12-1-1990

The search for branchio-oto-renal syndrome on human chromosomes 1 and 8.

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THE SEARCH FOR BRANCHIO-OTO-RENAL SYNDROME ON HUMAN CHROMOSOMES 1 AND 8

A Thesis

Presented to the

Department of Biology

and the

Faculty of the Graduate College

University of Nebraska

In Partial Fulfillment

of the Requirements for the Degree

Master of Arts

University of Nebraska at Omaha

by

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December 1990

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THESIS ACCEPTANCE

Acceptance for the faculty of the Graduate College, University of Nebraska, in partial fulfillment of the requirements of the degree Master of Arts, University of Nebraska at Omaha.

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December 4, 1990
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ACKNOWLEDGEMENTS

First, I would like to thank Dr. William J. Kimberling for offering me the opportunity to pursue this area of academic research. He has introduced me to a field which I have found very rewarding in every way.

I would also like to thank Dr. Thomas A. Weber and Dr. David F. Conway for serving as members of my committee.

The various members of Boys Town National Research Hospital's Genetics Department have been of infinite help to me: Mike Weston, Kathleen Brennan, Judy Kenyon, Yin Shugart, Sue Tinley and Kathy McBrown.

Finally, I would like to thank my family and friends whose unflagging support and understanding have sustained me.

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ABSTRACT

Branchio-oto-renal (BOR) syndrome is an autosomal dominant disorder in which affected individuals may have conductive, sensorineural or mixed hearing loss, preauricular pits, structural defects of the outer, middle or inner ear, fistulas and cysts of the second branchial arch and renal anomalies ranging from mild hypoplasia to complete agenesis. There is wide variation in expression of the symptoms and not all symptoms are present in all carriers of the gene. Less frequent symptoms (e.g. lacrimal duct stenosis) have been described.

The purpose of this study is to attempt to localize the gene for branchio-oto-renal syndrome using the linkage analysis approach. This syndrome had been tentatively mapped to chromosome 8 at 8q13.3 or 8q21.13. Confirmation of this finding was one of the goals of this research. Chromosome 1 was also explored as a candidate chromosome because of the large number of DNA markers available. Localization of the disease gene is accomplished by using classical markers and probe DNA segments which are homologous to the region of interest on chromosomes 1 or 8. Both probe and classical marker results can be used in concert with clinical findings to determine linkage to the BOR gene.

Four large 4 generation families were typed with the following DNA probes pJA110, pTH154, pL1.22, pN8C6, EB8, pEKH7.4, pDR78 and pHRN1 and classical markers RH, PGM1, PGD and FY for chromosome 1. DNA markers utilized for chromosome 8 included pYNM3, pMCT128.2, pHHH171, pCRF H-8, pH11F3b, pH25-3.8, p380-8A and pCHT 16/8, however the majority of these were utilized for only one family. Linkage analysis was performed on the polymorphic results

presented by family members. The purpose of the linkage analysis is to eliminate the likelihood of the BOR gene being linked to the various markers presented. Branchio-oto-renal syndrome has not been localized to any region of chromosome 1 or 8, although it has been excluded from some areas of both of these chromosomes.

INTRODUCTION

Branchio-oto-renal (BOR) syndrome is an autosomal dominant disorder in which affected individuals may have sensorineural, conductive or mixed hearing loss, preauricular pits, structural defects of the outer, middle or inner ear, lacrimal duct stenosis, branchial fistulas or cysts of the second branchial arch, and renal anomalies ranging from mild hypoplasia to complete absence (Fraser et al., 1978). The frequency of the syndrome has been estimated as one in 40,000 (Fraser et al., 1980). BOR is reported to occur in 2% of profoundly deaf children (Heimler & Lieber, 1986).

Heusinger, in 1864, first described a condition in which preauricular pits, branchial fistulas and hearing impairment occurred together. The first report of a family with branchial anomalies, hearing impairment and kidney problems was given by Fara et al., (1967). This was considered an isolated finding because the renal involvement occurred in only one family member. Melnick (et al., 1975) presented a study which confirmed the status of a syndromic disorder on BOR because the kidney anomalies occurred in more than one family member. Because male-to-male transmission of the disease was present in the pedigree, this supported an autosomal dominant mode of inheritance. This syndrome was originally termed autosomal dominant branchiootorenal dysplasia designating a defect in the growth and differentiation of the branchial arches, oocyst and renal primordia.

DELINEATION OF THE SYNDROME

Over the years, there has been a considerable amount of debate about the etiology of branchio-oto-renal syndrome. McKusick, 1971, suggested that preauricular pits and branchial cleft anomalies represent separate autosomal dominant mutations because most families present one condition or the other. However, several families exhibit both traits being inherited together in an autosomal dominant fashion (Hunter 1974, Martins 1961, Schull & Fumata, 1957). Several additional families (Fourman & Fourman 1955, McLaurin et al., 1966, Wildervanck 1962, Rowley 1969, Bailleul et al., 1972, Bourguet et al., 1966) exhibited branchial cleft anomalies, preauricular pits and malformed auricles associated with deafness. In these cases, examination by X-ray or IVP for renal anomalies was not performed or not noted. Hilson, 1957, noted an autosomal dominant mutation that caused malfunctions of the external ear and kidney, but no branchial anomalies or hearing loss. Melnick (et al., 1978) reported three families; one with branchio-oto-renal dysplasia and two with branchio-oto dysplasia. They characterize branchio-oto-renal dysplasia (BOR) as persons with pits, branchial fistulas and hearing loss with renal anomalies and branchio-oto dysplasia (BO) in persons which have all of the above components except renal anomalies. This suggests the existence of two separate genetic diseases; one in which the gene product is critical for both renal and auditory development, and the other in which it is critical for auditory development alone. Konigsmark and Gorlin (1976) considered BOR to be divided into two separate entities on the basis of hearing loss; one disorder with sensorineural hearing loss and one with mixed or conductive hearing loss. However, Fraser (et al., 1978) showed that hearing loss could be sensorineural in one ear and conductive in the other ear of

the same individual so this separation on the basis of hearing loss seems unjustified. Fraser (et al., 1983) reported a family with preauricular pits, branchial fistulas, hearing loss and duplication of the ureters suggesting that this be a distinct disorder from BOR and be called the BOU syndrome. However, Heimler and Lieber (1986) demonstrated, in one family, that some affected members have duplication of the collecting system, while others have the normal renal malformations. It is this author's opinion that BO and BOU are simply variants of the BOR syndrome and that the BOR gene shows a high degree of variable expression.

SYMPTOMS OF BRANCHIO-OTO-RENAL SYNDROME

The main symptoms of branchio-oto-renal syndrome are preauricular pits; conductive, sensorineural or mixed hearing loss; branchial clefts or fistulas; ear anomalies and renal anomalies (See Table I). Not all features of the syndrome are expressed in all carriers of the gene; however, very few carriers lack all the features. Preauricular pits, branchial clefts and hearing loss are the most frequently expressed symptoms (Melnick et. al, 1978). The penetrance of BOR appears to be high, as almost all known carriers exhibit some manifestation of the syndrome.

Table I. Ranges of Frequencies of Symptoms and Associated Anomalies of Branchio-Oto-Renal Syndrome.*

	Frequency Range
Preauricular pit	70-95%
Hearing loss	73-100%
Branchial fistula/cleft	60-84%
Anomalous pinna	37-62%
Renal anomaly (I.V.P.)	9-75%
Renal function anomaly	2-33%
Lacrimal duct block	5-9%
Retrognathia	7-16%
Facial paralysis	4%

*Adapted from Cremers & Fikkers-van Noord (1980)

PREAURICULAR PITS

Typically, preauricular pits are shallow, pinhead size depressions in the helix of the ear near its upper attachment; cartilaginous preauricular appendages (tags) may occur as well or instead in some individuals (Fraser et al., 1978). Preauricular pits can be bilateral or unilateral, however the former is more common. These occur in about 1% of the general population, although preauricular pits are much more common in certain racial groups, particularly blacks (Ewing, 1946). There is a highly significant ($P<0.001$) increase in preauricular pits in a sample of white children with profound hearing loss (4.5%) over a sample of newborn white infants (0.7%) (Fraser et al, 1980). The sample from the newborn population has a significant excess of females with pits (0.28), there is no difference in the ratio of males to females with pits in the BOR syndrome, this suggests a causal difference between the usual sort of ear pit and those associated with the BOR syndrome (Fraser et al., 1980). Cremers

and Fikkers-van Noord (1980) estimated that 95% of persons with BOR exhibit preauricular pits.

Preauricular pits are thought to represent incomplete fusion of the first and second branchial mesodermal hillocks (Melnick et al., 1976). However, since these sinuses are not necessarily at points of fusion, this explanation is not satisfactory (Hunter, 1974). Some investigators have suggested that the sinuses may be related to a defective closure of the most dorsal part of the first branchial cleft (Moore, 1973), while others argue that they represent ectodermal folds that are sequestered during auricle formation (Aronsohn et al., 1976).

HEARING LOSS

The second symptom of BOR is hearing loss which can be sensorineural, conductive or (most frequently) mixed. The hearing loss may be any of the three types and may range from a mild to profound loss. This loss may be sensorineural in one ear and conductive in the other ear of the same individual. It is not usually described as progressive, but one report (Bourguet et al., 1966) presents two individuals in which there was a sudden hearing loss after intense exertion or trauma. Since BOR is a congenital disorder the hearing loss is present at birth.

Conductive

A conductive hearing loss is caused by a malfunction of the ossicles in the middle ear. The ossicles may be displaced and malformed; the stapes and incus may be fused, unconnected or normal (Fraser et al., 1978). One case reported by Cremers and Fikkers-van Noord (1980) demonstrated an individual who completely lacked ossicles. Abnormalities of the incus or malleus are most often due to incomplete or atypical differentiation of the dorsal end of the first arch cartilage.

This dysplasia is seen as malformed individual ossicles, the bony union of two otherwise normally formed ossicles or fusion of two malformed ossicles. Failure of differentiation of the fibrous annular ligaments attached to the footplate of the stapes results in bony fixation of the footplate of the stapes to the otic capsule (Melnick et al., 1980). In summary, the etiology of the conductive deafness in this syndrome seems to be due to malformations and fusion of the ossicles (Fitch & Srolovitz, 1976).

If an individual's audiogram shows reduced hearing sensitivity by air conduction (AC) and normal sensitivity by bone conduction (BC), the result is a conductive hearing loss. This type of loss can usually be corrected by surgery or improved by hearing aids. Persons with BOR have a conductive hearing loss 30% of the time (Fraser et al., 1980).

Sensorineural

In BOR the sensorineural hearing loss is produced by the maldevelopment of the cochlea or neural connections or both. The cochlea is normally coiled in a spiral of 2 1/2 turns, however persons with BOR have a Mondini type cochlear malformation which means that the cochlea is reduced to 1-1 1/2 turns (Melnick et al., 1976). This type of malformation is due to incomplete development of the cochlea which leads to a hypoplastic apex (Melnick et al., 1976). An audiogram of a person with a sensorineural hearing loss shows air conduction and bone conduction to be the same (±5dB) up to a point (See Figure 1). This type of loss is considered more serious than conductive because if sensory hair cells in the cochlea or nerve fibers are degenerated or absent they cannot be restored. Sensorineural hearing loss occurs about 21% of the time in affected individuals (Fraser et al., 1980). BOR is reported to occur in 2% of profoundly deaf children (Heimler & Lieber, 1986).

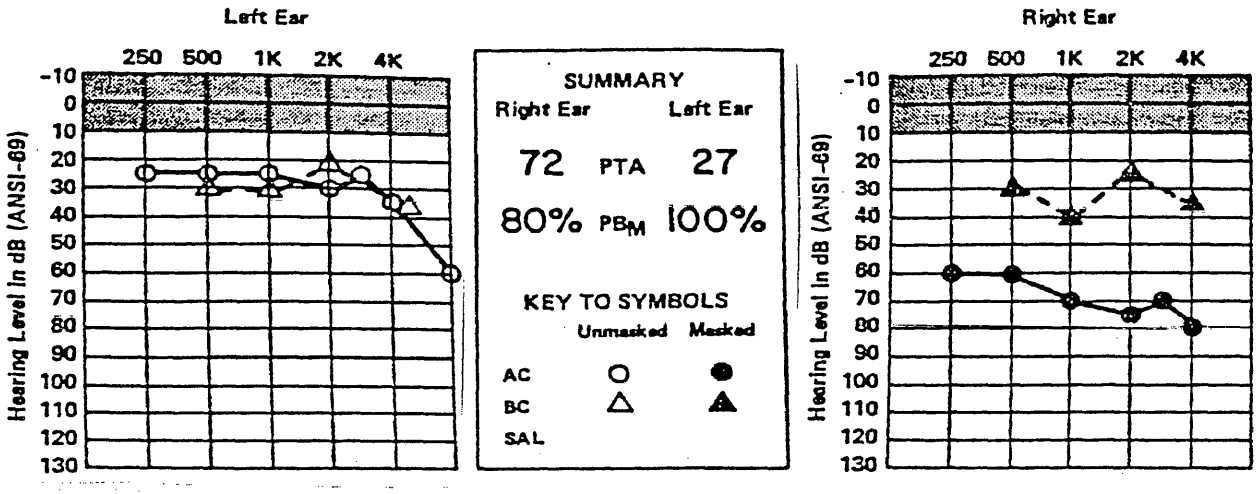

FIGURE 1. Audiogram of a patient with BOR. There is a mild sensorineural hearing loss in the left ear and mixed loss in the right ear.

Mixed

A mixed hearing loss is a combination of both conductive and sensorineural hearing losses. A mixed hearing loss is produced by a combination of stapedial fixation (conductive component) and a Mondini type cochlear malformation (sensorineural component) (Melnick et al., 1976). Mixed hearing loss is the most common type of loss in individuals with BOR since it occurs about 48% of the time (Fraser et al., 1980). A mixed hearing loss audiogram shows both AC and BC thresholds as being reduced, but BC yields better results than AC (See Figure 1).

BRANCHIAL FISTULAS AND CLEFTS

The third critical feature of branchio-oto-renal syndrome is branchial sinuses, fistulas or clefts. Branchial cleft fistulas open externally in the lower third of the neck, usually on the medial border of the sternomastoid muscle and may open internally into the tonsillar fossa. The cutaneous opening, if present, is usually quite inconspicuous, but the sinus may ooze fluid or become infected. The developmental

defect may also lead to a cyst with no opening or a cartilaginous mass (Fraser et al., 1978). Branchial fistulas or cysts can be bilateral or unilateral, but are usually the former. The frequency of hearing loss in those with bilateral fistulas (98%) was higher than in those with one (82%) or none (82%) and this difference is statistically significant ($P<0.01$) (Fraser et al., 1980). Branchial anomalies are reported to occur in 84% of persons affected with BOR (Cremers & Fikkers-van Noord, 1980).

Branchial sinuses, fistulas and cysts originate from an incomplete obliteration of the second pharyngeal cleft (Hunter, 1974) so that vestiges of the cleft epithelium form a tract, which may have an orifice in the skin, the tonsillar fossa, in both of the sites, or no orifice at all. In this way an external or internal sinus, a fistula or a cyst is formed (Gimsing & Dyrmose, 1986).

EXTERNAL EAR

Defects of the external ear occur 62% of the time in persons affected with BOR (Cremers & Fikkers-van Noord, 1980). These may range from severe microtia to minor anomalies of the pinna, variously described as cup, flap, lop, flattened or hypoplastic (Fraser et al., 1978). The external canal may be narrow, malformed or slanted upwards, making otoscopic examination difficult (Cremers & Fikkers- van Noord, 1980). The ear malformations in the syndrome are variable among and within families and may even be different between right and left sides of the same person.

Malformations of the external ear result from disturbances of growth and differentiation of the first and second branchial arches which can result in varying degrees of microtia and malposition of the pinna (Melnick et al., 1976). The most

critical period of development for the external and middle ear is between the 5th and 8th week of embryonic life (Melnick et al., 1980).

RENAL ANOMALIES

The other prominent symptom of branchio-oto-renal syndrome are renal anomalies which may range from mild hypoplasia to complete agenesis (Cremers & Fikkers-van Noord, 1980). Renal function is assessed by intravenous pyelography (IVP). Upon examination by this method it is estimated that 75% of the individuals with BOR have some sort of renal anomaly (Cremers & Fikkers-van Noord, 1980). However, earlier reports (Fraser et al., 1978) put this figure at 12%, this may be because renal anomalies are so subtle they can be missed by routine screening unless specifically searched for (Heimler & Lieber, 1986). In any case, the renal anomalies as a rule remain asymptomatic being medically significant in only 10-25% of cases (Cremers & Fikkers-van Noord, 1980). Urinary tract infections (UTIs) occur in individuals with BOR at an incidence higher than that in the general population and a history of recurrent UTIs might represent expression of the BOR gene. Perhaps these recurrent UTIs represent expression of the BOR gene on the basis of a renal anomaly undetectable by currently available diagnostic methods (Heimler & Lieber, 1986).

As with the external and middle ear the most critical period for renal development is between the 5th and 8th weeks. The metanephros (permanent kidney) begins to develop early in the 5th week from two embryonic structures; the ureteric bud and the metanephrogenic mesodermal mass. The ureteric bud arises from the mesonephric duct near its entry to the cloaca and gives rise to the ureter, renal pelvis, major and minor calyces and collecting tubules. Renal anomalies of the

BOR syndrome can be explained as variable failures of ureteric bud differentiation and can be thought of as a progressive continuum. Renal agenesis can be explained by the absence of ureteric bud. Renal aplasia results from failure of the ureteric bud to grow. Hypoplastic kidneys are the consequence of generalized hypoplasia of the ureteric bud. Bifid ureters or pelvis is the outcome of precocious division of the ureteric bud. Dysplasias of calyces and/or collecting tubules is the aftermath of incomplete or failed end-stage division of the ureteric bud. In summary, the common denominator for all of these anomalies rests with the variable ability of the ureteric bud to grow and divide into its required component parts.

OTHER FINDINGS

Other less frequently described symptoms of Branchio-oto-renal syndrome include aplasia or stenosis of the lacrimal duct (5%), facial paralysis (4%) and retrognathia (16%) (Cremers & Fikkers-van Noord, 1980). Hemifacial microsomia (HFM) has also been described associated with BOR (Rollnick & Kayo, 1985, Heimler & Lieber, 1986) and suggests that the HFM phenotype may constitute a severe form of BOR in some families.

RELATED SYNDROMES

In addition to branchio-oto-renal syndrome several other syndromes are recognized in which dysplastic kidneys are associated with ear malformations. These are the Muckle-Wells syndrome, Townes-Brock syndrome and Epstein syndrome which are autosomal dominant and Oto-renal-genital syndrome and Renal tubular acidosis with deafness, both of which are known to be recessive.

SUMMARY

The available knowledge of BOR may be summarized in terms of information useful in genetic counseling of family members with one or more of the primary features of branchio-oto-renal syndrome. First, a person who has preauricular pits only and no affected relatives is likely to have a nongenetic defect or occasionally may represent a fresh mutation of the normal allele of the gene for auricular pits. The chance that this person carries a gene for the BOR syndrome is very small. Second, a person with the preauricular pits-branchial cleft-hearing loss (P-F-D) triad is very likely to be a carrier of the mutant gene for this syndrome (See Table II). Each of the children then runs a 50% risk of inheriting this disorder. Third, in families in which the P-F-D triad is segregating, those offspring of affected persons who have pits, fistulas or both, run a high risk of hearing loss (80-100%) and of renal anomalies (50%). The risk of having a renal anomaly serious enough to be medically significant is in the range of 10-25%. Fourth, offspring of a carrier who shows no pits, fistulas or other features except hearing loss at birth, run only a small risk (<5%) that they are carriers and that the hearing loss will be expressed (Cremers & Fikkers-van Noord, 1980).

Table II. Frequency of Association of Preauricular Pits (P), Branchial Fistulas (F) and Hearing Loss (D).*

Association of traits			Frequency Range
P	F	D	37-73%
P	F		5-12%
P		D	15-18%
	F	D	5-11%
P			7-12%
	F		1-2%
		D	5-7%

* Adapted from Cremers & Fikkers-van Noord (1980).

BOR SYNDROME IS A SINGLE GENE DISORDER

Branchio-oto-renal syndrome is a genetic disease which shows considerable variation in expression. Due to the variable expression of the syndrome, it might be hypothesized that it is the result of the combined effect of two or more genes, each being responsible for the lesions in one or more organs (Gimsing & Dyrmose, 1986). From the literature it can be concluded that the conductive and sensorineural hearing losses are inherited together, suggesting that the malformations of the middle and inner ear and of the branchial derivatives are produced by the same gene. Thus, if more than one gene were involved, the second gene would be expected to cause the renal anomalies. However, the existence of a BO syndrome without renal involvement as proposed by Melnick et al. (1980) has been discounted. Another possibility is that the differentiation of the organs involved is under the control of the same inducing or organizing mechanism so that a single gene disturbing this mechanism would have a multi-organ effect (Gimsing & Dyrmose, 1986).

It is this author's belief that the single gene explanation is the most reasonable. The specific gene product would necessarily be an element common to the morphogenesis of the branchial, auditory and renal systems. Among other possibilities, this gene product might either be the cell-surface glycoprotein or a proteolytic or biosynthetic enzyme involved in the post-transcriptional modification of the protein (Melnick et al., 1978). Additional insight into these possibilities could be obtained by employing an animal model system for demonstrating the extent of a immunological similarity, if any, between cell-surface recognition antigens on those cells responsible for branchial, auditory and renal pattern formations (Melnick et al., 1978).

Using immunochemical and immunohistochemical techniques in the rat and guinea pig Quick et al., (1973) were able to demonstrate evidence of a shared antigen between the kidney and the cochlea. This relationship appears to be particularly strong between the stria vascularis and the renal glomeruli (Arnold et al., 1976).

Our hypothesis is that there is a single defective gene responsible for BOR. Therefore, the ultimate goal of this research is to locate the gene responsible for the defect using a method called linkage analysis. For linkage analysis to be successful several requirements must be met. First, an accurate diagnosis of the disease must be made. Second, the family histories of individuals must be recorded and pedigrees developed. Third, testing of individuals must be performed; in the case of BOR syndrome it is advised that a complete auditory battery of tests be performed as well as tests of renal function. Also, blood samples on all informative individuals must be obtained. Fourth, laboratory tests on the DNA must be performed. Finally, the data must be analyzed and reported.

LINKAGE ANALYSIS

How does one go about locating a particular disease gene in the human genome which consists of approximately 100,000 genes on 23 chromosomes? The linkage analysis strategy exploits the way genes are inherited. An ordinary human cell contains 23 pairs of homologous chromosomes, one of the pair is inherited from the mother and one from the father. In meiosis, the series of cell divisions that gives rise to egg or sperm cells, the homologous chromosomes are duplicated and distributed among four egg or sperm cells each of which receive 23 single, unpaired chromosomes. However, in the course of meiosis crossing-over takes place, that is, the exchange of DNA segments of equal length between homologous chromosomes (White & Lalouel, 1988). This phenomenon is known as recombination. The closer two loci are together on a chromosome the less often they will be separated during recombination and are thus said to be linked. Conversely, loci which are on different chromosomes are inherited randomly with regard to one another and are thus said to be unlinked. These two loci can be imagined as one being the particular disease gene which we are attempting to locate and the other belonging to a set of genes whose location on a particular chromosome is known. The ultimate goal here is to identify markers which are linked to the disease gene thus localizing the gene to a specific region on a chromosome. Correlating the inheritance of a marker and a disease gene requires two things. The marker must be readily detectable and it must be found in a number of distinguishable variants throughout the population (polymorphic) (White & Lalouel, 1988).

Before 1978, only a limited set of markers met both of these criteria. The genes coding for certain enzymes, blood-group antigens and other proteins which demonstrate polymorphisms were the only markers available for studying their

possible linkage to disease genes. This group of 25 to 30 classical marker systems covered only a small region of a few chromosomes.

The advent of recombinant DNA technology has made it theoretically possible to explore any region of the human genome which may be suspect for harboring a disease gene. This linkage strategy gains its power from the high level of normal polymorphisms that can be found in the sequence of base pairs which make up DNA (White & Lalouel, 1988). Cloned DNA segments, derived from either known gene loci (cDNA) or from randomly cloned DNA fragments which detect these variations are used in the strategy of linkage analysis. Each cloned single-copy DNA segment represents a new genetic locus, whose localization to specific regions on human chromosomes is determined by methods such as in-situ hybridization, deletion mapping, somatic cell hybridization and linkage analysis with other mapped DNA sequences.

There are two main types of DNA polymorphisms which are detectable; restriction fragment length polymorphisms (RFLPs) and variable number tandem repeats (VNTRs).

DNA restriction enzymes recognize specific sequences of base pairs in the DNA and catalyze endonucleolytic cleavages, yielding fragments of defined lengths. Restriction fragments can be displayed by electrophoresis on agarose gels, separating the fragments according to their molecular size. Differences among individuals in the lengths of a particular restriction fragment could result from many types of genotypic differences: one or more individual bases could differ, resulting in the loss of a cleavage site or creating a new one; alternatively, insertion or deletion of blocks of DNA within a fragment can alter its size (Bostein et al., 1980). The resulting fragments on the agarose gel are then transferred to a nylon membrane by a

technique known as Southern blotting (Southern, 1975). In Southern blotting the DNA fragments in the gel are first denatured by exposing then to a high pH which separates the double stranded DNA molecule. Next, these slightly separated strands are transferred to a nylon membrane. The nylon membrane is then exposed to a piece of radioactively labeled probe DNA which has a DNA sequence identical to the marker region of interest on the chromosome. The nylon membrane with the probe hybridized to it is exposed to X-ray film for 1-5 days and the result is an autoradiograph. This autoradiograph thus reveals the polymorphic patterns displayed by the DNA markers. Variable number tandem repeats (VNTRs) are detected in the same manner as RFLPs, the only difference between the two is that the span between cutting sites of the enzyme differs because of short, repeated DNA sequences which lie between them.

MATERIALS AND METHODS

FAMILIES

Four multi-generation families with branchio-oto-renal syndrome were included in this study (Figures A1-A4). Families #352 and #365 were supplied by Dr. C.W.R.J. Cremers of the University of Nijmegen, The Netherlands. Family #105 came to our attention when the proband registered with Boys Town National Research Hospital's Deaf Register which is a national clearing house for families with hereditary deafness who are interested in participating in research projects. The fourth family, family #2, was obtained from the proband being seen at Boys Town National Research Hospital (BTNRH). There were a total of 87 blood samples drawn from informative individuals in these families. There were 16 affected individuals in family #352, 8 in family #365, 7 in #105 and 11 in family #2. An in-depth clinical study was made of families #365 and #352 by Cremers and Fikkers-van Noord (1980) in which 19 members of these families underwent extensive testing. Members of families #105 and #2 were seen at BTNRH to assess their clinical characteristics.

Clinical studies performed on BOR patients fall into two areas; those which test audiological function and those that are used to measure renal function. Audiologic evaluation is performed by using a battery of tests which can determine crucial variables such as the degree of loss, location of the lesion and determine rehabilitative needs. Pure-tone audiometry measures are taken and the results are plotted on an audiogram (Figure 1). Next, a speech recognition test is given that measures the ability to hear and understand speech. Finally, an impedence test is

done to indicate the integrity of the tympanic membrane (tympanogram) and the acoustic reflex threshold which tests the ossicular chain. This battery of test was performed on affected members of all four families. Diagnosing renal function is typically done via intravenous pyelography (IVP) or by renal ultrasound. IVP's are performed by injecting a dye which quickly passes in to the urine, the quantity and quality recovered assess kidney efficiency. A preferred method is renal ultrasound which yields a visual account of the kidneys and is more useful in detecting subtle renal malformations. Families #352 and #365 were analyzed using IVP's, while renal ultrasound was used for families #105 and #2.

MOLECULAR METHODS

Two chromosomes were tested as candidates for the location of the gene for BOR. Chromosome 1 was explored first, mainly because our laboratory had a large number of DNA markers on this chromosome. Figure 2 shows a map of chromosome 1 with the location of the various DNA markers tested in addition to classical markers. The dotted lines indicate those markers whose exact physical location is not known, whereas the solid bars show the region of the chromosome a particular marker covers. Chromosome 8 was also considered as a possible location for the BOR gene (Figure 3). First, because of a high resolution cytogenetic study which tentatively mapped the BOR gene to either 8q13.3 or 8q21.13 (Haan et al., 1989). The second reason this was a candidate chromosome is because a mutation in the carbonic anhydrase gene located at 8q22 is known to cause renal tubular acidosis with deafness.

For chromosome 1, eight DNA markers were utilized to assess their possible linkage to the BOR gene. These included the probes pJA110, pTH154, pL1.22, pN8C6, pEB8, pEKH7.4, pDR78, and pHRN1 (See Table III).

FIGURE 2: Map of Chromosome 1

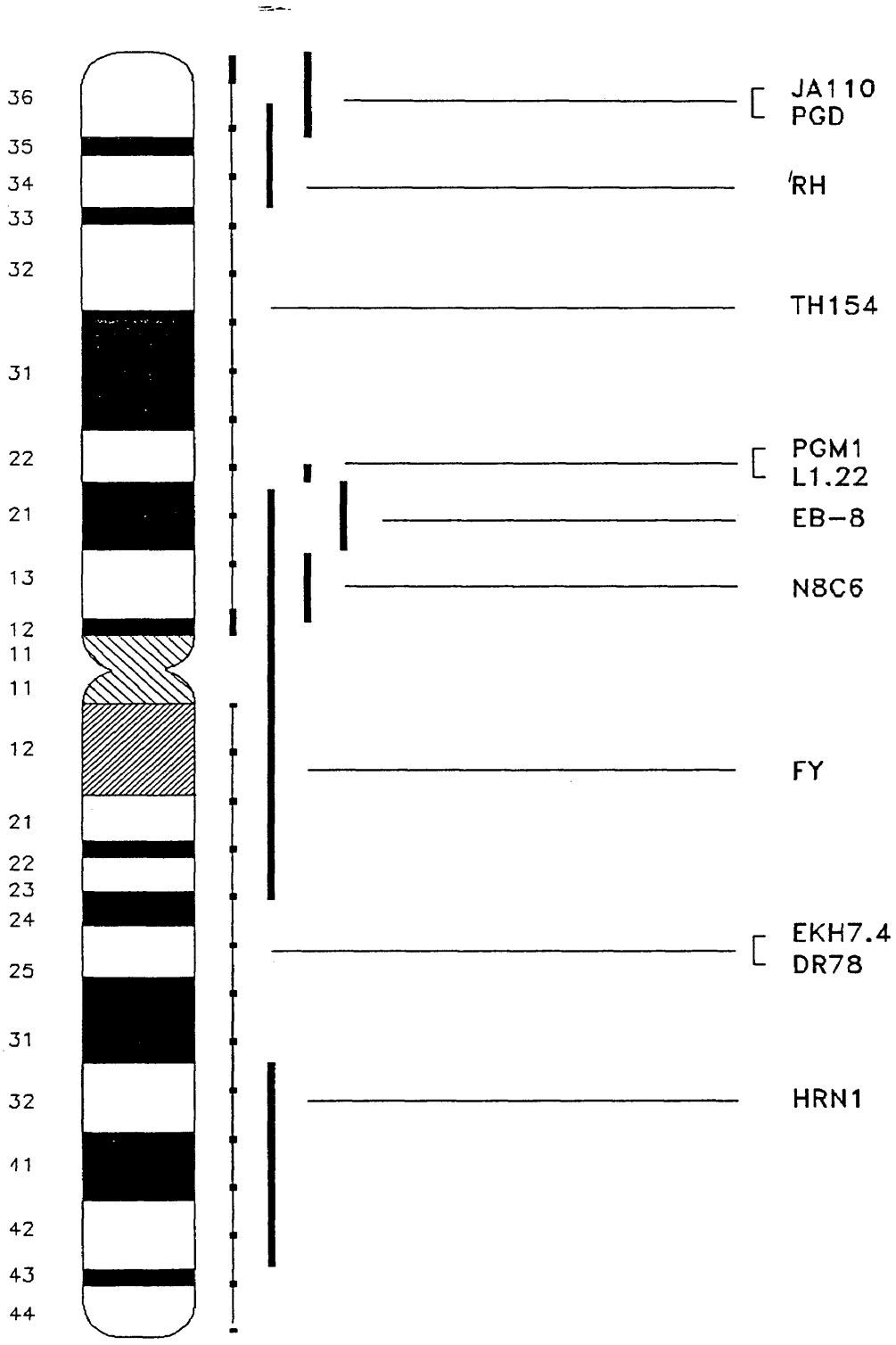

FIGURE 3: Map of Chromosome 8

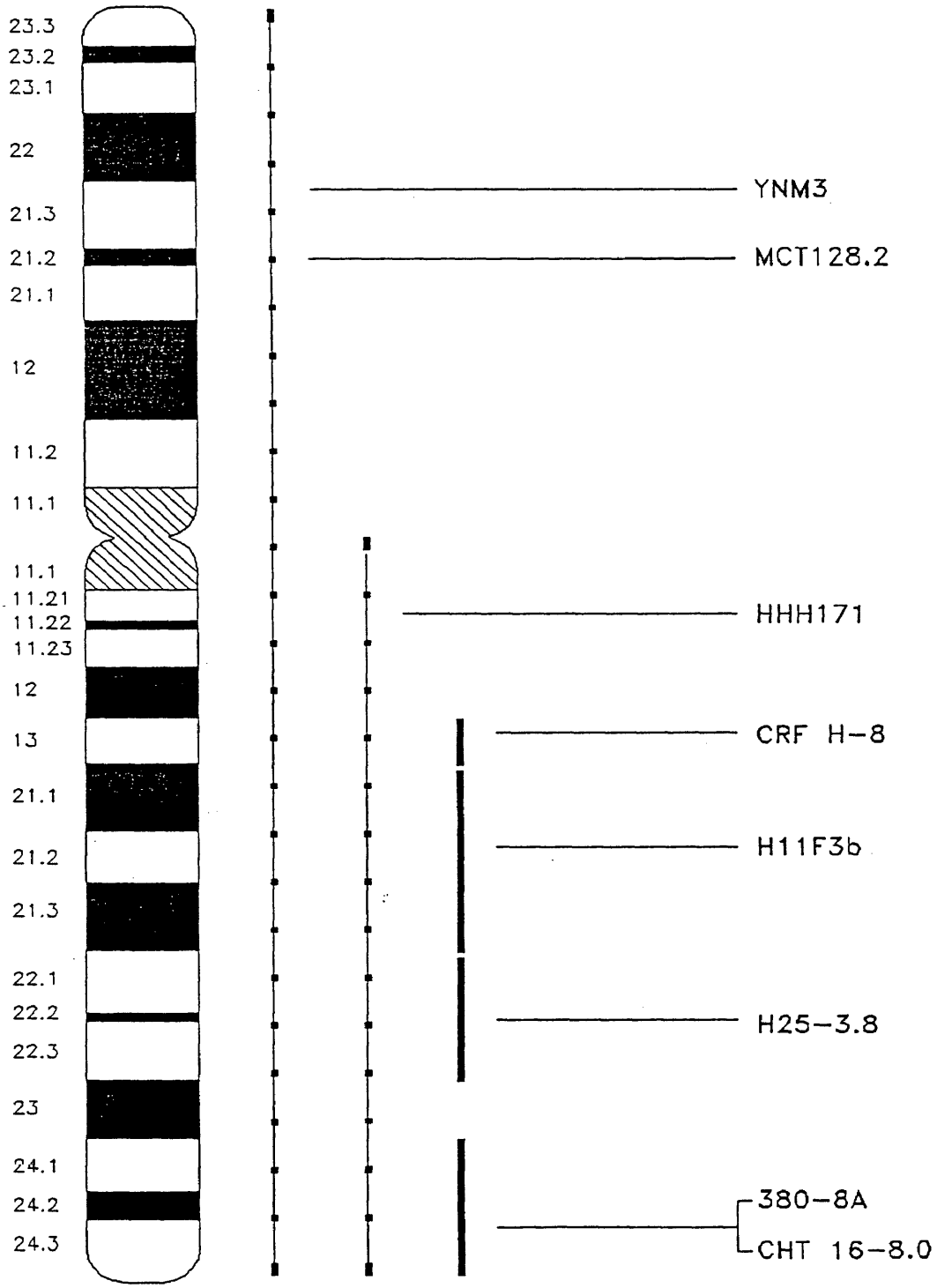

Table III. Chromosome 1 DNA Marker Summary Data.

Probe Name	Locus Symbol	Chromosome Region	Restriction Enzyme	Allele Size (kb)	Freq.
JA110	PND	1p36	BglI	10.0	0.87
				6.0	0.13
THI54	D1S62	1p	PvuII	6.0	0.51
				5.0	0.49
L1.22	D1S2	1pter-p31	BglII	10.0	0.81
				7.0	0.19
N8C6	NGFB	1p13	BglII	6.0	0.22
				4.1,1.9	0.78
EB-8	AMY	1p21	PstI	12.0	0.82
				8.0	0.18
EKH7.4	D1S65	1q	TaqI	5.0	0.47
				3.8	0.53
DR78	D1S4	1p21-qter	BglII	12.3	0.89
				5.9	0.11
HRN1	REN	1q32-1q42	BglII	20.0	0.72
				24.0	0.28

All of the above probes were obtained from American Type Culture Collection (ATCC) with the exception of pDR78 which was obtained from A. J. Driesel. All of these probes were inserted into bacterial plasmids and were purified using the large scale extraction procedure found in a protocol published by Promega, Inc. (Technical Bulletin #009).

In addition to DNA probes for chromosome 1, classical markers were also used (See Table IV).

TABLE IV. Chromosome 1 Classical Marker Summary Data.

Antigen/ Enzyme Name	Symbol	Chromosome Region	Allele Symbol	Freq.
Rhesus Blood Groups	RH	1p36.2-p34	R_0	0.02
			R_1	0.44
			R_2	0.14
			r	0.39
Phosphogulcomutase	PGM1	1p22.1	1+	0.65
			1-	0.12
			2+	0.18
			2-	0.05
Phosphoguconate Dehydrogenase	PGD	1p36.2-p36.13	A	0.98
			C	0.02
Duffy Blood Groups	FY	1p21-q23	a	0.43
			b	0.57

For chromosome 8, eight DNA markers were also used; these being pYNM3. pMCT128.2, pHHH171, pCRFH-8, pH11F3b, p380-8A, and pCHT16/8 (See Table V).

TABLE V. Chromosome 8 DNA Marker Summary Data.

Probe Name	Locus Symbol	Chromosome Region	Restrict. Enzyme	Allele Size (kb)	Freq.
YNM3	D8S17	8	PstI	(5) 3.6-3.2	VNTR
MCT128.2	D8S39	8	PstI	(3) 1.6-1.0	VNTR
HHH171	D8S19	8q	MspI	2.3	.74
				1.9	.26
CRF H-8	CRH	8q13	TaqI	17	.94
				8.2	.06
H11F3b	CYP11B1	8q21-22	MspI	1.7	.40
				1.2	.60
				1.4	.00
				0.9	.80
				0.6	.20
H25-3.8	CA2	8q22	TaqI	5.4	.50
				4.0, 1.4	.50
380-8A	MYC	8q24	TaqI	3.3	.47
				2.5	.44
				2.3	.09
CHT 16/8.0	TG	8q24	TaqI	5.8	.20
				5.2	.80

All of the above probes were obtained from ATCC except for CRFH-8 donated by Dr. M. Litt of the Oregon Health Sciences University; H11F3b contributed by Dr. Perrin White of New York Hospital, Cornell Medical Center; and 380-8A given by Dr. C. M. Croce of the Wistar Institute. In the case of these probes, they were large scale extracted using a polyethylene glycol precipitation procedure (Maniatis et al., 1980).

DNA TYPING

Total genomic DNA was isolated from blood samples by white blood cell separation using Lymphoprep as a density-specific reagent. Ten micrograms of DNA was digested with the proper restriction enzyme overnight. The resulting fragments from this digestion were separated on 0.6% to 1.0% agarose gels at 1.5 to 2.0 volts per centimeter. The gels were acid nicked, denatured, neutralized and transferred to Gene Screen Plus via the Southern blot method. Membranes were air-dried and stored until hybridization.

Plasmid DNA containing the probe of interest was nick-translated with dCTP$^{\alpha 32}$P to specific activities of 1-5 x 10^8 cpm/μg using New England Nuclear's NEK 004 Nick Translation Kit. Unincorporated radioactively labelled dCTP$^{\alpha 32}$P was removed using G50 Sephadex columns equilibrated in STE buffer. Gene Screen Plus nylon membranes were prehybridized with 50% formamide, 5xSSC, 1% SDS, 50mM phosphate buffer pH 6.5, 5x Denhardt's solution, and 250 μg/ml denatured salmon sperm in a 42°C shaking water bath for 16 to 24 hours. After removing the prehybridizing solution, the membranes were hybridized with the denatured probe using 1 to 2 x 10^6cpm/ml and a hybridization fluid consisting of 50% formamide, 5xSSC, 1.0% SDS, 20 mM phosphate buffer pH 6.5, 10% dextran sulfate and 100 μg/ml denatured salmon sperm and incubated as above. Following hybridization, the membranes were washed with 2xSSC and 0.1% SDS twice for 15 minutes and twice with 0.2xSSC and 0.1% SDS for 30 minutes in a shaking 65°C water bath. These post hybridization washes were performed to remove excess radioactivity. The membranes were then exposed to Kodak XAR film with DuPont Lightning Plus Intensifying Screens in cassettes for 1 to 5 days. The film was manually developed

using standard Kodak procedures and reagents. The resulting autoradiographs were scored for each individual, the autoradiographs labeled, and the data entered into a computer data bank.

TWO-POINT LINKAGE ANALYSIS

The goal of linkage analysis is to find linkage between a given marker and a disease gene, remember that the closer the two are located on a chromosome the greater the chance they will not be separated during recombination events and inherited together as a unit. The polymorphic information from each family member from the autoradiographs is entered into a computer data base along with data about the relationships between individuals in the pedigree, affected status and information about each marker. There is other information also taken into account; the male/female ratio, in the case of BOR we assumed that there was no sex difference; the mode of inheritance (autosomal dominant); penetrance which we assumed was full. A computer program known as MLINK from the LINKAGE program package (Lathrop et al., 1985) takes all of the above factors into account and translates the data for each marker and family into a lod score. A lod score is defined as the odds of linkage versus non-linkage for a particular marker and the BOR gene. Lod scores at different recombination fractions (θ) such as 0.00, 0.05, 0.10, 0.20, 0.30 and 0.40 are generated. These recombination fractions actually represent physical distances on the chromosome; for example a recombination fraction of 0.05 corresponds to 5cM (cM=centiMorgans) which is equal to 5 million base pairs. At lower recombination fractions the marker and the BOR gene are closer together, whereas at a higher fraction like 0.50 which corresponds 50cM or 50 million base pairs the two are so physically far apart that they recombine at rates equal to the recombination

observed between loci located on different chromosomes. A lod score of +3 indicates linkage, whereas a lod score of -2 indicates that the two are unlinked. Only a two-point analysis was performed on the data set versus a multipoint analysis because this type of analysis did not give any information that was useful.

RESULTS

DNA TYPING RESULTS

Figures 4-11 illustrate sample autoradiographs for each DNA marker which was used for chromosome 8. Sample blots are not shown for chromosome 1 markers because of space and budgetary constraints, however the figures of chromosome 8 are representative of these.

LINKAGE ANALYSIS RESULTS

Two-point linkage analysis results for chromosome 1 are reported in Table VI. All markers were tested against all four families. Asterisks in the table indicate these families were not informative for this particular marker. Lod scores are given at recombination fractions (θ) of 0.00, 0.05, 0.10, 0.20, 0.30 and 0.40. Total lod scores are summed across families for each marker. For chromosome 1 notably negative lod scores (-2 or greater) were found for the markers JA110, TH154, L1.22, EB8, DR78 and PGM1. A positive lod score of 1.36 was found for RH at the 0.20 recombination fraction. The other markers (N8C6, FY, EKH7.4 and HRN1) did not exhibit lod scores that were of any informative value.

Two-point linkage analysis results for chromosome 8 are given in Table VII. Recombination fractions are the same as those reported above. Markers which yielded significant negative lod scores for chromosome 8 were YNM3, 380-8A and CHT16/8. None of the other markers gave significantly negative or positive lod scores. All of the markers, with the exception of MCT128.2 and 380-8A, were only

tested against family #2. This was done in order to conserve the DNA of the other studies families. The marker CRFH-8 yielded no data as all family members were heterozygous.

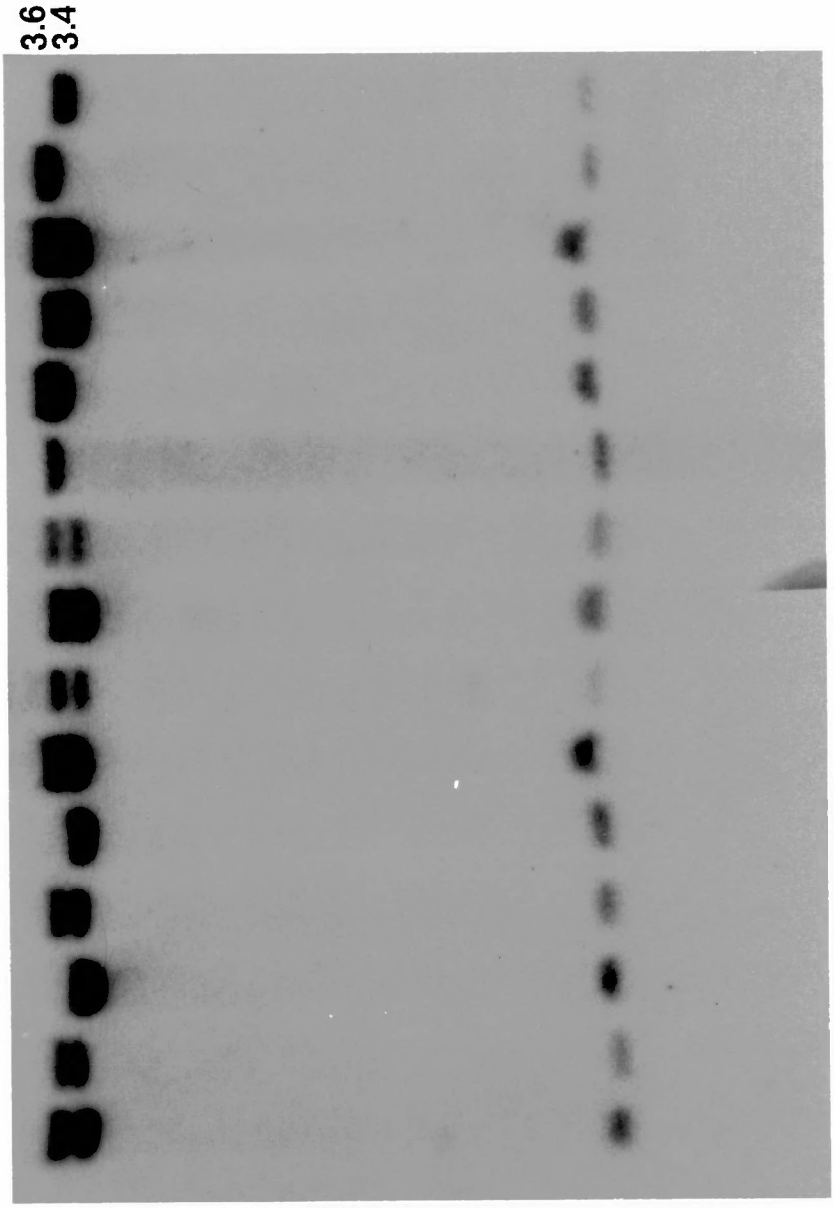

FIGURE 4. Autoradiograph of the pYMN3 PstI polymorphism. Band sizes are given in kilobase pairs(kb).

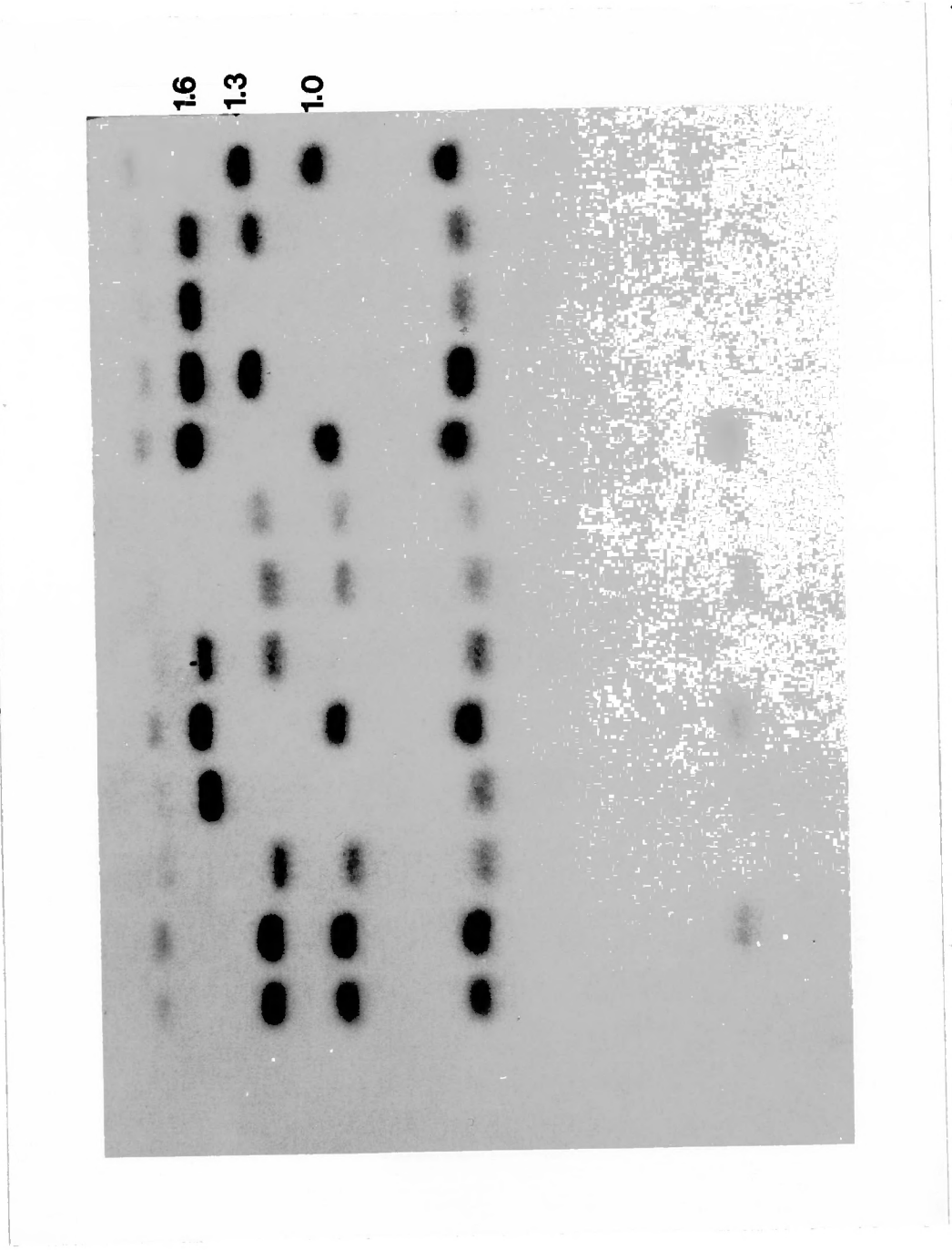

FIGURE 5. Autoradiograph of the pMCT128.2 PstI polymorphism. Band sizes are given in kilobase pairs(kb).

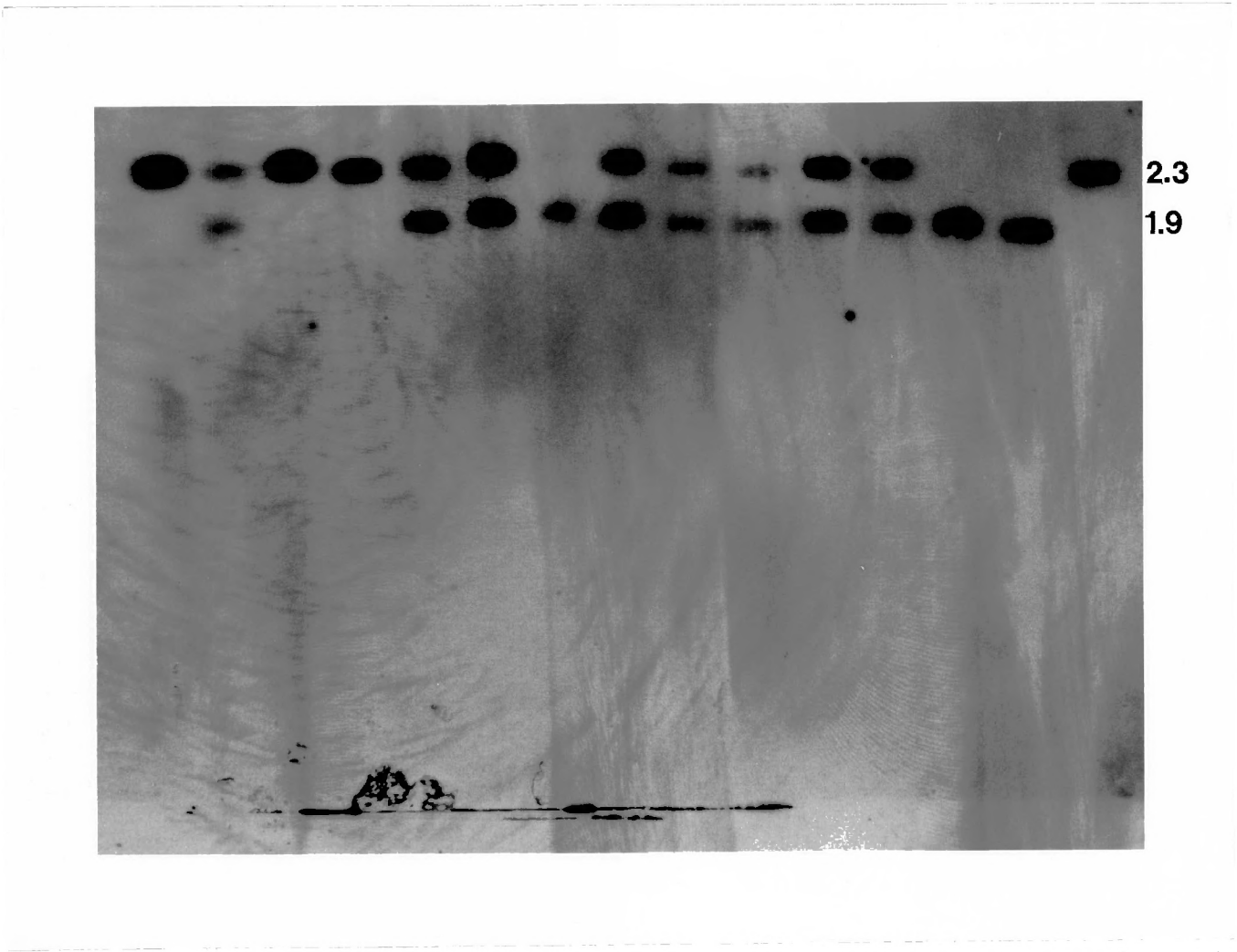

FIGURE 6. Autoradiograph of the pHHH171 MspI polymorphism. Band sizes are given in kilobase pairs(kb).

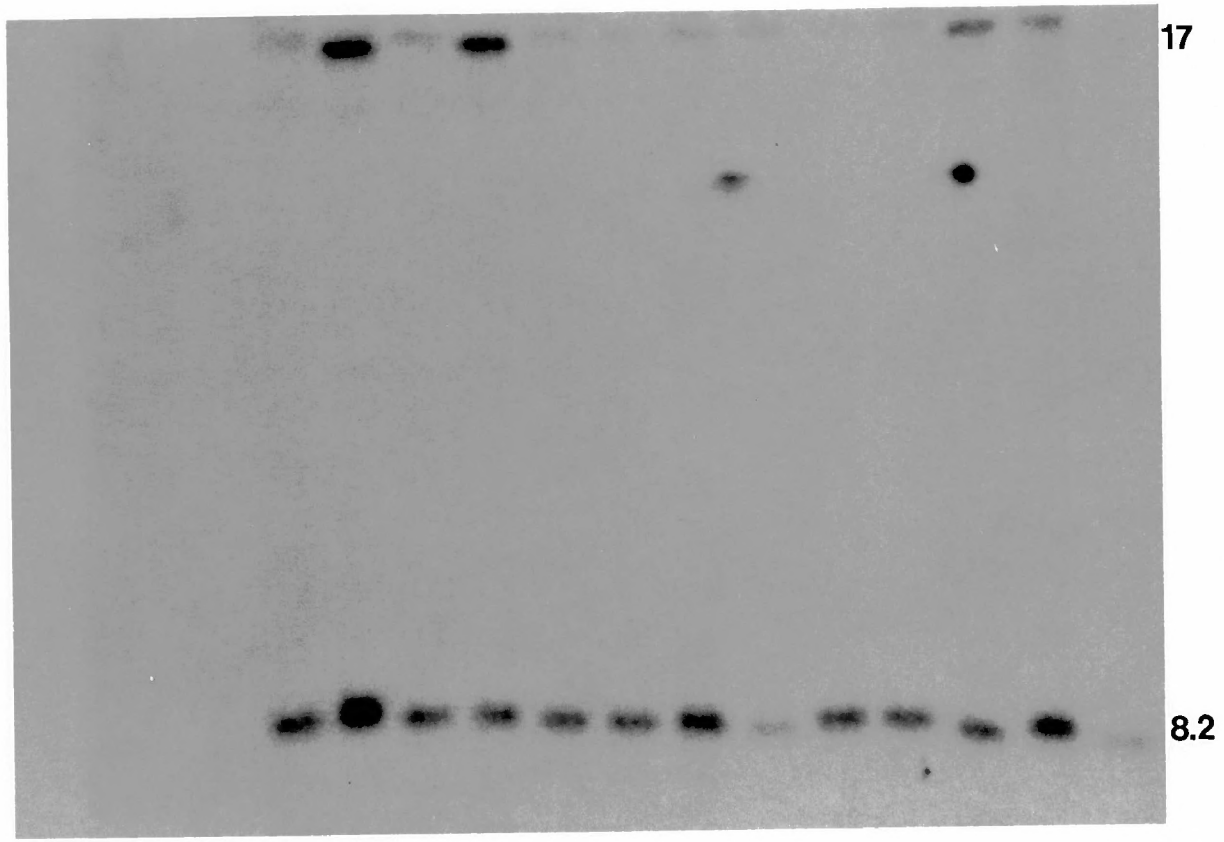

FIGURE 7. Autoradiograph of the pCRF H-8 TaqI polymorphism. Band sizes are given in kilobase pairs(kb).

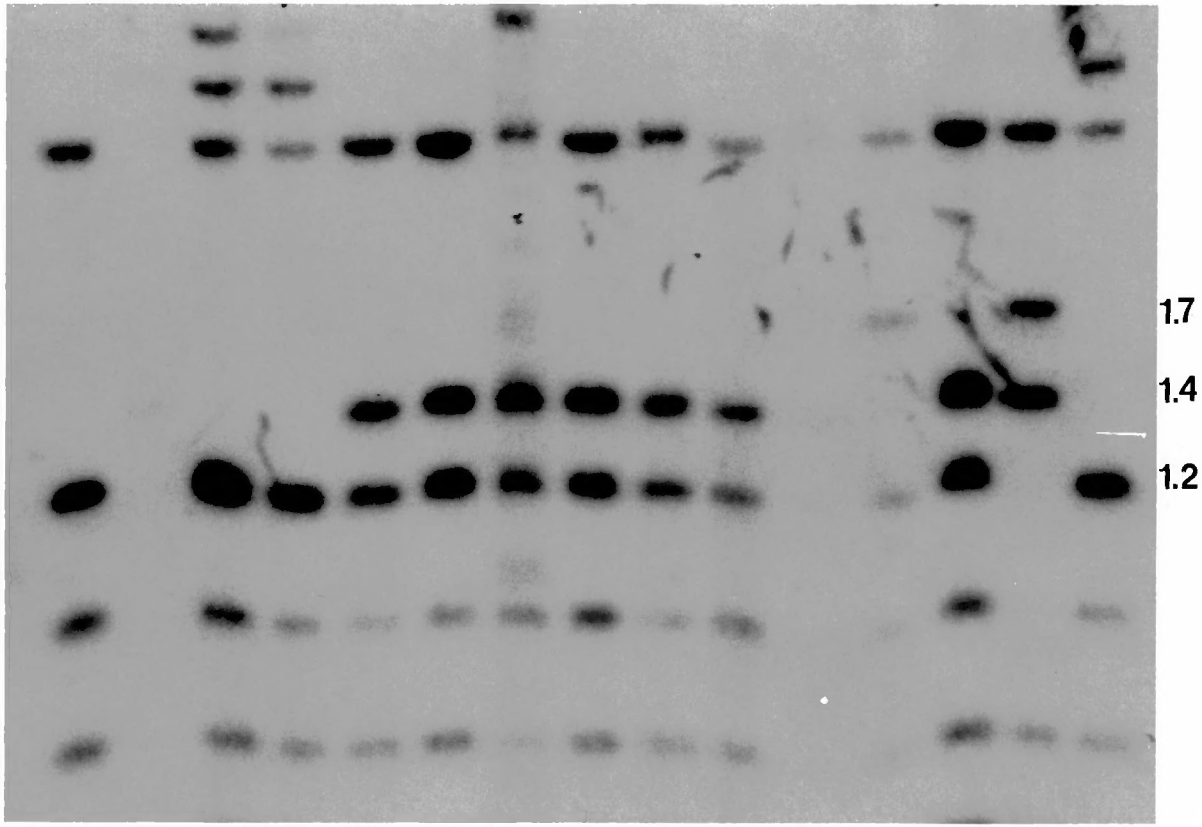

FIGURE 8. Autoradiograph of the pH11F3b MspI polymorphism. Band sizes are given in kilobase pairs(kb).

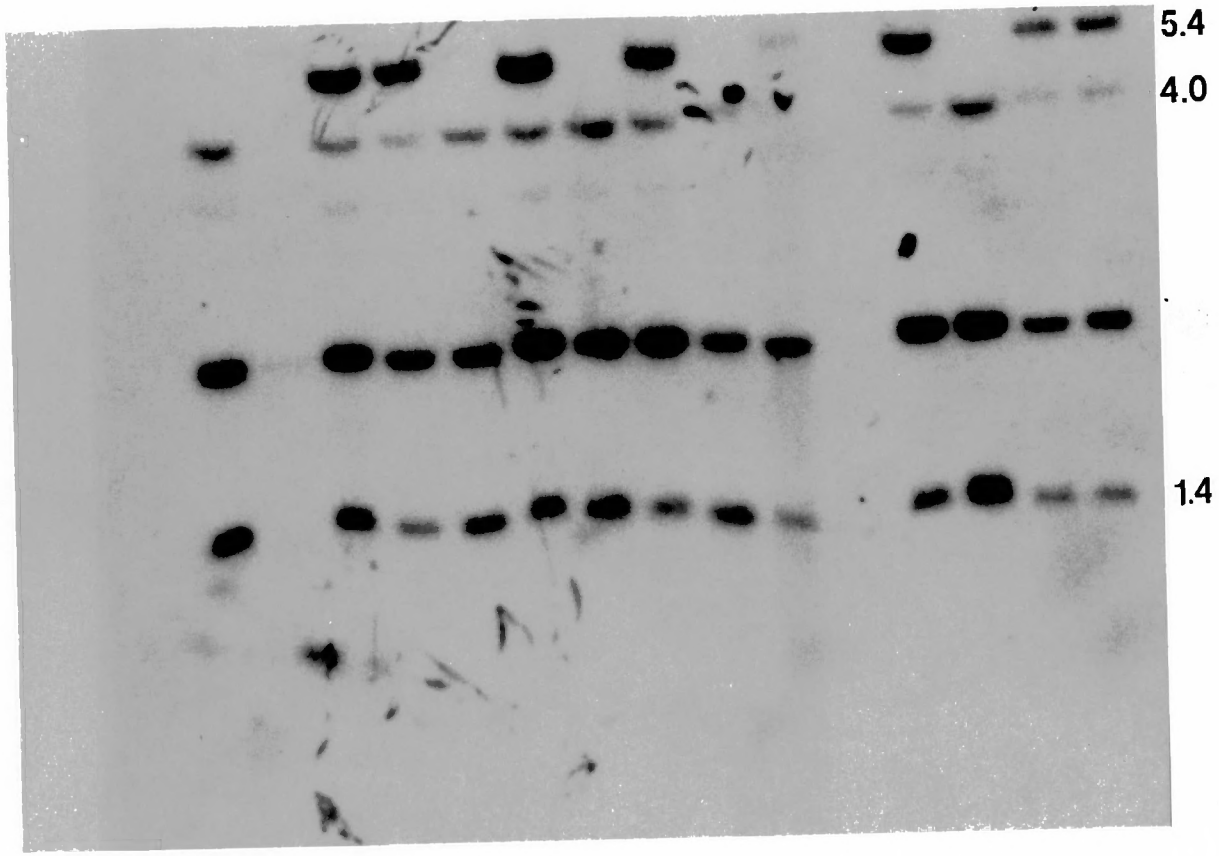

FIGURE 9. Autoradiograph of the pH25-3.8 TaqI polymorphism. Band sizes are given in kilobase pairs(kb).

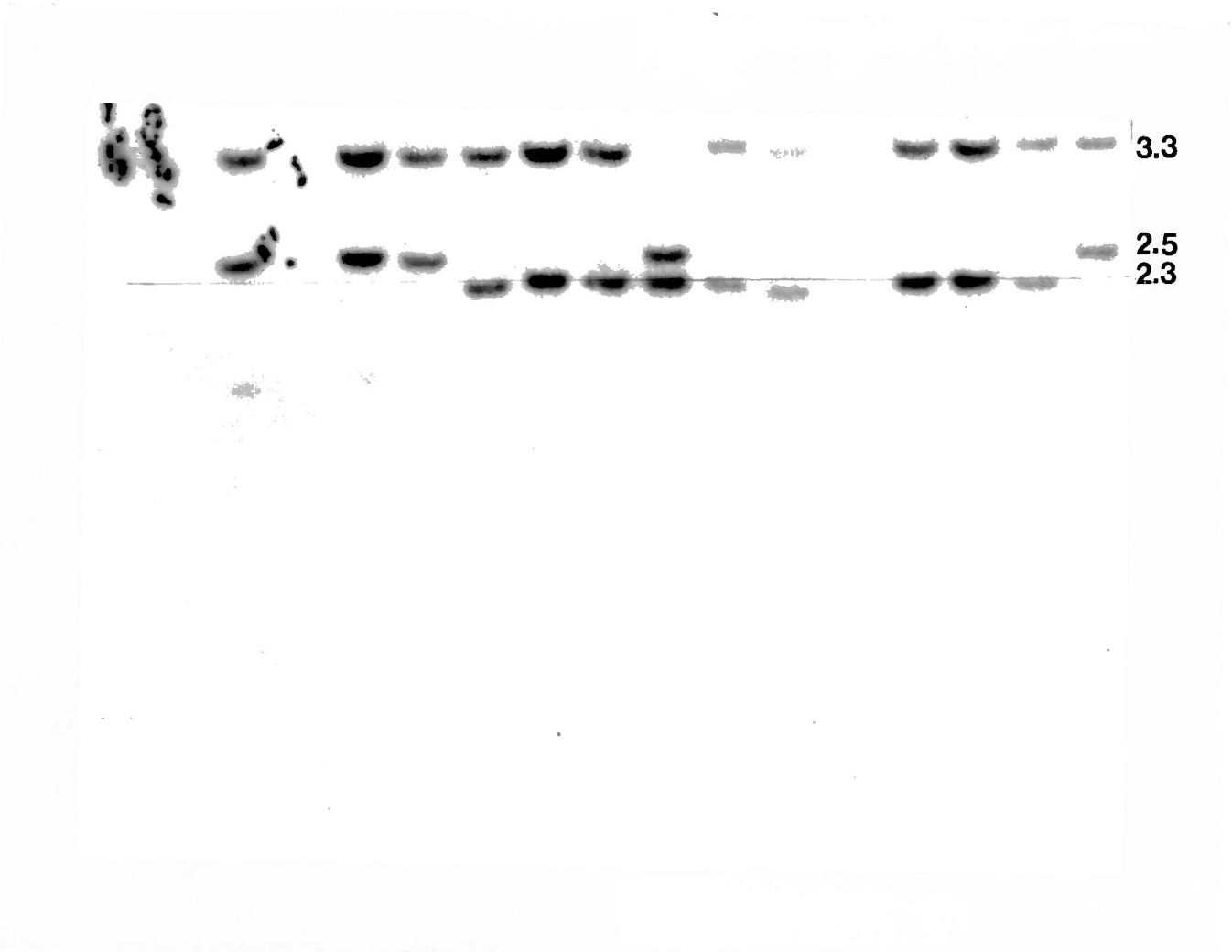

FIGURE 10. Autoradiograph of the p380-8A TaqI polymorphism. Band sizes are given in kilobase pairs(kb).

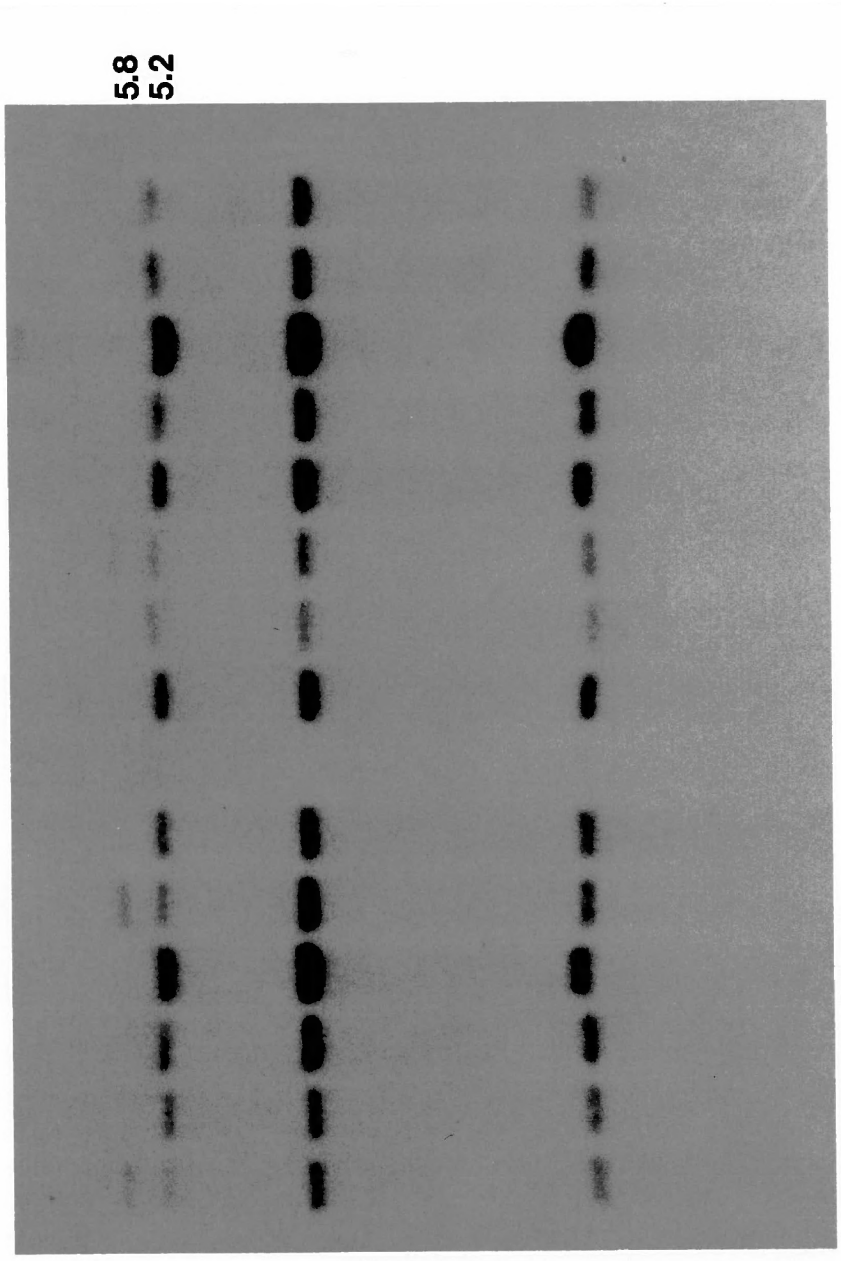

FIGURE 11. Autoradiograph of the pCHT 16/8 TaqI polymorphism. Band sizes are given in kilobase pairs(kb).

TABLE VI. Chromosome 1 lod scores.

		Recombination Factor θ					
Locus	Family	0.00	0.05	0.10	0.20	0.30	0.40
N8C6	2	-∞	-1.77	-1.23	-0.72	-0.42	-0.19
	105	0.05	0.42	0.35	0.20	0.08	0.01
	365	0.00	0.03	0.04	0.04	0.02	0.01
	352	0.18	0.15	0.12	0.07	0.03	0.10
Total		-	**-1.17**	**-0.72**	**-0.41**	**-0.29**	**-0.07**
EB8	2	0.00	0.16	0.16	0.15	0.12	0.07
	105	0.20	0.17	0.14	0.08	0.03	0.01
	365	-∞	-1.44	-0.88	-0.38	-0.15	-0.03
	352	-∞	-2.06	-1.29	-0.64	-0.33	-0.13
Total		-	**-3.17**	**-1.87**	**-0.79**	**-0.33**	**-0.08**
Duffy (FY)	2	0.12	0.12	0.12	0.10	0.08	0.04
	105	*	*	*	*	*	*
	365	*	*	*	*	*	*
	352	-∞	-1.62	-1.06	-0.55	-0.28	-0.11
Total		-	**-1.50**	**-0.94**	**-0.45**	**-0.20**	**-0.07**
EKH7.4	2	*	*	*	*	*	*
	105	*	*	*	*	*	*
	365	-∞	-0.72	-0.44	-0.19	-0.07	-0.01
	352	-∞	-0.87	-0.59	-0.30	-0.13	-0.03
Total		-	**-1.59**	**-1.03**	**-0.49**	**-0.20**	**-0.04**
DR78	2	-∞	-2.10	-1.11	-0.33	-0.05	0.02
	105	-2.22	-0.52	-0.27	-0.08	-0.01	-0.01
	365	-∞	0.00	0.00	0.00	0.00	0.00
	352	-∞	0.00	0.00	0.00	0.00	0.00
Total		-	**-2.62**	**-1.38**	**-0.41**	**-0.06**	**0.01**
JA110	2	-∞	-0.01	-0.01	-0.01	-0.01	-0.01
	105	-∞	0.20	0.17	0.14	0.08	0.03
	365	-∞	-2.72	-1.84	-0.98	-0.51	-0.21
	352	-∞	0.19	0.16	0.13	0.08	0.04
Total		-	**-2.34**	**-1.52**	**-0.72**	**-0.36**	**-0.15**

RH	2	-∞	0.09	0.27	0.33	0.25	0.13
	105	-∞	-1.17	-0.66	-0.25	-0.08	-0.01
	365	0.60	0.55	0.51	0.40	0.29	0.15
	352	-1.02	0.64	0.85	0.87	0.68	0.37
Total		**-**	**0.11**	**0.97**	**1.35**	**1.14**	**0.64**
THI54	2	-∞	-2.16	-1.33	-0.58	-0.22	-0.05
	105	-∞	-0.56	-0.29	-0.08	-0.01	-0.01
	365	-∞	-0.28	-0.04	0.12	0.14	0.10
	352	-∞	-3.29	-2.09	-0.98	-0.43	-0.13
Total		**-∞**	**-6.29**	**-3.75**	**-1.52**	**-0.52**	**-0.09**
L1.22	2	0.35	-0.31	-0.26	-0.15	-0.06	-0.01
	105	0.45	0.43	0.39	0.28	0.15	0.04
	365	-∞	-1.99	-1.39	-0.79	-0.44	-0.19
	352	-∞	-1.69	-1.08	-0.49	-0.20	-0.05
Total		**-∞**	**-3.56**	**-2.34**	**-1.15**	**-0.55**	**-0.21**
PGM1	2	-∞	-7.02	-4.67	-2.42	-1.21	-0.46
	105	-∞	-2.15	-1.32	-0.58	-0.22	-0.05
	365	-∞	-4.16	-2.72	-1.37	-0.67	-0.24
	352	-∞	-2.22	-1.22	-0.39	-0.07	0.22
Total		**-∞**	**-15.55**	**-9.93**	**-4.76**	**-2.17**	**-0.53**
HRN1	2	*	*	*	*	*	*
	105	0.20	0.17	0.14	0.08	0.03	0.01
	365	*	*	*	*	*	*
	352	*	*	*	*	*	*
Total		**0.20**	**0.17**	**0.14**	**0.08**	**0.03**	**0.01**

TABLE VII. Chromosome 8 lod scores.

		Recombination Factor θ					
Locus	**Family**	**0.00**	**0.05**	**0.10**	**0.20**	**0.30**	**0.40**
YNM3	2	-∞	-2.60	-1.51	-0.57	-0.15	0.01
MCT128.2	2	0.00	0.42	0.73	0.75	0.51	0.20
	365	-∞	-0.90	-0.41	-0.04	0.05	0.06
	352	-∞	0.11	0.28	0.26	0.09	-0.03
Total		**-∞**	**-0.37**	**0.60**	**0.97**	**0.65**	**0.23**
HHH171	2	-∞	-0.80	-0.51	-0.23	-0.09	-0.02
CFRH-8	2	*	*	*	*	*	*
H11F3b	2	-∞	-0.05	0.15	0.26	0.23	0.14
H253.8	2	-0.17	-0.15	-0.13	-0.09	-0.06	-0.02
380 8A	2	-∞	-0.77	0.13	0.71	0.73	0.47
	365	-∞	-0.72	-0.44	-0.19	-0.07	-0.01
	352	-∞	-2.11	-1.24	-0.50	-0.18	-0.04
Total		**-∞**	**-3.60**	**-1.55**	**0.02**	**0.48**	**0.42**
CHT16/8	2	-∞	-2.93.	-1.64	-0.59	-0.19	-0.03

DISCUSSION

Chromosome 1 has become valuable in the research of branchio-oto-renal syndrome compared to chromosome 8 in that it serves as an exclusion map for BOR. Six of the 11 total markers run against all four families yielded negative lod scores. The marker JA110 gave a maximum lod score of -2.38 at 0.05 recombination fraction which means that we can exclude the BOR gene on either side of this marker to 2.5cM. For the marker TH154 the BOR gene can be excluded from 5cM on either side. However, it is interesting to note here that the RH marker is sandwiched between these two and has a positive lod score of 1.36 at 0.20 recombination fraction so further exploration around this locus could be of value. The probe L1.22 can be excluded as being linked to the BOR gene to 5cM on either side. The marker EB8 is not closely linked to the BOR gene at 2.5cM on either side. DR78 yielded similar results with BOR being excluded to 2.5cM on each side of this marker also. However, PGM1 gave the most valuable exclusion information for chromosome 1 because the BOR was found to be unlinked to this locus up to 20cM or 20 million base pairs on either side. The rest of the markers for chromosome 1 (N8C6, EKH7.4, HRN1, PGD and FY) did not give any remarkable results. Therefore, a total of 37.5cM was excluded as the location of the BOR gene on chromosome 1 which is 300cM long, in other words 14% of chromosome 1 was ruled out.

Chromosome 8 was much less informative than chromosome 1 since we were able to exclude only a very small region of this chromosome. The probe YNM3 was not linked to the BOR gene to 2.5cM on either side of the marker, however since this probe is only known to be somewhere on chromosome 8 this information is of little

value. The probes 380-8A and CHT16/8 are both located at the distal end of chromosome 8 at 8q24 and both yielded lod scores which exclude the BOR gene 2.5cM on either side of these markers. The rest of the markers for chromosome 8 were unremarkable (MCT128.2, HHH171, H11F3b and H25 3.8). Thus, only 5cM total have been excluded from chromosome 8 which is a total of 160cM long or 3% of the chromosome excluded. However, chromosome 8 is poorly mapped and not many informative markers are available, the availability of more markers would greatly enhance this endeavor.

The initial goal of the research was to localize the gene responsible for branchio-oto-renal syndrome in the hopes that this localization would lead to a means of prenatal diagnosis or ultimately a cure. Prenatal diagnosis could alert the family to the potential special needs of the child. Although the gene for branchio-oto-renal syndrome has not been localized, this study has offered information as to where the gene for BOR is not located. It is recommended that further work be done around the RH locus on chromosome 1 and that the other three families be run against the DNA markers YNM3, HHH171, H11F3b and H253.8 for chromosome 8 to give a more definite assessment as to the exclusion of BOR from these areas.

Using linkage analysis to localize disease genes has become a very powerful tool in molecular biology. Almost everyday some significant discovery is made towards this end. Diseases such as Duchenne Muscular Dystrophy, Huntington's Disease, Polycistic Kidney Disease and Cystic Fibrosis have been localized, which is the first step toward a cure. This author is confident that the gene for BOR will, indeed, be localized some time in the future.

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FIGURE A: LEGEND

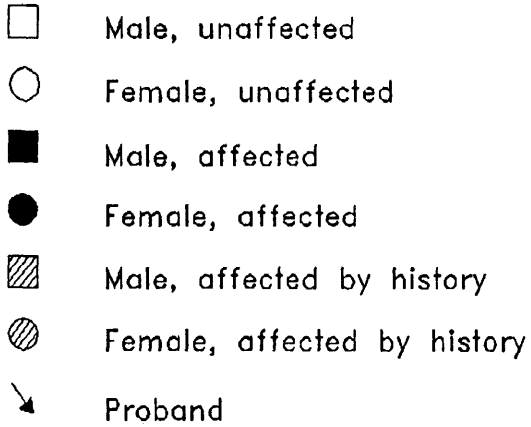


FIGURE A1

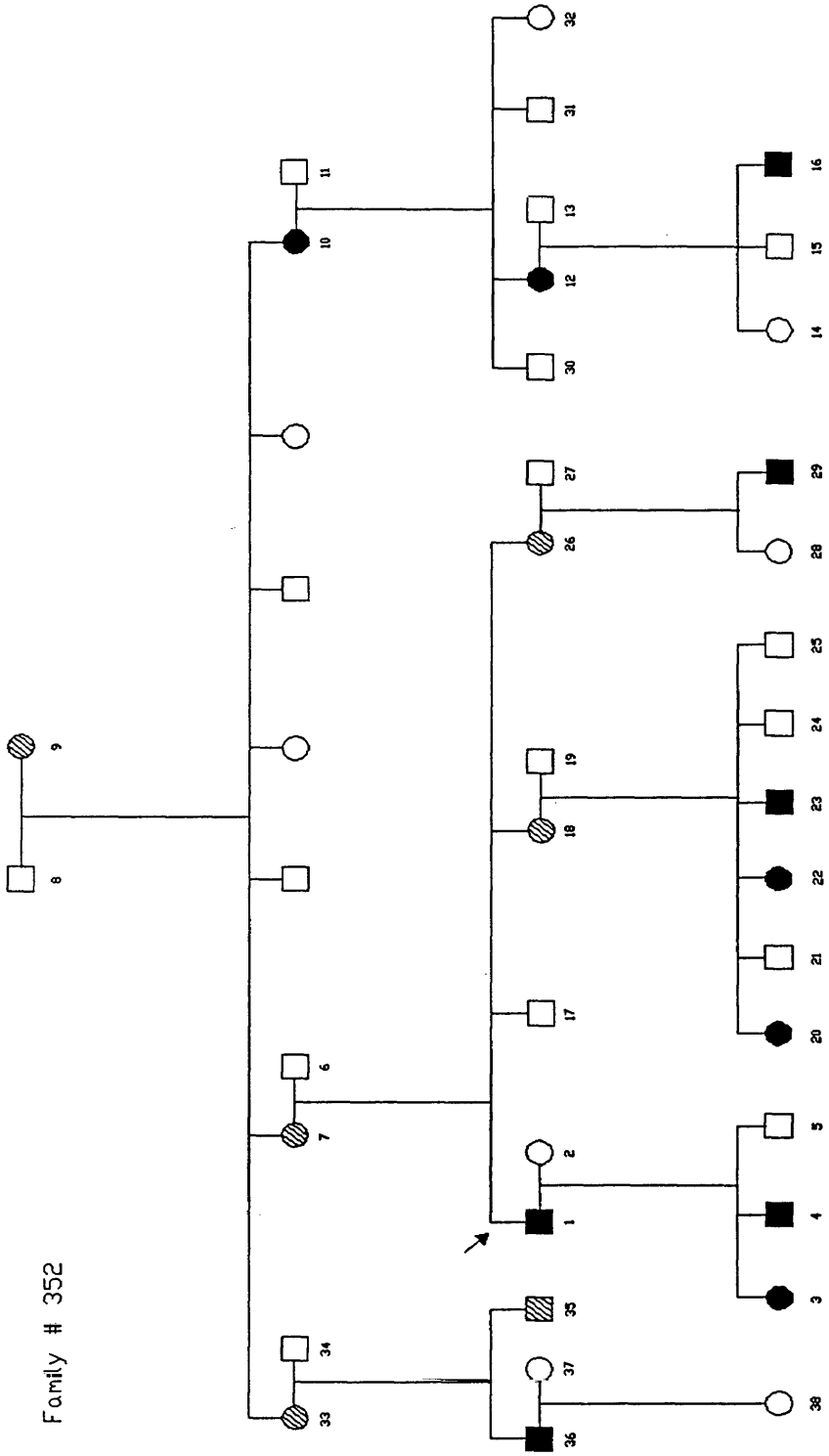
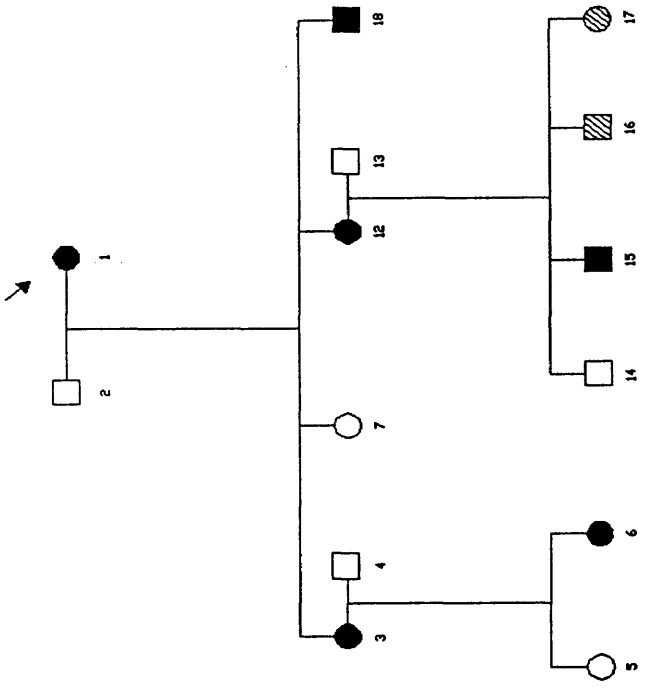


FIGURE A2



Family # 365

FIGURE A3

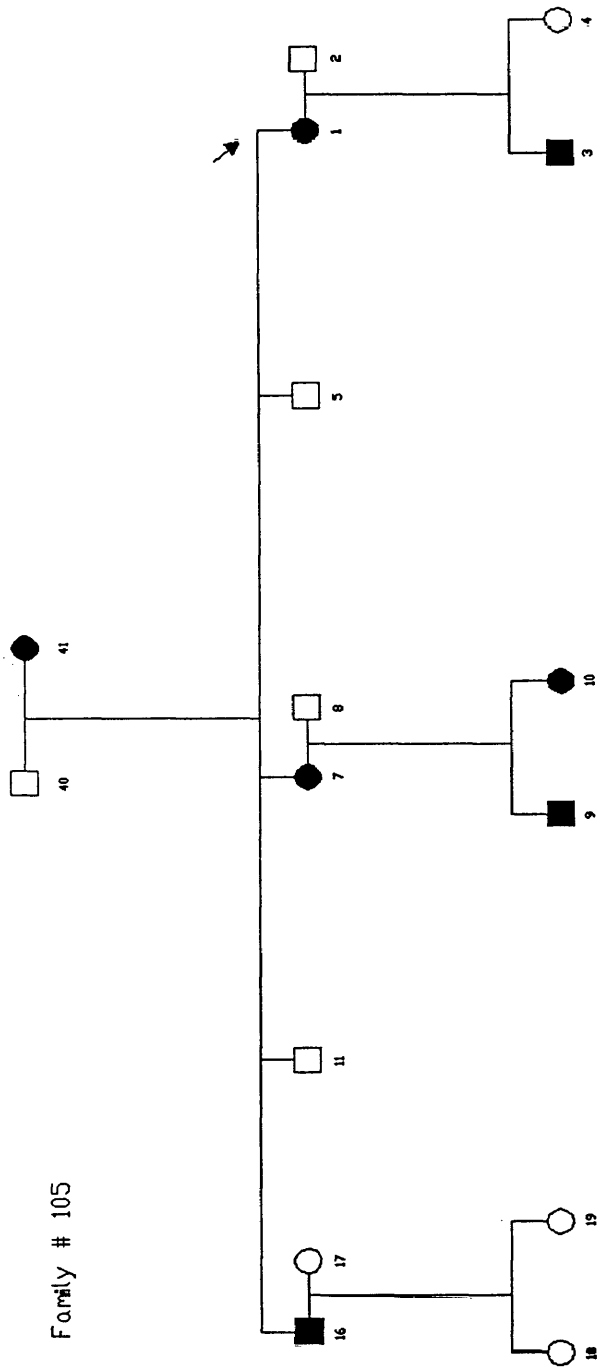


FIGURE A4