 1mM DTT), Buffer A plus 0.1% gelatin, or Buffer D (10mM Tris-Cl, pH 8.3, 50mM KCl, 1.5 mM MgCl$_2$), 0.25U of Taq polymerase (Gibco/BRL) and 2μCi α^{32}P-dCTP (Amersham). Table 1 shows template DNA, primers and dNTPs concentration used for each reaction along with primer sequences and buffer type.

2.5.2 PCR Program

PCR was completed in a PTC-100 Programmable Thermal Controller (MJ Research Inc.) with hot bonnet according to the following profiles. D18S40, D18S44 and D18S66 were initially heated to 94°C for three minutes followed by 30 rounds of 94°C for one minute, 57°C for two minutes, 72°C for two minutes, with a final extension at 72°C for seven minutes and then held at 10°C indefinitely. D18S478, D18S1397 and D18S869 were heated to 96°C for five minutes followed by 30 rounds of 96°C for thirty seconds, 55°C for forty seconds, 72°C for one minute and a final extension at 72°C for ten minutes and then held at 10°C indefinitely. D18S1107, D18S480, D18S1108 and D18S1101 were heated to 95°C for three minutes followed by 27 rounds of 94°C for one

Table 1: Chromosome 18 microsatellite PCR conditions

The 15 polymorphic microsatellite markers located on chromosome 18 that were used for linkage analysis are listed. For each marker, forward and reverse primer sequence, DNA, primer and dNTP concentrations and type of buffer used is provided.

Table 1

Microsatellite Marker	Primer Sequence	DNA Conc. (ng)	Primer Conc.	dNTP final Conc. (μM)	Buffer
D18S40	5'-CAAGATAGATGCATTCAGT-3' 5'-CATCCAAAGGGTGAATGTGT-3'	50	50nM	200	A + 0.1% gelatin
D18S1104	5'-GACATCACGCCACTCAC-3' 5'-TTATTCTAGATACTCTTAGGTCCCC-3'	50	50nM	200	A + 0.1% gelatin
D18S1398	5'-TGGGTGACAAGAGCGAAAC-3' 5'-CAGCCTGGGAAACAGAGTG-3'	250	10μM	400	A
D18S1397	5'-AAGAGCGAGACTCCGTCAC-3' 5'-CCCCAAGAAAGTAGAAATAGTGG-3'	250	20μM	400	A + 5% glycerol
D18S1396	5'-GCCATTTGTGTGTGTGTATG-3' 5'-CTGGGTGACAGAGTGAGACT-3'	250	10μM	400	A
D18S869	5'-GAGTGAATGCTGTACAAACAGC-3' 5'-TGTTTATTTGTTTGACTCAATGG-3'	250	100μM	400	D
D18S44	5'-AAACGCTAAATAATATTCCTGTGTTG-3' 5'-TTGACGGATGAATGGACAAA-3'	50	50nM	200	A + 0.1% gelatin
D18S1101	5'-GACACATTTGGGACACAAGA-3' 5'-GTAGGTGCTTCAGCCTGACT-3'	250	20μM	400	D
D18S1108	5'-CTGTCACATGGTCCTCT-3' 5'-GAACTGTGTCCTCCAAAAA-3'	250	50μM	400	A
D18S480	5'-TTTATTACAAAGTCTGAGGGGTTCC-3' 5'-TGGGCGAGCCAATTCCT-3'	250	20μM	400	D
D18S1107	5'-AGAACCCAATAGAATACAAACAC-3' 5'-CAAAAAATGACAAAGGTACTTG-3'	250	100μM	400	D
D18S975	5'-GTTGTATGCCTAGCACTGCA-3' 5'-ACCTCATAAAATAAGCCTGGC-3'	250	50μM	400	D
D18S66	5'-CAGCCTCGGAGAAACG-3' 5'-AGAGCAAGTCCCTGCC-3'	50	50nM	200	A + 0.1% gelatin
D18S478	5'-ACATCATCAAGTAGATTTCCATT-3 5'GACCAACTCAAGTGTTCCAC-3'	250	10μM	400	D
D18S1151	5'-AAGGCTGATCTGAGCCTATAC-3' 5'-ATGGCAAAGGTGATACAACT-3'	250	20μM	400	D

minute, 55°C for two minutes, 72°C for one minute and a final extension at 72°C for seven minutes and held at 10°C indefinitely. D18S1151 and D18S975 were heated to 96°C for five minutes followed by 30 rounds of 96°C for thirty seconds, 57°C for forty seconds, 72°C for one minute with a final extension at 72°C for ten minutes and held at 10°C indefinitely. D18S1396 and D18S1398 were heated to 95°C for five minutes, followed by 30 rounds at 95°C for 55 seconds, 56°C for 45 seconds and 72°C for 75 seconds with a final extension and 72°C for ten minutes and then held at 10°C indefinately.

2.5.2 Electrophoresis of PCR Product

The sequencing gel electrophoresis apparatus S2 or SA-32 (Gibco/BRL) was assembled according to manufacturer's directions. PCR products were separated on a 0.4mm, 8% denaturing polyacrylamide gel (7.9M urea, 8% acrylamide (ratio of bis:acrylamide was 1:19), (Boehringer Mannheim, Gibco BRL), 1X TBE, 10% ammonium persulfate (APS) (BioRad) and 0.02% (v/v) TEMED (BioRad). Urea pellets were added to a small volume of dH_2O and microwaved for 20-30 seconds. The solution was allowed to cool until warm to touch and then the bis-acrylamide and TBE was added and the solution was brought to its final volume with water. Finally APS and TEMED were added and the gel solution was gently swirled and taken up in a 60cc syringe. The assembled plates were placed at a 45° angle on their right hand corner and the gel solution was slowly poured between the plates. The flat surface of sharks tooth combs were then placed in between the top of the plates and the gel was left to polymerize. After polymerization, a piece of moistened filter paper was placed over top of the gel which

was then wrapped in saran wrap. The gel was then used no earlier than 2 hours and no later than 24 hours after polymerization.

After polymerization, the gel was heated at 75 watts for the S2, 50 well model, or 40 watts for the SA-32, 25 well model for approximately 1 hour or until it reached a temperature of 50-54°C. Once the gel had reached a desired temperature, any excess acrylamide that may have accumulated in the wells was removed by squirting 0.5X TBE buffer in the wells. The sharks tooth comb(s) were then place tooth-side down into the wells so that they penetrated the gel 1-2 mm. Two μl of each ^{35}S-ATP labelled M13 size ladder nucleotides A,C,G and T were loaded respectively from left to right followed by 3μl of the PCR product diluted with 16μl of loading dye (95% formamide, 10mM EDTA, 0.05% bromphenol blue, 0.05% xylene cyanol FF). Both the PCR products and M13 nucleotides were heated to 90°C for 2.5 minutes and then quick chilled on ice prior to loading. The gel was electrophoresed between 40-80 watts depending on model, for 2-7 hours depending of size of fragment amplified. The gel apparatus was disassembled according to manufacturer's protocol and the two plates were separated. The glass plate on which the gel remained, was then placed in 10% methanol for 5-10 minutes. The gel was then removed from the plate with whatmann 3MM filter paper and dried at 80°C for one hour on a gel dryer (Bio-Rad) and then exposed to X-ray film (Kodak X-OMAT) in an electrophoresis cassette with intensifying screens for 12-72 hours at -70°C. The X-ray film was then developed according to manufacturer's directions.

2.53 Radioactive labeling of co-migratory M13 ladder

The protocol for radioactively labelling M13mp18 single stranded DNA was that

provided by United States Biochemical (USB) Sequenase Version 2.0 DNA sequencing

kit. Briefly, 5μl of M13mp18 DNA, 1μl of universal primer, 2μl of Sequenase buffer and

2μl of dH$_2$O were combined in a microfuge tube and heated to 65°C for two minutes. The

mixture was then allowed to cool to room temperature (approximately 30 minutes) and

then placed on ice. While the mixture was cooling, the 5X labelling mix (7.5μM dGTP,

7.5μM dCTP, 7.5μM dTTP) was diluted 5-fold with dH$_2$O. In four fresh microfuge tubes

2.5μl of each termination mixture (80μM dGTP, 80μM dATP, 80μM dCTP, 80μM

dTTP, 50mM NaCl plus 8μM ddGTP, ddATP, ddCTP or ddTTP) was added and

prewarmed in a 37°C water bath. To the annealed template-primer 1μl of 0.1M DTT, 2μl

diluted labeling mix, 0.5μl α-^{35}SdATP and 2μl diluted sequenase (T7 DNA polymerase in

20mM KPO$_4$, pH 7.4, 1mM DTT, 0.1mM EDTA, 50% glycerol diluted 1:8 in 10mM

Tris-Cl, pH 7.5, 5mM DTT and 0.5mg/ml BSA) were added for a final volume of 15.5μl,

mixed and incubated at room temperature for 3-5 minutes. 3.5μl of the labeling reaction

was then added to each of the termination (A,C,T,G) tubes and incubation was continued

at 37°C for an additional five minutes. Reactions were stopped by the addition of 4μl of

stop solution (95% formamide, 20mM EDTA, 0.05% Bromphenol Blue, 0.05% Xylene

Cyanol FF).

2.6 MEASUREMENT OF CHOLESTEROL ESTERIFICATION TO ASSESS CARRIER STATUS

This assay was performed at the Atlantic Research Centre, Halifax, NS. Briefly, cholesterol esterification in Epstein Barr virus transformed lymphoblasts was determined by measuring incorporation of [^{3}H]oleic acid into cholesteryl-[^{3}H]oleate according to the protocol outlined by Byers et al. (1994). 12×10^6 lymphoblasts were seeded into 150 cm^2 flasks and grown for four days at 37°C with 5% CO_2 in RPMI 1640 containing 5% delipidated serum. On the day of experiment, cells were seeded in 35-mm dishes at a density of 3.5×10^6 cells per dish with 2 ml of the above medium with or without 10% FBS. Four hours later, [^{3}H]oleic acid (3.6 μCi, 0.2 μmol/dish) was added to each dish. Cells were pulsed with [^{3}H]oleate for two hours and harvested at 4°C by centrifugation (1 500 × g, 5 min), washed once with 2 ml ice-cold phosphate-buffered saline, and resuspended in 2 ml of hexane:isopropanol (3:2 v/v) for lipid extraction (Liscum and Faust, 1987). Lipid extracts were separated by thin layer chromatography and identified by comparison with standard mixtures and cholesterol-[^{3}H]-oleate was quantified using a BioScan 200 radioimaging detector (Byers et al, 1994).

2.7 ANALYSIS OF HALF BROTHER-SISTER SIBSHIP IN ONE FAMILY

To asses the possibility that the parents of an affected NPD boy were half brother-sister, fourteen polymorphic loci from across the genome were studied. Three of the markers, (Apolipoprotein B (ApoB), PYNZ222, and PMCT118) were variable number tandem repeat markers (VNTRs), while six, (D1S104, D3S1614, D5S207, D5S112, D7S492 andD21S213) were polymorphic microsatellite markers and the remaining five

(HLA-A, HLA-B, HLA-C, HLA-DRB1 and HLA-DQB1), were variable HLA alleles linked together on chromosome 6.

2.7.1 PCR reaction

All VNTR loci were amplified in a 50µl reaction containing 1X buffer 1.5 (50mM KCl, 10mM Tris-Cl, pH 8.4, 1.5mM MgCl$_2$, 1mg/ml BSA, 0.2mM of each dNTP, 10mM DTT), 100ng/µl of forward and reverse primers, and 0.5U Taq polymerase (Gibco/BRL). Microsatellite markers were amplified in a 25µl reaction which contained 1X buffer A plus 0.1% gelatin, 200µM dNTPs, 0.2U Taq polymerase, and 2µl of α^{32}P-dCTP. For specifications on primer sequence, DNA, primer and dNTP concentrations, buffer type, and chromosome location, refer to table 2.

HLA typing was performed at the HLA lab located at the Queen Elizabeth II Health Centre, VG Site.

2.6.2 PCR program

The PCR reaction profile for each locus was as follows. ApoB was initially heated to 94°C for six minutes followed by 25 rounds of 94°C for one minute and 58°C for six minutes and then held at 10°C indefinitely. pYNZ22 was heated to 95°C for nine minutes followed by 30 rounds of 95°C for one minute, 57°C for one minute and 72°C for one minute followed by a final extension at 72°C for seven minutes and then held at 10°C indefinitely. pMCT118 was initially heated to 95°C for nine minutes followed by 25 rounds of 95°C for one minute, 65°C for one minute and 70°C for two minutes followed

Table 2: Random genomic marker PCR conditions

The 9 randomly distributed genomic, polymorphic markers used to evaluate a possible half brother-sister sibship are listed. For each marker, forward and reverse primer sequence, NDA, primer and dNTP concentrations, type of buffer used and chromosomal location (#) is provided.

Table 2

Marker	Primer sequence	DNA conc.	Primer conc. (ng)	dNTP conc. (μM)	Buffer	Chrm, #
ApoB	5'-ATGGAAACGGAGAAATTATG-3' 5'-CCTTCTCACTTGGCAAATAC-3'	300ng	100ng	200	1.5	2
pMCT118	5'-GAAACTGGCCTCCAAACACTGCCCGCCGT-3' 5'-GCAAGGGGCACGTGCATCTCCAACAAGAC-3'	200ng	100ng	200	1.5	17
pYNZ22	5'-CGAAGAGTGAAGTGCACAGG-3' 5'-CACAGTCTTTATTCTTCAGCG-3'	1μg	100ng	200	1.5	1
D1S104	5'-ATCCTGCCCTTATGGAGTGC-3' 5'-CCCACTCCTCTGTCATTGTA-3'	50ng	50ng	200	A	1
D3S1614	5'-TTCCAAGATATGTGTGACTTACAG-3' 5'-GATATTTAAGCCTTGACCCTGA-3'	50ng	50ng	200	A	3
D5S207	5'-TTGGAAGCCTTAGGAAGTGC-3' 5'-AAGAATTCTAGTTTCAATACCG-3'	50ng	50ng	200	A	5
D5S112	5'-TGTTCTTGGCATCACTGC-3' 5'-TTTGAAGCCCTGGAATAT-3'	50ng	50ng	200	A	5
D7S492	5'-ATCTTGGATTTAGGGTTGGC-3' 5'-GGCTCTGCTCCATCTTCATA-3'	50ng	50ng	200	A	7
D21S213	5'-TAGAGGCTTGAATTGGCTGG-3' 5'-GTGTTTCATTAGACACACACCC-3'	50ng	50ng	200	A	21

by a final extension at 72°C for seven minutes and then held at 10°C indefinitely. All of the microsatellite marker reactions were heated to 94°C for three minutes followed by 30 rounds of 94°C for one minute, 57°C for two minutes and 72°C for two minutes, with a final extension at 72°C for seven minutes and then held at 10°C indefinitely.

2.6.3 Electrophoresis of PCR product

The VNTR amplicons were separated on a mini-Protean gel electrophoresis system (BioRad) which was assembled according to manufacturer's directions. For a 0.75mm gel a 10% polyacrylamide gel solution was prepared (10% bis:acrylamide (1:30), 0.055% ammonium persulfate, 0.05% TEMED (v/v). The gel solution was poured between the glass plates using a pasteur pipette and a 10-15 well comb was inserted tooth-face down. The gel was allowed to polymerize for at least one hour before using. PCR products were diluted with 5µl of fast-slow dye (50% glycerol, 0.1M EDTA, 0.05% bromphenol blue, 0.05% xylene cyanol FF) and 10-15µl of this mixture was loaded on the gel along with a 100 bp size ladder (Gibco/BRL). The gel was electrophoresed in 1X TBE at 150V for 2 hours, stained with ethidium bromide (EtBr) and then observed on a UV light box. All microsatellites were separated on an 8% denaturing polyacrylamide electrophoresis gel as described previously.

2.8 RETROFITTING OF YACs WITH NEOMYCIN

2.8.1 Preparation of pLNA-1 plasmid for retrofitting

The pLNA-1 plasmid was a generous gift donated by Dr. Rakash Anand, ICI Pharmaceuticals. Isolation and purification of the pLNA-1 plasmid was done following manufacturer's instructions from the Wizard™ Megapreps Purification System (Promega). In brief, 2ml of 2xYT (1.6% tryptone, 1% yeast extract, 0.5% NaCl) broth was inoculated with pLNA-1 and grown overnight shaking at 37°C. The next day, 1L of YT broth was inoculated with the 2ml culture and allowed to grow for another 24 hours at 37°C. The grown culture was centrifuged at 10 000 rpm in 2 x 500ml Beckman centrifuge tubes for 15 minutes at 4°C. The supernatant was discarded and both pellets were resuspended and combined in 15ml of cell resuspension solution (50mM Tris-Cl, pH 7.5, 10mM EDTA, 100µg/ml RNase A). Thirty ml of cell lysis solution (0.2M NaOH, 1% SDS) was added and the suspension was left at room temperature for 20 minutes with occasional shaking. Thirty ml of neutralization solution (1.32M K acetate, pH4.8) was added, the solution was mixed thoroughly and then centrifuged for 15 minutes at 13 000 rpm at 4°C. The supernatant was mixed with 0.6 volume of isopropanol in a new 250ml tube and then centrifuged at 13 000 rpm for 15 minutes, 4°C. The DNA pellet was resuspended in 5 ml TE, pH 7.5. Twenty ml of DNA purification resin was added and the solution was transferred into the Megacolumn supplied by the manufacturer, and a vacuum was applied. Twenty-five ml of column wash solution was added to the DNA/resin mix bottle and this was then transferred to the Megacolumn and pulled through. The Megacolumn was then washed with an additional 25 ml of column wash solution and 10ml of 80% EtOH. The Megacolumn was placed in a 50ml screw

cap tube and centrifuged for 5 minutes at 2 500 rpm. The supernatant was discarded and 3ml of prewarmed (65-70°C) TE was applied to the Megacolumn, incubated for 1 minute and then centrifuged a final time for 5 minutes at 2 500 rpm. The Megacolumn was discarded and the plasmid was stored at 4°C indefinitely.

2.8.2 Restriction digest of pLNA-1

In order to insert the neomycin gene into YACs by homologous recombination, the plasmid was linearized to create recombinogenic ends (Figure 6) (Riley et al., 1992). This was done by digesting it with the restriction enzyme Aat II (Pharmacia Biotech) which recognizes the site GACGT↓C. The restriction digest mixture contained 1U of enzyme/μg of DNA, 1X dilution of the 10X "one phor all buffer" supplied by the manufacturer (100mM Tris-Cl, pH7.5, 100mM Mg acetate, 500 mM K acetate) and 0.2mM spermidine. The reaction was terminated by heating to 85°C for 30 minutes. Digested DNA samples were size separated electrophoretically on a 1% agarose gel along with 100bp size ladder (Gibco/BRL) to confirm presence of linear DNA.

2.8.3 Purification of carrier herring sperm DNA for retrofitting

Prior to retrofitting, it was necessary to purify herring sperm DNA to be used as a carrier to increase transformation efficiencies with YACs. This was done according to the standard protocol outlined in Sambrook (1989). Equal volume of TE saturated phenol (Boehringer Mannheim) was added to the herring sperm DNA and centrifuged for 3

Figure 6: Retrofitting of candidate YACs with the neomycin resistant gene by homologous recombination

The pLNA-1 plasmid which contains the neomycin resistant gene is linearized with the restriction enzyme, AatII to create recombinogenic ends. The plasmid then inserts into the left hand arm of the YAC by homologous recombination. Shaded areas represent regions of homology between plasmid and YAC. The retrofitted YAC now contains the mammalian selectable marker, neomycin. Figure modified from Riley et al., (1992).
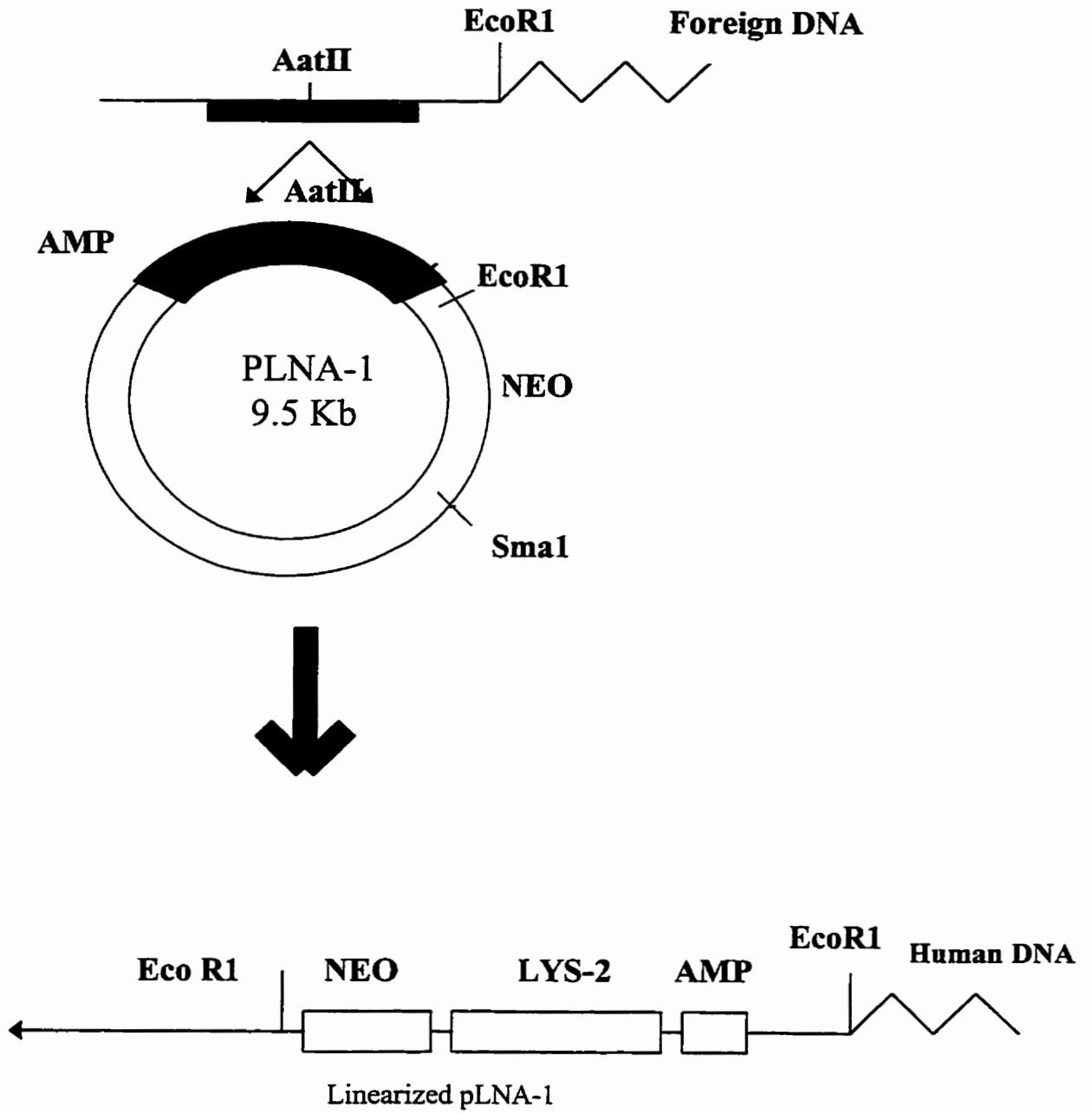
Figure 6

minutes at room temperature. The top aqueous layer was transferred to a fresh 1.5ml tube and equal volumes of phenol:chloroform were added. The solution was centrifuged for another 3 minutes. The top, aqueous layer was removed and transferred to a new 1.5ml tube.

2.8.4 Precipitation of carrier and plasmid DNA

Both the herring sperm (carrier) and pLNA-1 (plasmid) DNA were precipitated according to standard protocol outlined in Sambrook (1989). One tenth volume of 3M sodium acetate was added to the DNA solution. Two volumes of ice cold EtOH was added and the tube was placed on ice for 1 hour. Tube(s) were then centrifuged at 10 000 rpm for 10 minutes at $0^{\circ}C$. The pellet was resuspended in $750\mu l$ 70% EtOH and centrifuged at 10 000 rpm for another 2 minutes at $4^{\circ}C$. The pellet was left to air dry overnight and was resuspended in $50-100\mu l$ of TE.

2.7.5 Integration of neomycin resistant gene into intact YACs

The following method of integrating the neomycin resistance gene into intact YACs was modified from Schiestl and Gietz (1988) and Gietz et al. (1995). YACs (877F12, 906C12, 911D5) were grown in 25-50ml of synthetic complete (SC) media (0.67% yeast nitrogen base, 2% glucose, 1% casamino acids, 0.2% adenine) overnight at $30^{\circ}C$. The following morning, cells were counted on a haemocytometer to ensure that there were at least $1-2 \times 10^8$ cells/ml and then diluted to 2×10^6 cells/ml in fresh, warm YPAD (1% yeast extract, 2% peptone, 2% glucose, 0.2% adenine). Cells were grown for three cell divisions, to approximately $6 \times 10^6 - 1 \times 10^7$ cells/ml. Once cells reached

desired concentration, they were centrifuged at 2500pm for 5 minutes at room temperature. The pellet was washed twice in 10ml dH_2O and once in 10ml 1X TE/LiAC and was resuspended in 1X LiAc to a final concentration of 2×10^9 cells/ml, and then incubated at 30°C for 15 minutes without agitation. Fifty μl of cell suspension was then aliquoted into 1.5ml tubes containing 50μg of purified herring sperm DNA, and boiled for five minutes prior to adding 100-1000ng of pLNA-1 plasmid and 300μl of 40% PEG 4000 (BDH)/1XLiAc/1XTE. For positive and negative controls, a separate plasmid, pSA4, (known to work), donated by Dr. Melanie Dobson, and no plasmid was added to mixture respectively. Tubes were vortexed and incubated at 30°C for 30 minutes without agitation and then heat shocked at 42°C for 20 minutes. Tubes were then centrifuged for 1-2 minutes in a TOMMY capsule HP-120 and supernatant was removed and discarded. The tubes were centrifuged for an additional minute to remove excess supernatant. Pellets were resuspended in 1ml dH_2O and 100-200μl aliquots of suspension were pipetted and spread on to selective plates (SC-ura-trp-lys) (0.67% yeast nitrogen base, 2% glucose, 2% agar, 0.2% adenine, 1% histidine, 1% arginine, 1% methionine, 0.2% tyrosine, 1% leucine, 1% isoleucine, 0.1% phenylalanine, 0.1% valine, 0.1% threonine). Because the YACs contain the URA1 and TRP3 genes as yeast selectable markers, and the neomycin gene carries the LYS2 gene as its marker, only those YACs retrofitted with neomycin should grow on the -ura-trp-lys selective plates. The positive control was plated on SC plates without adenine. Plates were allowed to dry and then were incubated, inverted, at 30°C for 3-5 days until positive colonies appeared.

2.8.6 Preparation of agarose plugs for pulse field gel electrophoresis

Retrofitted YACs were grown in 5ml of selective media(SC-ura-trp-lys) overnight at $30^{\circ}C$ or until there were $6-8 \times 10^{7}$ cells/ml. Cultures were then centrifuged at 2000 rpm for 5 minutes at room temperature. The pellet was resuspended in $250\mu l$ of sorbitol solution (1.2M sorbitol, 10mM Tris-Cl, pH 7.5, 20mM EDTA and 14mM β-mercaptoethanol, $100\mu g/ml$ Zymolase 20T). $2.5\mu l$ of 1.5% of low melt preparative grade agarose (BioRad) was then added to each yeast suspension and $100\mu l$ of solution was added to each plug mold set on ice. Plugs were allowed to polymerize and then were pushed out of plug molds into 15ml tubes with fresh sorbitol solution and incubated at $37^{\circ}C$ for 1-2 hours to allow the formation of spheroplasts. Sorbitol solution was decanted off and 5ml of yeast lysis solution (1% LDS, 0.1M EDTA, 10mM Tris-Cl, pH 8.0) was added and tubes were incubated for 2 hours at $37^{\circ}C$ with occasional shaking. Plugs were replaced with fresh lysis solution and left overnight at $37^{\circ}C$. The following day, the plugs were replaced once more with fresh lysis buffer and left at room temperature indefinitely.

2.8.7 Pulse field gel electrophoresis

0.5X TBE was placed in the buffer chamber of the pulse field gel electrophoresis apparatus and allowed to circulate and cool to $14^{\circ}C$ for 1-2 hours before loading. While buffer was cooling, 1/3 of each plug was placed in 1.5ml tubes and washed 3×30 minutes with 0.5X TBE. Pulse field certified agarose (1%) (BioRad) was cast in a gel tray and left to polymerize. The remaining 10-15ml of agarose was set aside in a $56^{\circ}C$ waterbath. After polymerization, each well was filled with 0.5X TBE and YAC plugs

along with *Saccharomyces cerevisiae* size ladder and AB1380 negative control were loaded into wells and sealed over with remaining agarose. The gel was removed from the casting tray, placed in the buffer chamber and was electrophoresed at 6v for 20 hours with initial switch time of 50s and final switch time of 90s.

2.8.8 Preparation for Southern blotting

After electrophoresis, the gel was stained in EtBr for 30 minutes and then destained in dH_2O for at least 30 minutes. A polaroid picture was then taken. The gel was treated once in 0.25M HCl for 15 minutes and then denatured twice in 0.4M NaOH, 1.5M NaCl for 15 minutes. DNA was then transferred for three days onto a nylon membrane (BioRad) according to the standard capillary method of Southern (1975), using 0.4M NaOH as transfer buffer. Each day blotting pads and Kim towels were changed and buffer refilled. After three days, the transfer setup was disassembled and the nylon membrane was neutralized in 2X SSC(0.3M NaCl, 0.03M trisodium citrate), 0.2M Tris-Cl, pH7.5 for 15 minutes and then washed in 2X SSC for an additional 15 minutes. The membrane was allowed to air dry overnight.

2.8.9 Preparation of neomycin and alu probe

The pLNA-1 plasmid was double digested with EcoRI and SmaI, to recover an approximate 2 kb fragment containing the neomycin gene to be used as a probe to identify DNA fragments that contain the inserted neomycin gene. pLNA-1 was first digested with 1U SmaI (Gibco/BRL)/μg DNA and 1X React 4 buffer (20mM Tris-Cl, pH 7.4, 5mM $MgCl_2$, 50mM KCl) in a 60μl volume overnight at 30°C. 1U EcoRI/μg DNA

and 1X Buffer (5mM Tris-Cl, pH 8.0, 1mM MgCl₂, 10mM NaCl) were then added to the digested SmaI reaction, to a final volume of 100μl (to dilute out the Sma buffer) and the reaction was incubated at 37°C overnight. This produced an approximate 2 kb neo probe.

The plasmid pBlur8, required for the preparation of the alu probe, was donated by Dr. Duane Guernsey. pBlur8 was digested with 1U Bam HI/μg DNA, 1X buffer B (1mM Tris-Cl, pH 7.5, 1mM MgCl₂, 10mM NaCl, 0.1mM EDTA) and 0.2M spermidine and incubated at 37°C for 2 hours. This produced an approximate 300 bp alu probe.

Both digested pLNA-1 and pBlur8 were then separated on a 1.2% low melt agarose (Gibco/BRL) with the 1kb size ladder (Gibco/BRL) or the 100 bp size ladder (Gibco/BRL) respectively. The appropriate band was then cut out, melted and used for random primed labeling.

2.8.10 Random primed labeling of neo and alu probe

Random primed labeling of probes utilized the Random Primed DNA Labeling Kit from Boehringer Mannheim. Approximately 25ng DNA (maximum volume of 9μl) was heated to 95°C for 10 minutes. To the DNA, 1μl of each dATP, dGTP and dTTP, 2μl hexanucleotide mixture, 1μl Klenow and 5μl α-³²PdCTP were added. The mixture was incubated at 37°C for a minimum of 30 minutes after which 40μl of stop solution (10mM Tris-Cl, pH 7.6, 1mM EDTA, 0.1% SDS) was added. After labeling, probes were heated to 65°C to ensure that the agarose remained melted, and then purified using the Quick Spin™ Columns (Boehringer Mannheim). One μl of the probe was diluted

into 5ml of scintillation cocktail for direct counting of radiolabeled nucleotide incorporation ($\geq$ 100 000 cpm) to assess the extent of α-^{32}PdCTP incorporation.

2.8.11 Hybridization of alu and neo probes to nylon membrane

Dried nylon membranes were prehybridized with 10ml of hybridization solution (0.25M Na$_2$PO$_4$, 1mM EDTA, 7% SDS, 10mg/ml BSA) overnight at 65°C in a Hybaid oven at 65°C overnight. The following morning 50-100µl of $\geq$100 000 cpm labeled probe was added to the hybridization tube containing the membranes. Hybridization continued for 4-6 hours. Membranes were then washed for 20 minutes with 2X SSC and then 20 minutes with 0.5X SSC and 0.1X SSC only if high levels of residual radioactivity was detected. Membranes were wrapped in Saran wrap and placed in an electrophoresis cassette with X-ray film at -70°C overnight. Film was developed according to manufacturer's directions.

2.9 IDENTIFICATION OF A 60kDa PROTEIN BY PHOTOAFFINITY LABELING

On day 0, normal and NPC/NPD fibroblasts were seeded in 24 well plates at a density of 2-2.5 × 10^4 cells/well in DMEM + 10% FBS and incubated at 37°C + 5% CO$_2$. On day 1, cell media was changed to DMEM + 5% delipidated serum (LPDS) and then cells were incubated for four days. 1,2,6,7-[^{3}H]-PRG (specific activity: 92Ci/mMol) (Amersham) was diluted in either DMEM + 10% FBS or DMEM + 5% LPDS to a final concentration of 10-75µCi/well. Cells were incubated with 300µl of diluted [^{3}H]-PRG at 37°C + 5% CO$_2$ for 6-36 hours. Two 5µl aliquots were taken before and after labeling

for direct liquid scintillation counting. After incubation, media was aspirated and cells were washed twice with 1 ml of cold TBS (20mM Tris, 0.1M NaCl, pH 7.5). Fresh TBS (300μl) was then added to each well and the uncovered plate(s) were placed in an ice filled container which was then placed in a UV Stratalinker 1800 (Statagene). Cells were irradiated for 5-20 minutes at 40W, 254 nm and a distance of 10cm. After crosslinking, TBS was aspirated and each well was then incubated at 4°C for ten minutes with 300μl of lysis buffer (TBS, 2mM EDTA, 1mM PMSF, 1μg/ml leupeptin, 1μg/ml pepstatin and μg/ml aprotinin), plus either 1% TritonX-100 (Pierce), .2mg/ml Digitonin (Sigma), or 1% Nonidet p40 (Boehringer Mannheim), 0.5% Na-deoxycholate, (RIPA buffer). After incubation with appropriate lysis buffer, cells were scraped, transferred to a 1.5 ml tube and then centrifuged at 10 000 rpm for 10 minutes at 4°C. The supernatant was transferred to a 2ml screwcap tube and 1.5 ml of ice cold acetone was added. Samples were vortexed and left at -20°C overnight. Precipitated proteins were recovered by centrifugation for 10 minutes at 10 000 rpm at 4°C and left to air dry for 10-20 minutes. Twenty μl of 1X SDS sample buffer (30mM Tris-Cl, pH 6.8, 12.5% glycerol, 1.25% SDS, 0.25% bromphenol blue) and 1μl of 5% β-mercaptoethanol (Sigma) was added to samples. Each tube was vortexed, centrifuged for 1 minute and then boiled for 90 seconds.

2.9.1 Preparation of SDS-polyacrylamide gel

Mini-Protean gel apparati (BioRad) were assembled with 1mm spacers according to manufacturer's directions. A 10% separating gel solution (0.375M Tris-Cl pH 8.8, 0.1% SDS, 10% bis-acrylamide (Biorad), 0.1% ammonium persulfate and .05% TEMED (v/v)) was prepared and poured between the plates with a pasteur pipette until 3/4 full. The gel was then overlaid with 0.1% SDS and allowed to polymerize for at least 45 minutes. After polymerization, excess SDS was removed from between plates and the gel was blotted with filter paper. A 4% stacking gel solution (0.3M Tris-Cl pH 6.8, 0.1% SDS, 4% bis-acrylamide, 0.1% ammonium persulfate and 0.1% TEMED (v/v)) was then made and poured on top of the separating gel. 10-15 well combs were placed between plates, tooth-face down and the gel was allowed to polymerize for another 30 minutes. Fifteen μl of each sample and 5μl of prestained SDS-PAGE broad range standards (BioRad) were loaded. The gel was electrophoresed in 1X running buffer (2.5mM Tris-Cl, 19.2mM glycine, 0.1% SDS) at 150V for approximately 1 hour. Gels were stained with Coomassie blue (0.1% Coomassie blue, 40% MeOH, 10% HoAc) for 30 minutes with shaking at room temperature. Gel(s) were destained (40% MeOH, 10% HoAc) for 2-4 hours and then replaced with fresh destain at the end of day and left overnight. The following day gel(s) were placed in En³hance (Dupont) for 30 minutes, and then in a 5% glycerol solution for 5-10 minutes. Gel(s) were laid on top of dampened filter paper and covered with moistened cellophane and dried on a BioRad Model 443 slab dryer for 1 hour at 80°C. Dried gels were then placed in an electrophoresis cassette with preflashed X-ray film (Hyperfilm-MP, Amersham) for 10 days-6 weeks at -70°C. The film was developed according to manufacturer's instructions.

3.0 RESULTS

3.1 GENEALOGICAL DATA AND LINKAGE ANALYSIS

From the 90 Niemann-Pick Type II families contacted by mail, a total of 8 families responded, four of which with possible Acadian or Nova Scotian ancestry were pursued. The genealogy of each family was traced, and each family member was genotyped using between 10-15 polymorphic microsatellite markers which spanned a 15cM interval along the pericentric region of chromosome 18q (Figure 7).

The first family studied was from Salem, Massachusetts. Genealogical information provided by the family suggested that the maternal grandfather descended from Yarmouth County, Nova Scotia. Further documentation obtained from the Public Archives of Nova Scotia, Halifax, NS, and from the Centre of Acadian Studies, Moncton, NB, confirmed this link. The maternal grandfather can be traced to a French Acadian female who was born in Yarmouth County in c.1861. She is the great-great-grandmother of the affected boy. The only information known about the paternal heritage is that there may be French Canadian (Quebec) ancestry (anecdotal information), however the surnames provided do not resemble any of the common Acadian surnames, suggesting no direct link to Yarmouth County, NS. It was brought to our attention by one of the family members that the parents of the affected boy may have had a consanguineous relationship and that they may in fact share the same father (that is, the maternal grandfather of the affected boy may also be the illegitimate paternal grandfather). Evidence in support of

57
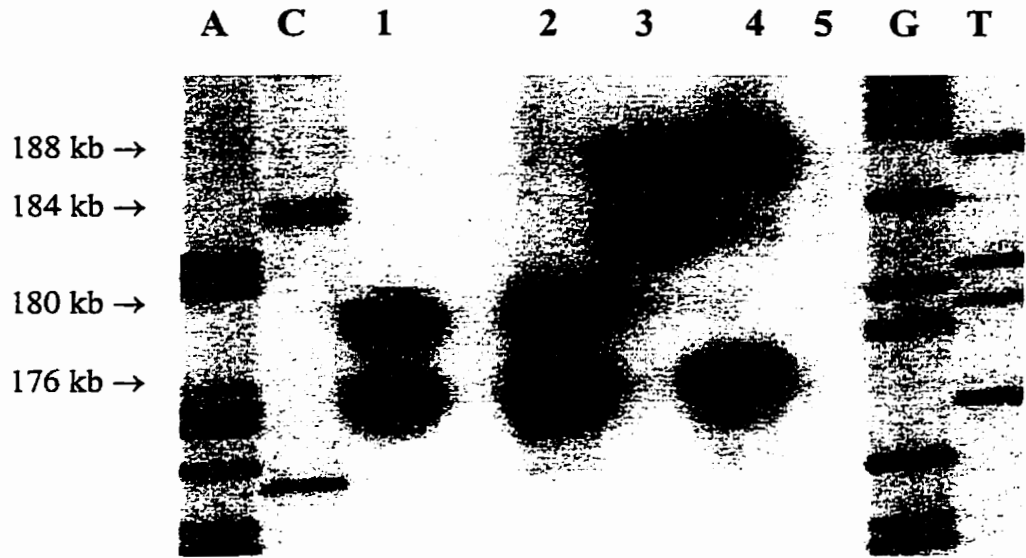
Figure 7: Representative autoradiograph of PCR amplified polymorphic microsatellite markers.

DNA from each patient was PCR amplified with primers specific for microsatellite marker D18S869. Lanes 1, 2, 3 and 4 are the allele patterns for 4 different individuals, lane 5 is the negative control. Samples were electrophoresed with a co-migratory M13 ladder, represented in lanes A, C, G, T to determine allele sizes in base pairs.

this possibility would indicate French Acadian ancestry from Yarmouth County on both sides and a diagnosis of NPD. This possibility was assessed by genotyping the family at 14 loci randomly distributed throughout the genome to determine if the father shared a greater proportion of paternal alleles with his wife than his sister. After genotyping each family member with the 13 randomly distributed markers, it was found that the father resembled his assumed sister and wife almost equally (Figure 8). He shares 5 alleles with his sister, in addition to the ones received from their mother, and only 4 with his wife. These results neither confirm or exclude the possibility that the parents of the affected boy are half brother-sister.

Haplotype analysis of this family with 12 polymorphic microsatellite markers from 18q11-12 is shown in Figure 9. The affected boy was homozygous for every marker except the most distal D18S1151. It is interesting to note that he inherited the grandpaternal haplotype from his father, which lends support to the hypothesis that his parents share the same father. Furthermore, he shares the same common haplotype between microsatellite markers D18S40 and D18S975 with the NPD affected individuals from Yarmouth County (Figure 10). Due to the fact that the NPD families are in linkage disequilibrium, these haplotype results together with the existing genealogical information, support that this affected boy has Niemann-Pick Type D disease.

The second family investigated was from St. Louis, Missouri. They had two affected boys who were diagnosed with NPC. Some of the family ancestors had French surnames, a few of which resembled those on the common Acadian surname list sent out to the families. There was, however, no genealogical evidence found that directly linked this family to Nova Scotia or French Acadians. Nevertheless, since our lab has shown

Figure 8: Analysis of half brother-sister relationship between the parents of the NPD patient from Massachusetts.

The NPD family from Massachusetts was genotyped at 13 unlinked polymorphic markers distributed randomly throughout the genome. Genotyping was performed to assess the possibility of half brother-sister relationship between the parents of the affected boy by comparison of the number of alleles shared between the father, his wife (putative half sister) and his sister. Allele sizes in base pairs at each locus are indicated. The name of each marker is located in the text box. Markers 1-9 do not form haplotypes and are in no particular order, markers 10-14 form the HLA haplotype on chromosome 6.

Markers	Alleles
apoB	1, 2, 3
P118	1, 2
YNZ22	1, 2
D1S104	1, 2, 3
D3S1614	1, 2, 3, 4
D5S207	1, 2, 3
D7S492	1, 2
D21S213	1, 2, 3
D5S112	1, 2, 3
HLA-A	1, 2, 3, 4
HLA-B	1, 2, 3, 4, 5
HLA-C	1, 2, 3, 4
HLA-DRB1	1, 2, 3, 4, 5
HLA-DQB1	1, 2, 3, 4
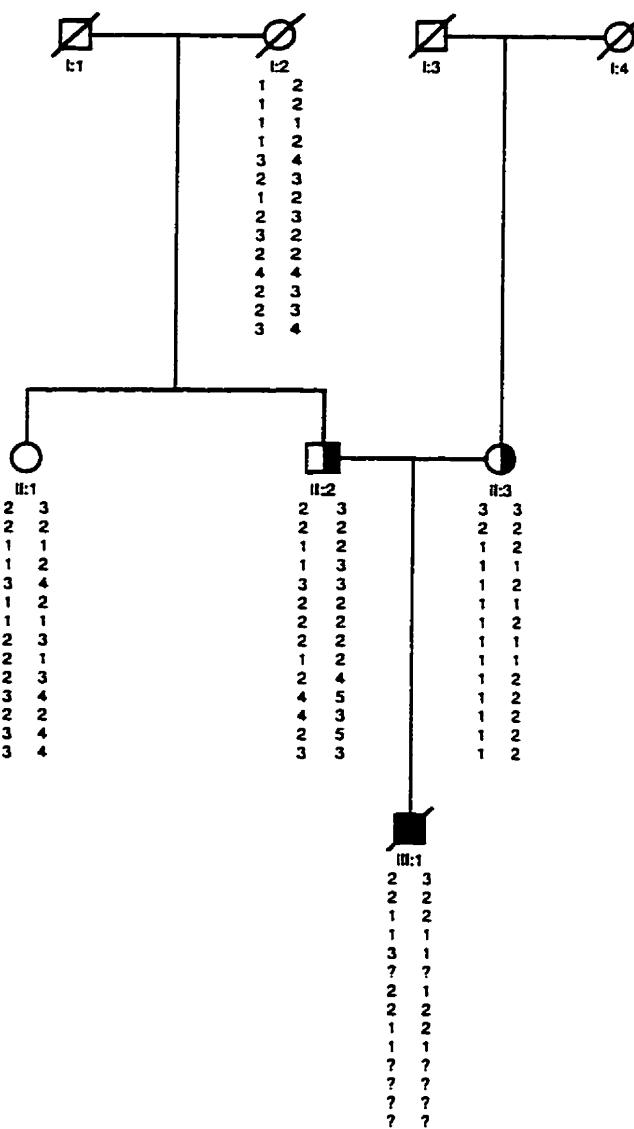
Figure 8

Figure 9:   Segregation pattern of 12 polymorphic microsatellite markers from proximal chromosome 18q in the NPD family from Massachusetts.

The order of markers as they occur on proximal chromosome 18q (proximally to distally) is indicated in the text box.   Allele sizes in base pairs at each locus are indicated. Haplotypes have been deduced from segregation patterns.   Paternally and maternally derived haplotypes are represented on the left and right respectively.

Markers	Alleles
D18S40	Multiple
D18S1104	Multiple
D18S869	Multiple
D18S44	Multiple
D18S1101	269, 271, 273, 275, 277
D18S1108	Multiple
D18S480	Multiple
D18S1107	Multiple
D18S975	Multiple
D18S66	Multiple
D18S478	Multiple
D18S1151	211, 213, 215, 217, 219, 221
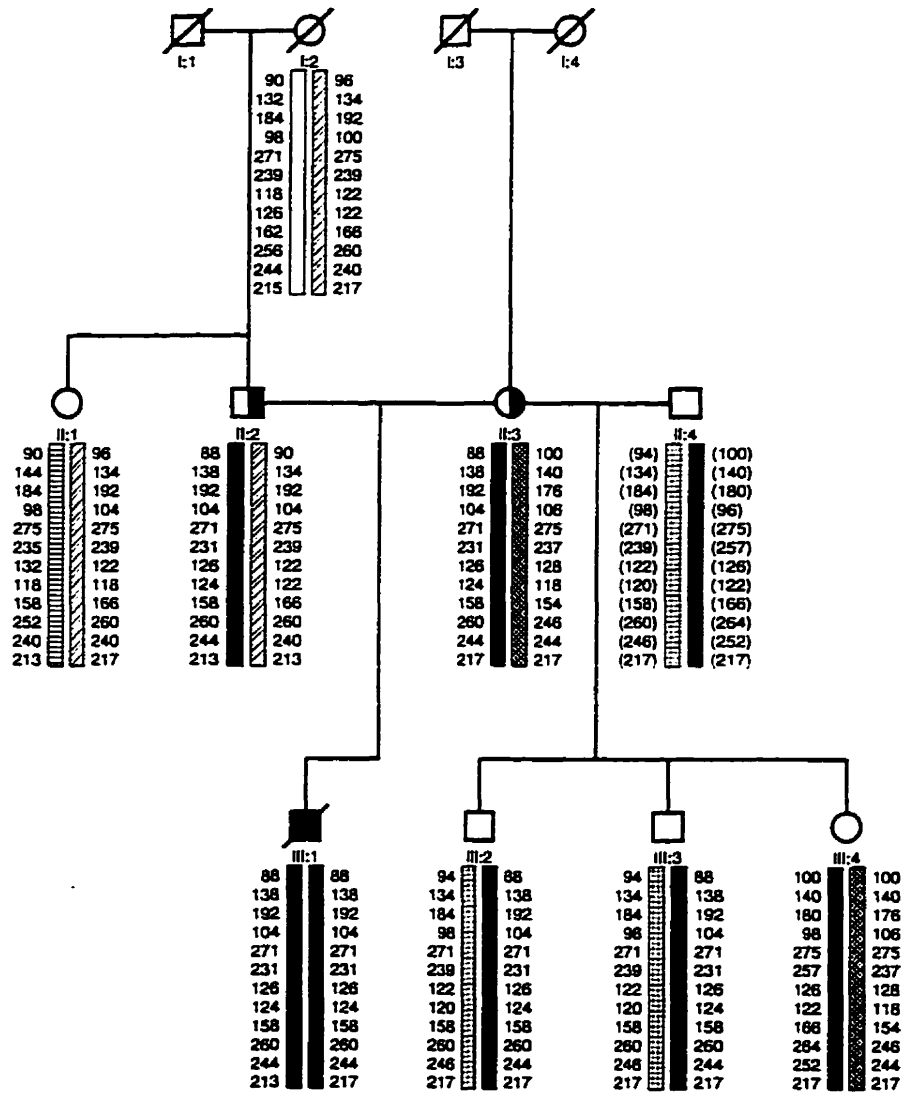
Figure 9

Figure 10: Haplotypes of NPD patients from Yarmouth County compared to a NPD patient from Massachusetts and a NPC patient from New Brunswick.

Haplotypes of the NPD patient from Massachusetts were compared to the common NPD haplotypes of patients from Yarmouth County, NS. It can be seen that all the NPD patients share the same haplotype between microsatellite markers D18S44 and D18S975. A NPC patient is shown on the right to illustrate the completely different haplotype.

Markers	Alleles
D18S40	Multiple
D18S869	Multiple
D18S44	Multiple
D18S1101	269, 271, 273, 275, 277
D18S1108	Multiple
D18S480	Multiple
GATA-P19280	Multiple
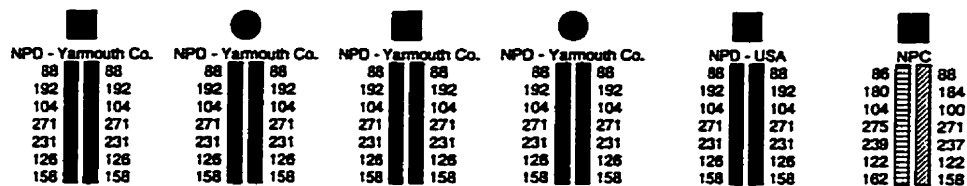
Figure 10

that NPC and NPD are likely allelic variants (Greer et al., 1997a), this large NPC family still had the potential of narrowing the NPC/NPD critical region if a recombinant was identified. In total, 22 family members provided blood samples for our study. Genotyping at 11 polymorphic microsatellite loci from the NPC/NPC critical region on chromosome 18, showed that the haplotypes of the two NPC boys (IV-1, IV-2) greatly differ from those of all of the NPD affected individuals (Figure 11). This confirms that this is a NPC family. Phase of the markers on the maternal side could not be established. More than 4 parental alleles appear to be segregating in the family. This shows questionable paternity of one or more of the siblings.

The third family was a French Acadian family from Tracadie, New Brunswick. This family was particularly interesting because it was a French Acadian family that resides in an adjacent province so could quite possibly be directly related to the Yarmouth County kindred, and thus provide an additional NPD affected individual for our study. Genealogical information provided by the family dated back several generations but revealed no evidence of Nova Scotian Acadian ancestry. Haplotype analysis with 11 polymorphic markers confirmed this (Figure 12). Both haplotypes of the affected boy (II-1) differ considerably from the common haplotype shared by NPD patients. This indicates that he is a NPC patient.

The final family investigated was another family from New Brunswick with a son diagnosed as having NPC. The limited genealogical information provided by the family, showed no evidence of Nova Scotian Acadian ancestry. As with the previous two families, haplotype analysis of 13 polymorphic microsatellite markers confirm that the affected individual is a NPC patient (Figure 13). Complementation studies from this

Figure 11: Segregation pattern of 11 polymorphic microsatellite markers in a NPC family from Missouri.

The order of markers as they occur on proximal chromosome 18q (proximally to distally) is indicated in the text box. Allele sizes (in base pairs) at each locus are indicated. Haplotypes have been deduced from segregation patterns. Paternally and maternally derived haplotypes are represented on the left and right respectively. '?' represents loci where markers were not tested. The lack of haplotype bars on the maternal side reflects the inability to establish phase in some of the siblings.
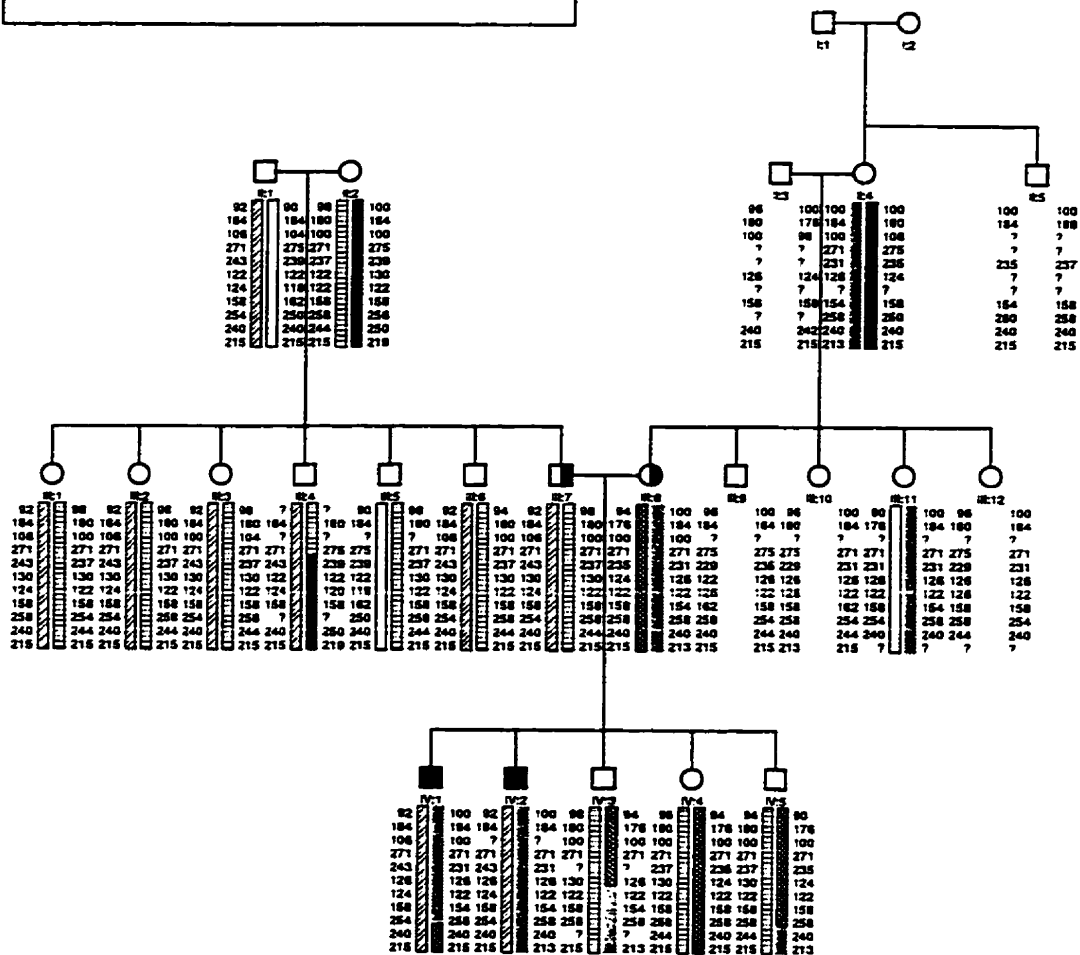
Figure 11

Figure 12: Segregation pattern of 11 polymorphic microsatellite markers in a NPC family from New Brunswick

The order of markers as they occur on proximal chromosome 18q (proximally to distally) is indicated in the text box. Allele sizes in base pairs at each locus are indicated. Haplotypes have been deduced from segregation patterns. Paternally and maternally derived haplotypes are represented on the left and right respectively. '?' represent loci not genotyped.

Markers	Alleles
D18S40	Multiple
D18S869	Multiple
D18S44	Multiple
D18S1101	269, 271, 273, 275, 277
D18S1108	Multiple
D18S480	Multiple
D18S1107	Multiple
D18S975	Multiple
D18S66	Multiple
D18S478	Multiple
D18S1151	211, 213, 215, 217, 219, 221

I:1

88	88
184	180
98	104
275	271
231	241
126	130
112	126
162	162
254	254
240	240
215	215

I:2

96	100
192	184
104	104
271	271
235	239
124	122
114	122
154	158
246	254
240	244
219	215

II:1

?	?
184	184
98	104
275	271
?	?
126	122
112	122
162	158
254	254
240	244
215	215
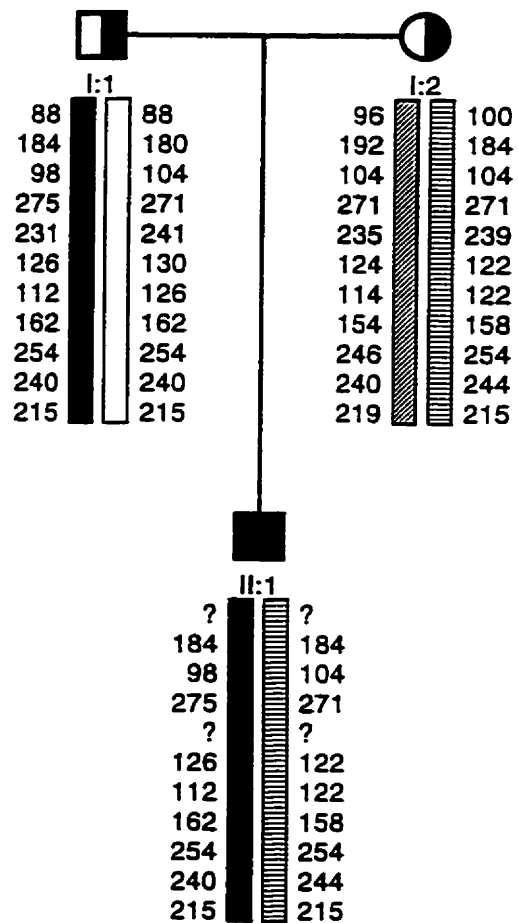
Figure 12

Figure 13: Segregation pattern of 12 polymorphic microsatellite markers in a second NPC family from New Brunswick.

The order of markers as they occur on proximal chromosome 18q (proximally to distally) is indicated in the text box. Allele sizes (in base pairs) at each locus are indicated. Haplotypes have been deduced from segregation patterns. Paternally and maternally derived haplotypes are represented on the left and right respectively. A meiotic recombination event is present between microsatellite markers D18S1397 and D18S1101, and is arbitrarily represented in individual II-2

Markers	Alleles
D18S40	Multiple
D18S869	Multiple
D18S44	Multiple
D18S1398	178, 174
D18S1397	128, 130, 134
D18S1396	141, 143, 145
D18S1101	269, 271, 273, 275, 277
D18S1108	Multiple
D18S480	Multiple
GATA-P19280	Multiple
D18S66	Multiple
D18S478	Multiple
D18S1151	211, 213, 215, 217, 219, 221

I:1

86	84
180	188
104	104
178	174
130	134
141	143
275	271
239	235
122	130
162	154
258	252
240	240
215	215

I:2

88	88
192	184
104	100
174	174
130	128
143	143
273	271
235	237
122	122
158	158
254	254
244	240
215	215

II:1

86	88
180	184
104	100
(178)	(174)
130	128
141	143
275	271
239	237
122	122
162	158
258	254
240	240
215	215

II:2

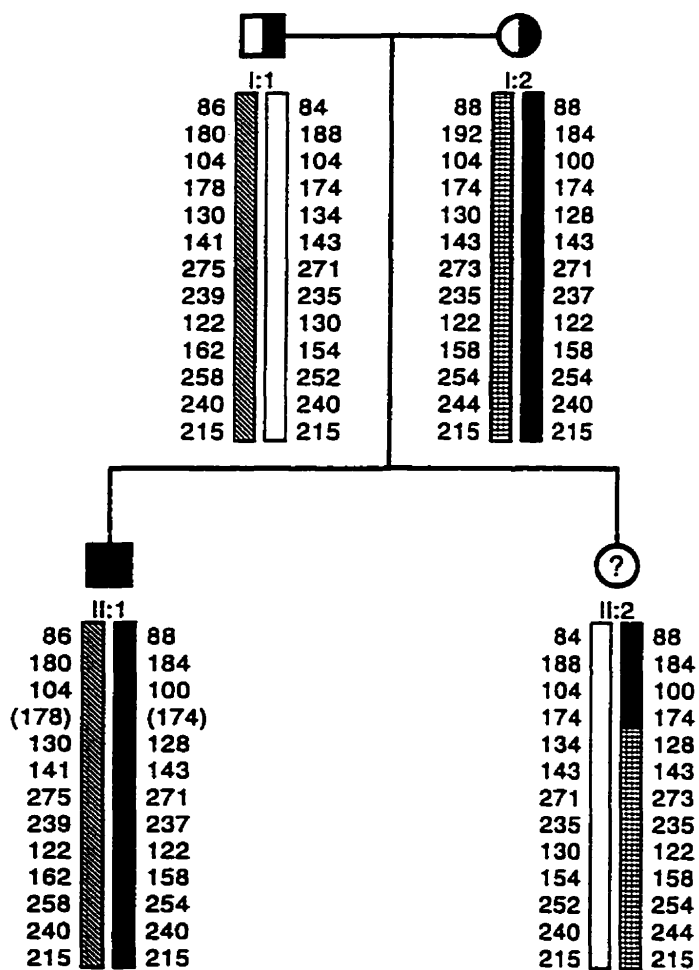|	
84	88
188	184
104	100
174	174
134	128
143	143
271	273
235	235
130	122
154	158
252	254
240	244
215	215

Figure 13

laboratory (Gillan et al., 1997) indicates that this patient is from the NPC major complementation group with a defect that maps to chromosome 18. Interestingly, the deduced haplotypes indicate that either the affected son (II-1) or the unaffected daughter (II-2) has inherited a recombinant maternal chromosome with the breakpoint within the NPC/NPD critical region between microsatellite markers D18S44 and D18S1101. The daughter has arbitrarily been represented as the recombinant. She and her affected brother share the same maternal haplotype proximal to D18S1101, but they differ at D18S1101 and distal loci. In order to position the gene with respect to the breakpoint, it was necessary to determine the carrier status of II-2. The cholesterol esterification assay, illustrated in figure 14, indicates that individual II-2 is a carrier for the NP Type II gene. The level of esterification in her cells more closely resembles her heterozygous mother from whom she received the recombinant chromosome, than the normal control. Based on these results, we conclude that the NP Type II gene lies above the microsatellite marker D18S1101 and somewhere within the 1 cM interval between D18S1101 and D18S44.

Recently, three new polymorphic microsatellite markers between D18S44 and D18S1101 were isolated in our lab. The marker D18S1397 lowers the recombination breakpoint to below D18S44. The daughter has inherited the affected allele from her mother at this locus, however since this locus is above the recombinant breakpoint it does not reduce the critical region. The other two markers, D18S1398 and D18S1396 are uninformative for this family, as they are homozygous in both the mother and daughter. Despite the addition of these new polymorphic markers, the critical region of the NP Type II gene still includes the region between D18S44 and D18S1101.

Figure 14: ACAT activity in cultured lymphoblasts from the second NPC family from New Brunswick

Solid bars represent cholesterol esterification (nmol/h/mg protein) in response to 6 hour incubation with FBS. Clear bars represent negative controls without FBS.
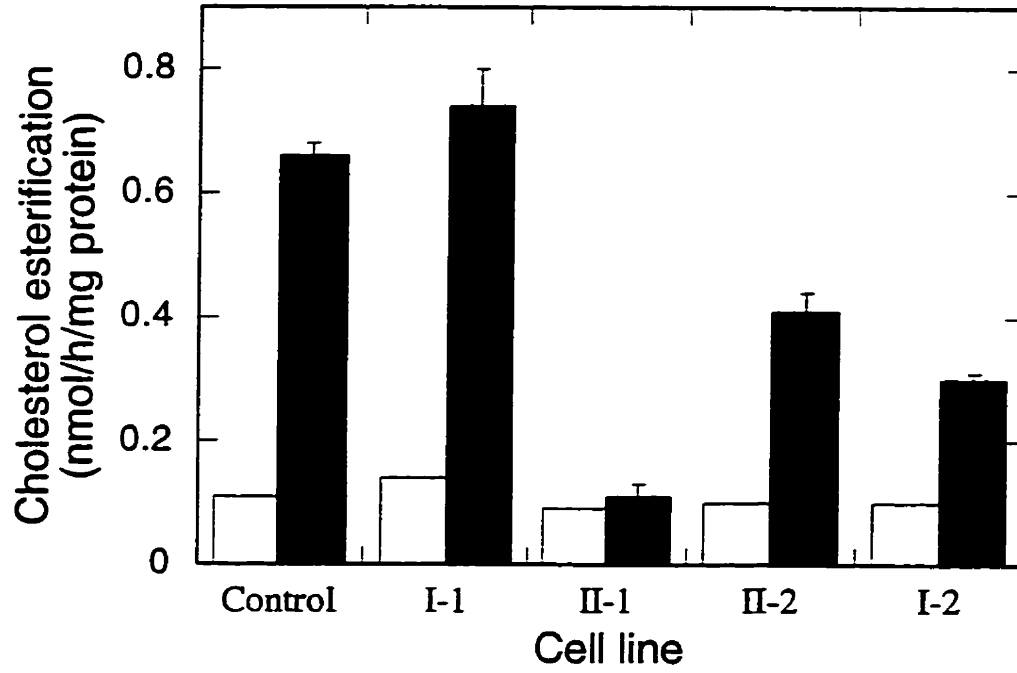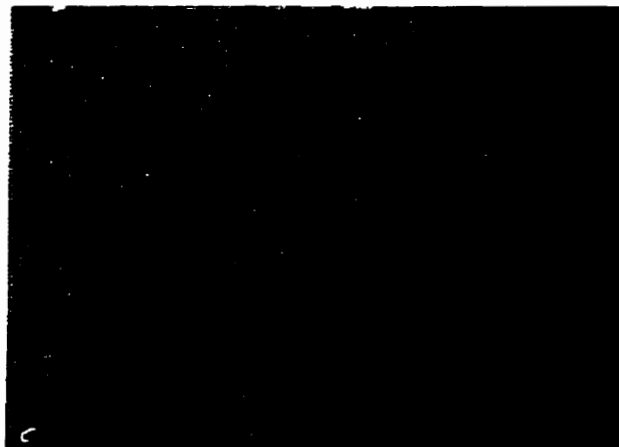
Figure 14

3.2 RETROFITTING YACS WITH THE NEOMYCIN RESISTANT GENE

In preparation for complementation studies where CHO cells with a NP Type II phenotype are fused with yeast containing YACs with human inserts from the NP Type II critical region, YACs were retrofitted with a mammalian selectable marker. A neomycin resistant gene, which confers resistance to the drug geneticin (G418) was used. Insertion of the neo^r gene was done by homologous recombination between the neo^r plasmid and YACs 877F12, 906C12 and 911D5 which span the NPC/NPD critical region. The number of successfully retrofitted yeast colonies that grew on the selective plates within the appropriate time frame of 3-5 days varied with each transformation. As few as one colony per plate, and as many as >50 colonies per plate were observed. When only a few positive colonies grew, each one was harvested; however, when there were a large number of colonies, a random sampling of colonies were harvested and chromosomes were separated by pulse field gel electrophoresis (Figure 15). Retrofitting of the YAC 877F12 produced >50 positive colonies with neomycin resistance. Of these, ten were screened for integration of the neo^r gene into the left hand arm of the YAC which can be seen as a 750 kb band which hybridizes to both neo (Figure 16) and alu (Figure 17) probes. Only 3 of the colonies, represented in lanes 6, 7 and 8 show correct insertion of the neo^r gene. The remaining 7 colonies had neo^r inserted elsewhere in the yeast genome as seen by neo^r chromosomal bands running at a molecular weight of 850 kb.

Retrofitting of 911D5 with neomycin was not as efficient as 877F12. Only 10 positive colonies were observed on the selective plates. When DNA from each of these colonies was separated by pulse field gel electrophoresis and probed for the presence of

906C12 911D5

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
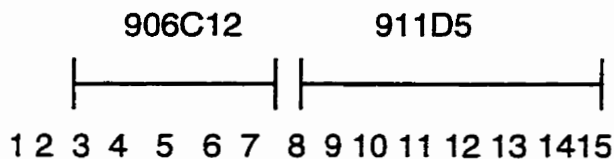
Figure 15: Representative picture of neomycin retrofitted YACs electrophoresed on a pulse field gel.

This figure shows an ethidium bromide stained pulse field agarose gel of YACs 906C12 and 911D5 retrofitted with the neomycin gene. Lane 1 is the *Saccharomyces cerevisiae* size ladder, lane 2 is the *S. cerevisiae* AB1380 yeast negative control, lanes 3-7 depict 5 independent colonies of 906C12 + neo and lanese 7-13 are 7 independent colonies of 911D5 + neo.

Lanes   1 2 3 4 5   6 7 8 9 10 11

850 kb →
750 kb →
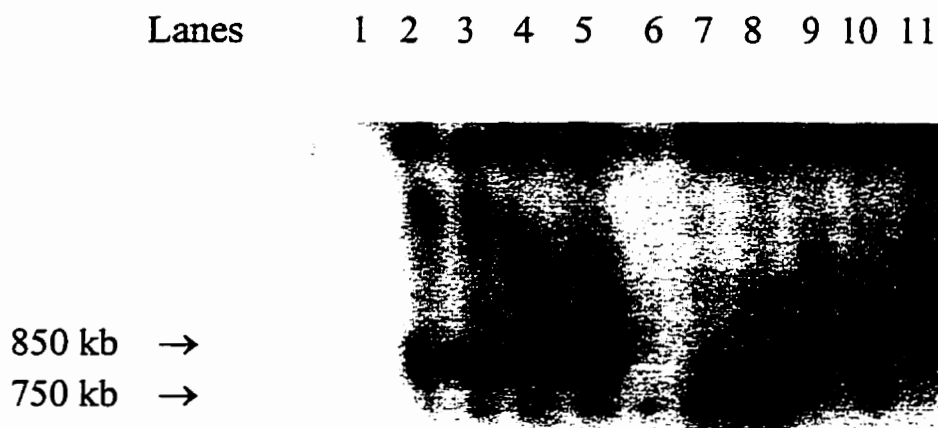
Figure 16 An autoradiograph showing the correct insertion of the neomycin gene into the left hand arm of YAC 877F12

Insertion of the neomycin resistance gene into YAC 877F12 was evaluated by probing Southern blots containing 877F12 YAC DNA retrofitted with neomycin, with a neo specific probe.   Fragment sizes, in kb, are indicated on the left hand side of the autoradiograph.   Lane 1 is the AB1380 negative control, and lanes 2-11 depict 10 independent colonies of 877F12 retrofitted with neomycin. Only lanes 6, 7and 8 indicate colonies where neomycin had correctly inserted in the left hand arm of the YAC, represented by the neomycin probe hybridizing to a ~750 kb band. The remaining lanes represent colonies where neomycin was integrated into other parts of the yeast genome. Fragment sizes were determined by comparison to *S. cerevisiae* size ladder.

Lanes   1 2 3 4 5 6 7 8 9 10 11

750 kb →
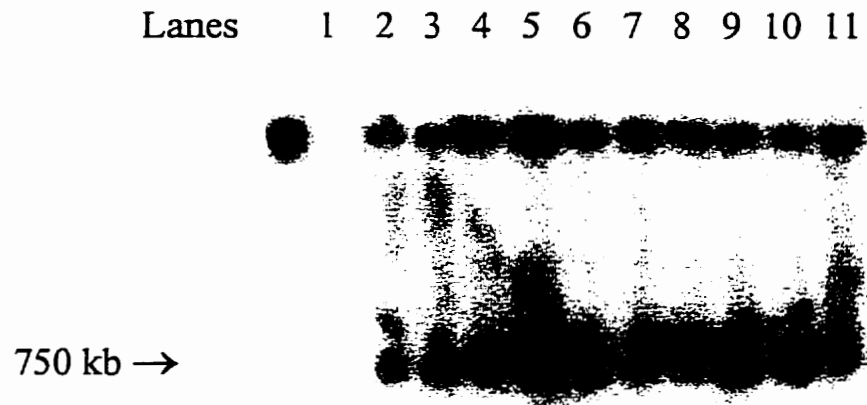
Figure 17: Autoradiograph showing the correct size of the YAC 877F12 by probing with the human repeat alu.

The actual size of the retrofitted 877F12 YAC was evaluated by probing Southern blots containing 877F12 YAC DNA retrofitted with neomycin, with the human repeat, alu. Fragment sizes in kb are indicated on the left hand side of the autoradiograph. Lane 1 is the AB1380 negative control, and lanes 2-11 depict 10 independent colonies of 877F12 retrofitted with neomycin. Fragment sizes were determined by comparison to *S. cerevisiae* size ladder

the neo^r gene, a chromosomal band of approximately 785 kb was seen, however the correct fragment size of 911D5 is about 590 kb (Figure 18). This suggests that the neo gene preferentially integrated into another portion of the yeast genome. Reprobing with alu identified a band of the correct size of 590 kb (Figure 19). Retrofitting of the YAC 906C12 was the least efficient of all 3 YACs. The most successful targeting experiment produced only 6 positive colonies. 906C12 has been found to be extremely unstable. It yields human inserts of 1100 kb, which is the reported size, yet often breaks down into a smaller product of 750 kb. Probing with neo identified a fragment of approximately 750 kb, which likely represents neomycin insertion into the left hand arm of the smaller YAC fragment (Figure 18). Reprobing with Alu produced 2 bands representing the expected 1100 kb fragment and the smaller 750 kb fragment where neomycin actually integrated (Figure 19). Efficient and successful targeting of neomycin into the correct arm of the YAC seems quite low in stable YACs, and may prove even more difficult in unstable YACs such as 906C12.

3.3 PHOTOAFFINITY LABELING OF PROGESTERONE TO NORMAL AND NP TYPE II CELLS

Progesterone has been shown to induce a Niemann-Pick Type II-like phenotype in human fibroblasts in vitro. It is hypothesized that progesterone may bind to and inactivate the same protein that is defective or absent in NP Type II cells. It has further been shown that progesterone can be covalently linked to proteins by ultraviolet light crosslinking. Therefore, the technique of photoaffinity labeling normal and NP Type II

906C12   911D5

Lanes 1   2   3   4   5   6   7   8   9

785 kb →
750kb →
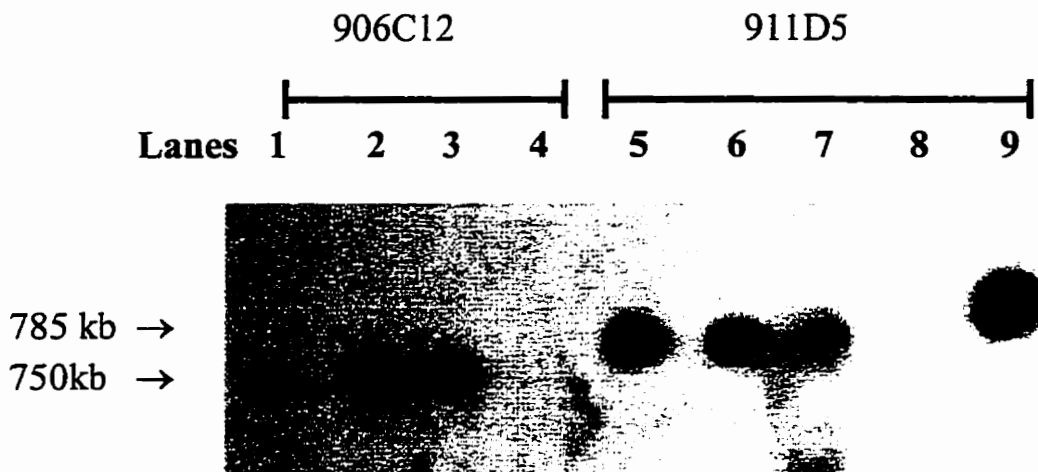
Figure 18: Autoradiograph showing insertion of the neomycin gene into YACs 906C12 and 911D5

Insertion of the neomycin resistant gene into YACs 906C12 and 911D5 was evaluated by probing Southern blots containing 906C12 and 911D5 YAC DNA retrofitted with neomycin, with a neo specific probe. Sizes in kb are listed on the left hand side of the autoradiograph. Lanes 1-4 depict 4 independent colonies of 906C12 retrofitted with neomycin, represented by a ~750 kb band (no band is observed in lane 4 likely due to the fact that neomycin did not integrated into the YAC). Lanes 5-9 represent 5 independent colonies of 911D5 retrofitted with neomycin, represented by a 750 kb band (no band is observed in lane 8 likely due to the fact that neomycin was not integrated into the YAC). Fragment sizes were determined by comparision to *S. cerevisiae* size ladder.

Figure 19: Autoradiograph showing the correct size of YACs 906C12 and 911D5 by probing with the human repeat alu.

The actual size of the retrofitted YACs 906C12 and 911D5 was evaluated by probing Southern blots containing YAC DNA retrofitted with neomycin, with the human repeat alu. Fragment sizes in kb are listed on the left hand side of the autoradiograph.. Lane 1 is the AB1380 negative control, lanes 2-6 depict 5 independent colonies of 906C12 and lanes 7-14 represent 8 independent colonies of YAC 911D5 retrofitted with neomycin. Fragment sizes were determined by comparison to *S. cerevisiae* size ladder.

cells with [³H]-progesterone ([³H]-PRG) as a novel approach to try to identify a candidate

NP Type II protein was conducted. Initial experiments identified a progesterone bound

60 kDa protein in the normal cell line, F8, which was absent in the Niemann-Pick C cell

line, GM3123 (Figure 20). A series of experiments were conducted to determine the

optimum conditions for visualizing this protein. A time course with 6 hour intervals of

[³H]-PRG labeling up to 36 hours was performed and there was no significant difference

among time points (data not shown). For consistency, the 18 hour time point was used

for most experiments. Five, 10 and 20 minute UV-crosslinking times were also tested

and again it was found that there was no significant difference with each crosslinking time

and the 20 minutes time point was chosen for most experiments (data not shown). Cells

were also incubated with or without LDL to see if there was a different response in

stressed cells, (ie. NP Type II cells incubated with LDL, and therefore with high levels of

lysosomal cholesterol) than in unstressed cells (cells not incubated with LDL). Again

there appeared to be very little difference whether the cells were incubated with or

without LDL. For most experiments, cells were labeled in media with delipidated serum.

3.3.1 Evaluation of p60 in other cell lines

The 60 kDa protein was originally seen in the F8 normal but not the GM3123

NPC cell line. To confirm that the protein was not an artifact of this particular cell line,

but was actually present in normal and not NP Type II cells, additional cell lines were

investigated. The 60 kDa protein was also absent in GM0110A (NPC) and TB (NPD)

cells and was present in another normal cell line, F2. However, the intensity of the band
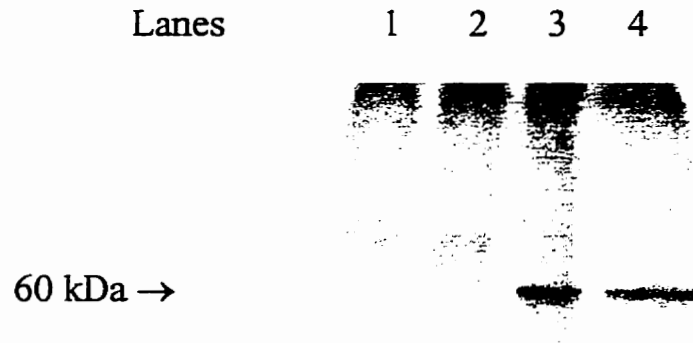
Figure 20: Identification of a 60kDa protein crosslinked with [³H]-PRG in normal and not NP Type II cells

Proteins crosslinked to [³H[-PRG in the NPC (Gm3123) cell line (lanes 1 and 2) and normal (F8) cell line (lanes 3 and 4) were separated by SDS-PAGE and visualized by fluorography. Protein size, in kDa is indicated along the left hand side.

in the F2 lanes was significantly less than the F8 cells (data not shown).

3.3.2 Fractionation studies of p60

Initial experiments that identified p60 used the Triton X-100 extraction method which would extract all cytosolic and membrane proteins. A fractionation study was executed to determine a more accurate subcellular location of the protein, either in membrane, cytosol, or nucleus. Cells were sequentially extracted with digitonin to obtain cytosolic proteins, Triton X-100 to obtain membrane proteins, and then with RIPA buffer to extract the remaining nuclear and/or cytoskeletal proteins. p60 was found exclusively in the digitonin extracts of normal F8 cells indicating that this is a cytosolic protein (Figure 21). The Triton X-100 fraction showed a band of slightly lower molecular weight present in both normal and NPC cells. No labeled proteins were detected in the RIPA fraction.
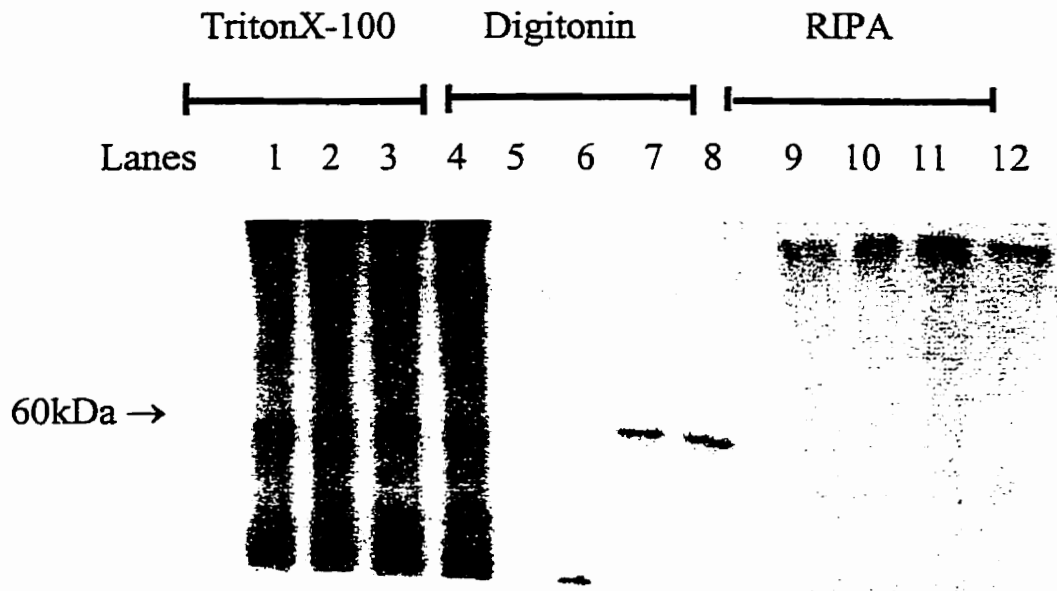
Figure 21: Analysis of p60 in membrane, cytosol and nuclear fractions of F8 cells.

The localization of p60 was determined by sequential protein extraction with Digitonin (cytosol), Triton X-100 (membrane) and RIPA (nuclear) buffers. Lanes 1, 2, 5, 6, 9, 10 represent duplicate samples from the NPC cell line GM3123 and lanes 3, 4, 7, 8, 11, 12 represent duplicate samples of the F8 normal cell line.

4.0 DISCUSSION

Research efforts in our laboratory are directed towards isolating and characterizing the NP Type II gene defect through positional cloning and functional approaches. Previous studies by Carstea et al. (1993, 1994) localized the NPC gene to a 5 cM interval on the pericentromeric region of chromosome 18 between microsatellite markers D18S44 and D18S66. More recently, we have mapped NPD within this region, which suggests that NPC and NPD are allelic variants (Greer et al., 1997a) and have subsequently placed the NPD locus between a newly isolated marker D18S1397 and D18S1108 (unpublished data). Linkage analysis was used in the initial localization to chromosome 18. In order for this approach to be most informative, it is important to analyze as many families as possible. Therefore, the recruitment of NP Type II families, particularly NPD families, in addition to those already identified by our laboratory was initiated. It was anticipated that additional NPC/NPD carriers or affected individuals would be identified with recombinant chromosomes that would more accurately position the NPD gene through approaches of classic linkage analysis and linkage disequilibrium mapping.

Of the four families identified, two were of particular interest. One of these families resides in Salem, Massachusetts, and is believed to be a NPD family based on genealogical and haplotype data. This family provided one additional NPD patient that could be used in linkage disequilibrium mapping. The NPD boy shared common haplotypes with other NPD patients between microsatellite markers D18S40 and

D18S975, thus defining the NPD interval between these markers. These findings indicate that NPD families exist outside of Nova Scotia in other parts of North America and possibly the world, where members of the Nova Scotia kindred have emigrated. This supports previous studies by Fredrickson (1966) who identified additional NPD families residing in the United States.

The second family of interest was from New Brunswick and was diagnosed as NPC based on haplotypes which bore no resemblance to those common to NPD patients. It is assumed that NPC and NPD are allelic variants as indicated by complementation studies done in this laboratory (Sidhu et al., 1992 and Gillan et al., 1997). This family allowed the NPC critical region to be significantly narrowed. One of the children carried a recombinant chromosome that redefined the NPC critical region to a 1 cM interval between markers D18S44 and D18S1101. This together with the previously established NPD region between D18S1398 and D18S1108 (Greer et al., 1997b), places the NPC/NPD locus between D18S1398 and D18S1101. This represents approximately 1.1 Mb on the physical map. A significant portion of this interval could be potentially excluded from the critical region by further delineation of the recombination breakpoint reported here through the development and use of additional polymorphic markers.

Any conclusion concerning gene location derived from this meiotic recombination depends on accurate diagnosis of the carrier status of individual II-2. As with all biochemical carrier assays, measurement of ACAT activity in NP Type II families shows some overlap between heterozygotes and normals. The mean and SD for this assay was previously reported as 0.79 ± 0.21 for normal individuals and 0.45 ± 0.18 for

heterozygotes (Byers et al., 1994). Thus, the value of 0.41 in II-2 is well below normal. Her mother, from whom she has potentially inherited the gene defect, shows a similar level. Bayesian analysis, which takes ACAT activities into account, changes her carrier risk of 67% a priori to 98% a posteriori. It is interesting to note that ACAT activity in her father, on the other hand, was well within the normal range. Heterozygote levels that deviate from expected values have been reported previously by Roff et al. (1992).

Recently, the gene responsible for the major complementation group of NPC in both the human and mouse (C57BL/KsJ-*spm* and BALB/c-*npc*^{nih}) has been identified. NPC1 was identified by positional cloning techniques as a 4.9 kb cDNA encoding a 1278 amino acid protein with an estimated molecular weight of 142kDa (Carstea et al., 1997 and Loftus et al., 1997). The NPC1 gene lies within the NPC interval defined in this study, between markers D18S44 and D18S1101, and within the NPD interval between D18S1398 and D18S1108 reported by Greer et al. (1997b). Complementation studies of candidate YACs to NPC fibroblasts in conjunction with other positional cloning strategies such as exon trapping and mutation analysis were used by Carstea et al., (1997) to isolate the NPC gene. The approach of spheroplast fusion of candidate YAC 911D5 to NPC fibroblasts was used by Carstea et al., (1997) to localize the NPC gene to this 590 kb YAC. This confirms that our approach of spheroplast fusion of candidate YACs, 877F12, 906C12 and 911D5, to NPC or NPD fibroblasts and NP Type II mutant CHO cells was an appropriate and effective method of localizing the NP Type II gene. It would still be of interest to perform complementation studies between the YAC 911D5 and the mutant CHO cell line as a way to confirm that the CHO cell line is a true model for the NPC/NPD major complementation group. The CHO 2-2 mutant cell line could then be

used confidently to learn more about the NPC1 gene and its role in cholesterol homeostasis.

The structure of the NPC1 protein was found to have an NH$_2$-terminus containing 13 hydrophobic amino acids that are characteristic of signal peptides that target proteins to the endoplasmic reticulum. Analysis of regions of hydrophobicity and structural motif comparisons predict that the NPC1 protein may be an integral membrane protein with 13-16 possible transmembrane (TM) regions (Carstea et al., 1997).

The NPC1 protein was found to share extensive homology with the transmembrane regions of PATCHED, a morphogen receptor in *Drosophila* and the defective protein in basal cell nevus syndrome. In addition, the region between amino acids 615 to 797 shows homology with the sterol regulatory element binding protein (SREBP) cleavage-activating protein, SCAP, a modulator of cholesterol-regulation factor activation, and with 3-hydroxy-3-methyl-glutaryl coenzyme A (HMG-CoA) reductase, the regulatory enzyme of *de novo* cholesterol biosynthesis.

It has been suggested that the NPC phenotype may be due to the disruption of the pathway of cholesterol trafficking from the lysosome and plasma membrane to the ER, which may be modulated by a Golgi complex-mediated process (Neufeld et al., 1996 and Blanchette-Mackie et al., 1988). Analysis of three putative signal peptide and TM domains supports the idea that NPC1 is localized in the Golgi, ER or plasma membrane. NPC1 was also found to contain a COOH-terminal di-leucine motif which may act in signaling during endocytosis from the plasma membrane and lysosomal targeting. These findings imply that NPC1 is associated with one or more membrane structures, possibly plasma membrane, lysosome, golgi or ER, and may play a key role in intracellular

cholesterol homeostasis (Carstea et al., 1997 and Loftus et al., 1997). Based on the limited knowledge of the NPC1 gene product, it does support the existing hypothesis of a block in intracellular LDL-cholesterol trafficking.

Mutation analysis of nine unrelated NPC families identified the presence of eight distinct mutations which included a frameshift at codon 1205 that produced a premature termination, 2 multiple nucleotide deletions resulting in a frameshift that produced a premature termination at codon 632 and five missense mutations. The later includes two C→T transitions at codon 1036, an A→G transition at codon 1156, and two missense mutations that altered amino acids that are phylogenetically conserved.

The gene responsible for NPD is still unknown, however, with the identification of NPC1, the localization and identification of the NPD gene will be expedited. Since NPC and NPD are likely allelic variants, SSCP mutation analysis using primers designed from cDNA sequences or from inton sequences from the published NPC genomic sequence can be used to identify the mutation responsible for NPD. It is likely, since NPD disease occurs in a homogeneous population, that only a single mutation will be identified in all NPD patients, in contrast to the 9 different NPC mutations described to date. It is interesting to note that of the nine NPC patients analyzed in the study by Carstea et al. (1997), all but one were compound heterozygotes. The remaining patient had a homozygous C→ T missense mutation at codon 1036 which resulted in threonine being changed to methionine. It is possible that this particular NPC patient may actually be our NPD patient from Massachusetts. Mutation analysis on this patient and the other NPD patients will confirm this speculation.

With the identification of the NPC1 gene it is predicted that the gene product is a 142 kDa membrane protein not a 60 kDa cytosolic protein that was identified with the progesterone crosslinking experiments reported here. Nevertheless, this protein may still play a role in cholesterol trafficking and homeostasis.

Recent literature has reported the existence of three isoforms of the progesterone receptor (PR); PR-A, which has a molecular weight of 94 kDa, PR-B which has a molecular weight of 120 kDa and PR-C which has a molecular weight of 60 kDa (Wei et al., 1990, Weigel et al 1992 and Wei and Miner, 1994). All three PRs are nuclear proteins whereas the 60 kDa protein identified in this study is cytosolic, however, it is possible that the 60 kDa protein identified here may be an isoform or fragment of one of these PRs. It would be interesting to pursue this possible lead and determine whether the 60 kDa protein is PR-C, by obtaining an antibody directed against the C-terminal amino acids of human PR which would recognize PR-C. Western blotting with this antibody with the normal and NP Type II cells lines used in this study would identify if this PR is present. It is also possible that the protein identified in this study is one of the multiple drug resistant P-glycoproteins, which are required for cholesterol esterification, although it should be noted that these large (~170kDa) proteins are membrane bound rather than cytosolic. When progesterone is present, it interacts with both the PR and MDR P-glycoproteins. PRG binds directly to the MDR pump, inhibiting its activity and hence inhibiting esterification of LDL-derived cholesterol (Metherall, et al., 1996 and Debry, et al., 1997). It is therefore conceivable that the 60 kA protein identified by photoaffinity

labeling could be a soluble fragment of P-glycoproteins. It is also possible that the 60 kDa protein identified in this study may be a soluble fragment of the NPC1 protein.

Although this 60 kDa protein is not the NPC1 protein, and unlikely to be the NPD protein, it is still not ruled out as a protein somehow involved in cholesterol trafficking and homeostasis. The novel approach of crossing linking [^{3}H]-PRG to normal and NP Type II cells did identify a cytosolic protein present in the normal F8 cell line and, to a lesser extent normal F2 and F4 cell lines, (Byers, pers comm) and was not present in any of the NP Type II cell lines. The identification of this potentially new 60 kDa protein still leaves many unanswered questions. Further characterization of this protein is necessary to confirm that it is present in all normal cell lines, and not just an artifact of the particular cell lines used in this study. Further understanding of the mechanism by which progesterone induces a NP Type II-like phenotype in human fibroblasts, and identification of the 60 kDa protein that it binds to could provide valuable insight into cholesterol biosynthesis.

Although the gene responsible for the major complementation group of NPC has been identified, and the identification of the NPD gene mutation will soon follow, there are still many questions to be answered. We are now only beginning to understand the structure and function of the NP Type II protein and its role in cholesterol homeostasis. The immediate benefit of the discovery of the NP Type II gene is the accurate diagnosis of affected and carrier individuals. Until now, a positive diagnosis was confirmed by measurement of cholesterol esterification in fibroblasts or lymphoblasts in response to LDL-cholesterol and measurement of cholesterol storage within lysosomes. Mutation analysis of suspected NP Type II patients can now be used as a direct approach that is

much less time consuming and more cost effective. Soon accurate carrier diagnosis will be available to screen NPD family members, and couples with a family history of the disease who are interested in having children. This will be extremely beneficial in the Yarmouth County population which has the highest incidence of NP Type II disease in the world with an estimated carrier frequency between 10-26%.

To date there is no effective treatment or cure for individuals afflicted with this disease. However, with the identification of the NPC 1, insight into the function of this gene and subsequent gene product will provide valuable clues about this devastating disease. Hopefully, in the near future with this new knowledge, an effective treatment and ultimately a cure will be found.

APPENDIX I

INFORMATION SHEET FOR FAMILY MEMBERS

In 1978 some members of an "extended" family in Yarmouth County, Nova Scotia were identified as having the genetic condition known as Neimann-Pick D (NPD), a severe childhood disorder that causes gradually increasing physical and mental difficulties usually ending in death at 15-20 years of age. Both parents of an affected child each carry one abnormal NPD gene and one normal gene and have each contributed one copy of the abnormal NPD gene to that child. Also, all brothers and sisters of the parents have a 50% chance of carrying one copy of the abnormal NPD gene. Other close relatives also have a high risk of being "carriers" of the abnormal NPD gene.

The study of many families from Yarmouth County by scientists at the Atlantic Research Centre in Halifax, Nova Scotia showed that the gene causing the disorder in this area came from one person who came to Nova Scotia sometime in the 1600's. This study also showed that the number of carriers and the number of affected children in Yarmouth County is significantly higher than anywhere else in the world.

When the 1978 study was done, it was not possible to identify people who were carriers unless they had an affected child. However, we believe that it is now possible, in large families, to develop a "carrier" test by following the abnormal NPD gene through generations. We are presently trying to trace all individuals who have emigrated from Yarmouth County to the United States to participate in our study in hopes that a connection may be established between them and the Yarmouth County families. A large number of families from the Yarmouth area have already been contacted and have kindly supported and participated in our study. In order for this research to be successful we need as many members of the family to participate in the study as possible; the more members that contribute, the more accurate the results. Therefore it is essential that we contact as many close relatives as we can from both Yarmouth County and United States families. This includes affected individuals as well siblings, aunts, uncles, parents and grandparents of affected individuals.

We would like to ask for your help and participation in this study which would involve the contribution of a small blood sample of 20 ml (4 teaspoons). If you are willing to participate, we ask that you:

1) Make an appointment with your physician or a clinic for a 20 ml (4 teaspoons) blood sample to be taken
2) Sign enclosed consent form
3) Be sure to bring the attached sheet explaining the type of blood sample required and give to the nurse or technician taking your blood
4) Send the blood sample and consent form to our labs in Halifax according to the instructions on the sample type and delivery sheet. This can be done either by the physician or clinic or by yourself. (Please note that all costs of blood samples and shipping will be covered by us)
5) Call us collect at (902) 428-3691 or (902) 428-2873 to let us know the date the samples taken and to give us the way bill number from the courier slip

If there are any questions or confusion regarding this procedure please feel free to call Dr. Greer at (902) 428-3691 or Tanya Gillan at (902) 428-2873 and we will be happy to make the appropriate arrangements.The consent form simply indicates that you understand what the study is about and that you agree to take part in it. Dr. Greer will be available in Halifax at (902) 428-3691 to answer

any questions that you may have about the study. After your sample is collected, it will be shipped to the Victoria General Hospital where the DNA (the genetic material) will be isolated and stored. In addition, some of your donated sample may be specially treated ("transformed") in order to make the white blood cells grow continually in the lab. This is so that the researchers who need healthy cells for their biochemical work do not need to ask you for additional samples later on.

We understand that not everyone will want to participate in the study. If any member of the family chooses not to participate, it will not compromise his/her health care in the future.

It is hoped that your participation in this study will mean that you and other members of your extended family will be able to find out if they are a carrier of the abnormal NPD/NPC gene, and that this information will help all those involved to make informed family planning decisions. We hope that such a carrier test will be available within the next 12-18 months, and as well, that this study will give us further biochemical information that will lead us to a better understanding of how our body cells work.

If you are not sure about participating, but would simply like to get more information before deciding, please feel free to contact any of the people named below.

We hope that you will choose to participate in this important study, and thank you for your consideration.

Dr. Wenda Greer, PhD,
Victoria General Hospital
phone: (902) 428-3691
fax: (902) 428-4113
e-mail: wgreer@is.dal.ca

Tanya Gillan, Research Associate
Victoria General Hospital
phone: (902) 428-2873
fax: (902) 428-4113
e-mail: tlgillan@is.dal.ca

APPENDIX II

Common French Acadian Surnames

Amirault, Amereau, Amirau
Babin, Babineau
Belliveau
Boudreau, Bodrot
Bourque
Corporon
D'Entremont
Doucette, Doucet
Duon,
Inard, Hinard
LeBlanc
Moulaison
Muise, Muis, Meuse, Mius
Pothier, Poitier
Robicheau, Robichaud
Surette
Devillier, Deviller
Cothereau
Richard
Bertrand
Boucher
Clarmont, Clermont
Dulin
Frontain
Hebert, Hubbard, O'Bird
Jacquard
LeFavre

APPENDIX III

INFORMED CONSENT FOR KEY MEMBERS OF THE NIEMANN-PICK D FAMILY FOR
THE STUDY ENTITLED:
*LOCALIZING THE GENE DEFECT OF NEIMANN-PICK D DISEASE (NOVA SCOTIAN
VARIANT)*

I have read the information sheet describing this study and I would like to participate. I understand that I will be asked to donate a small blood sample (20 ml or approximately 4 teaspoons) and that the DNA (genetic material) obtained from this sample will be used to try and identify the Niemann-Pick D gene in family members. I understand that some of my white blood cells may be used to start a permanent laboratory cell line. This would mean that the researcher would have a continual supply of healthy cells from me without having to ask for additional samples.

I understand that there is no special risk associated with donating this small blood sample, although there may be slight discomfort during the collection procedure, and that there may be a small amount of bruising in the 24 hours following the collection.

I also understand that I may not directly benefit from taking part in this study, but that it is hoped that knowledge gained from this study will benefit others.

I understand that this study will take some time to complete, likely between 12 and 18 months. When the study is complete, I will be informed that the results are available and that, in order to receive them, I must contact the Atlantic Research Centre. This is to ensure that I receive the results with appropriate genetic counselling and learn how they might influence any family planning decisions which I may make in the future and implications for my children.

I understand that should I decide to withdraw from this study at any time, this decision will not influence in any way medical treatment which I may need in the future.

_____   _____
print name   *signature*

birthdate

_____   _____
name of closest affected relative   *your relationship to affected relative*

Witness _____   Date _____

I understand that any of my DNA sample which remains after the study is complete will be stored at the DNA bank at the IWK Children's Hospital. After the completion of this study, I give permission for this remaining material to be used for genetic studies related to my family, on the understanding that this wil be anonymous (i.e. my results will not be personally identified).

Permission granted _____ Date _____

APPENDIX IV

TYPE OF BLOOD SAMPLE REQUIRED FOR NIEMANN-PICK STUDY AND DELIVERY INFORMATION

Blood Sample:

-        20 ml of Peripheral Blood taken in lavender top-EDTA vacutainer tubes

-        10 ml of Peripheral Blood taken in green top-Heparin vacutainer tubes

Delivery Information:

-        Blood should be delivered by Federal Express or other courier service at room temperature

-        Send to the address below:

> ATT: Dr. Wenda Greer/ Tanya Gillan
> Room 309 West Annex
> Dr. D.J. Mackenzie Building
> Hematology Lab, Victoria General Hospital
> 1278 Tower Road
> Halifax, Nova Scotia
> Canada
> B3H 2Y9

-        Please phone us collect at (902) 428-3691 or (902) 428-2873 to let us know when the samples were delivered and their **way bill number**

-        **Remember:** all costs of blood samples and delivery will be covered by us; please be sure to enclose **all** bills so that you can be reimbursed.

-        Don't forget to enclose consent form!

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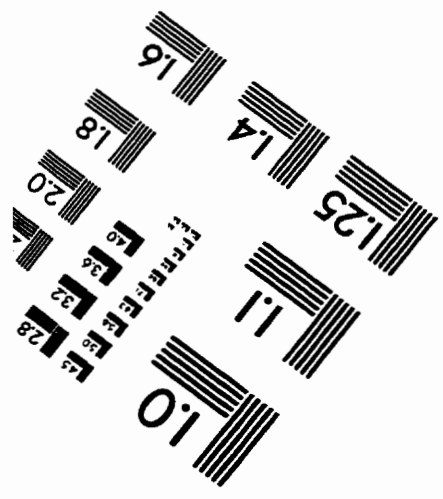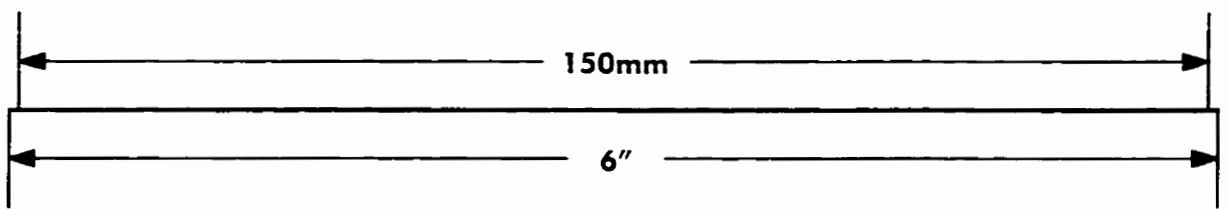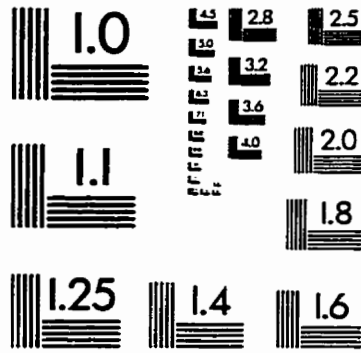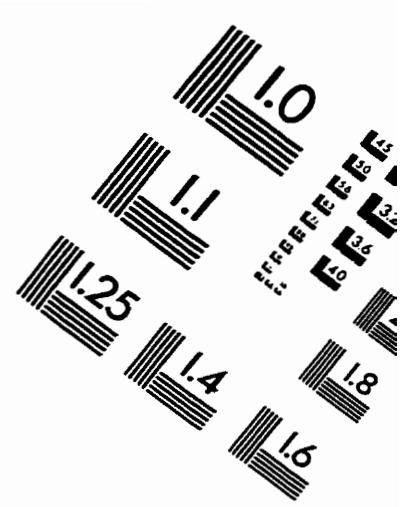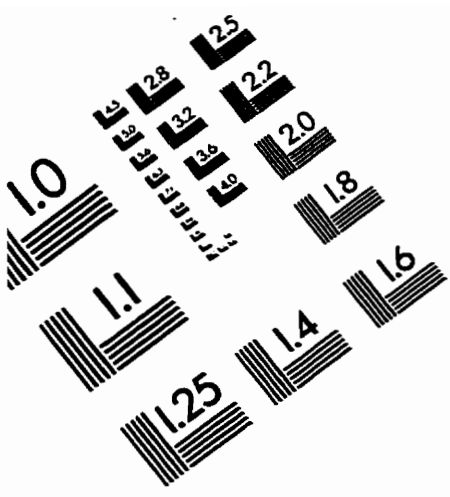
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IMAGE EVALUATION
TEST TARGET (QA-3)

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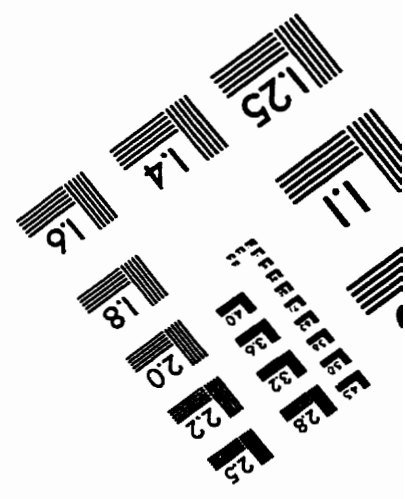
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