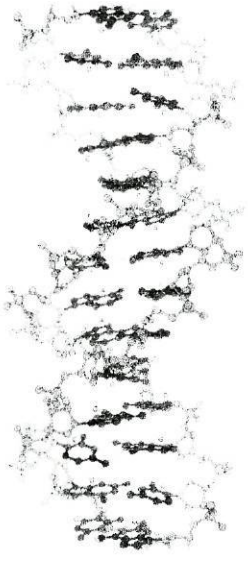
MIGUEL FILIPE TAVARES DA LUZ SOARES

TRINUCLEOTIDE REPEAT SCANNING IN PORTUGUESE FAMILIAL AMYLOIDOTIC POLYNEUROPATHY KINDREDS EXHIBITING GENETIC ANTICIPATION



PORTO & NEW YORK

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DISSERTAÇÃO DE CANDIDATURA AO GRAU DE MESTRE APRESENTADA À FACULDADE DE MEDICINA DA UNIVERSIDADE DO PORTO

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Abstract

Familial Amyloidotic Polyneuropathy is a lethal hereditary autosomal dominant disorder characterized by the deposition of a mutant form of the serum protein transthyretin (TTR) as fibrillar material in several tissues and organs. The most common form, FAP Type I or Portuguese, is characterized by a substitution of Met for Val at position 30 in the TTR molecule. It exhibits genetic anticipation, with clinical symptoms developing at an earlier age in succeeding generations.

Expansion of unstable trinucleotide repeats (TNRs) is the underlying mechanism in a growing number of inherited human disorders exhibiting anticipation, such as Fragile X Syndrome, Huntington's Disease, Myotonic Dystrophy, and Friedreich's Ataxia. The number of trinucleotide repeats appears to be correlated with the age of onset, the severity of symptoms, or both. Although the pathology of FAP is produced by the deposition of amyloid derived from a mutant TTR, rather than the expansion of unstable TNRs, we view it possible that the phenotypic variability manifested as anticipation, might be related to TNR expansion in a modifier gene(s), rather than TTR gene.

Using the Repeat Expansion Detection (RED) method we have scanned affected members of FAP type I families from the Portuguese population to determine whether anticipation within such families correlated with expansion of any of the ten possible trinucleotide repeats. Eight generational pairs were tested; in 7 (6 parent/child and 1 uncle/niece) the difference in age of onset was greater than 12 years. A mother/daughter pair, with identical ages of onset, served as control.

Eight of the ten possible trinucleotide repeats were successfully assayed. No major differences were found in the lengths of any of the repeats which could be analyzed. Expansion of the two untested trinucleotides as not been associated with any prior instance of anticipation. The observed distribution of the maximal triplet repeat sizes, as determined by the RED method, was consistent with previously reported studies in healthy unrelated individuals from several populations.

Our results are suggestive - within the sensitivity and limitations of the RED method - that trinucleotide repeat expansions are unlikely to be responsible for genetic anticipation in FAP kindreds in the Portuguese population.

Sumário

A Polineuropatia Amiloidótica Familiar (PAF) é uma doença hereditária autossómica dominante letal, que se caracteriza pela deposição de uma forma mutante da proteína plasmática transtirretina (TTR) como material fibrilar, em vários tecidos e órgãos. A forma mais comum da PAF - Tipo I ou Portuguesa - é caracterizada pela substituição de uma valina por uma metionina na posição 30 da molécula da TTR. Este tipo de PAF apresenta anticipação genética, desenvolvendo-se os sintomas clínicos em idades sucessivamente mais precoces nas gerações seguintes.

A expansão de repetições instáveis de trinucleotídeos ("trinucleotide repeats - TNRs") é um mecanismo associado a um número crescente de doenças humanas hereditárias que exibem anticipação, tais como o Síndroma do X Frágil, a Doença de Huntington, a Distrofia Miotónica, e a Ataxia de Friedreich. O número de repetições de trinucleotídeos parece estar correlacionado com a idade de início, a severidade dos sintomas, ou ambos. Embora a patologia da PAF tenha origem na deposição de TTR mutante como amilóide e não na expansão de "TNRs" instáveis, consideramos a possibilidade de que a variação fenotípica manifesta no fenómeno de anticipação possa estar relacionada com a expansão de "TNRs" em um ou mais genes modificadores.

Usando um método para a detecção de expansão de repetições ("Repeat Expansion Detection (RED) method "), o DNA de vários membros de famílias com PAF da população Portuguesa foi analisado, procurando determinar-se se existe uma correlação entre a anticipação observada e a expansão de repetições de qualquer um dos dez possíveis trinucleotídeos. Foram testados oito pares: em sete (6 progenitor/descendente e 1 tio/sobrinha) a diferença na idade de início da doença era superior a 12 anos. Um par mãe/filha, com idades de início idênticas, foi usado como controlo.

Oito das dez possíveis repetições de trinucleotídeos foram testadas com sucesso, não tendo sido encontradas diferenças significativas nos comprimentos de nenhuma das repetições analisadas. A expansão dos dois trinucleotídeos não testados não foi nunca associada a qualquer circunstância de anticipação. Além disso, a distribuição dos tamanhos máximos das repetições de trinucleotídeos determinada pelo método RED é consistente com estudos anteriores, realizados com indivíduos saudáveis não relacionados entre si, de várias populações. Os resultados deste trabalho sugerem, dentro da sensibilidade e limitações da técnica RED, que é

improvável que a expansão de trinucleotídeos repetidos seja responsável pela anticipação genética em famílias com PAF da população Portuguesa.

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Contents

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Abbreviations

Aβ	Amyloid β
AβPP	Amyloid β protein precursor
AA	Amino acid
ACD	Citric acid/trisodic citrate/dextrose
Apo AI	Apolipoprotein AI
Apo E	Apolipoprotein E
bp	Base-pairs
BSA	Bovine serum albumin
cAMP	Cyclic adenosyne-mono-phosphate
cDNA	Complementary Deoxyribonucleic acid
CNS	Central nervous system
Da	Dalton
dCTP	Deoxycytidine triphosphate
DM	Myotonic dystrophy
DMPK	Myotonic dystrophy protein kinase
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
DRPLA	Dentatorubral-pallidoluysian atrophy
EDTA	Ethylenediamine-N',N' N',N'-tetra acetate
EPM1	Progressive myoclonus epylepsy type 1
FA	Friedreich's ataxia
FAC	Familial amyloidotic cardiomyopathy
FAP	Familial amyloidotic polyneuropathy
FCS	Foetal calf serum
FMR	Fragile X mental retardation
FRAXA	Fragile X syndrome
HAP-1	Huntingtin-associated protein-1
HD	Huntington's disease

kb	Kilobase
MJD	Machado-Joseph disease
mRNA	Messenger ribonucleic acid
PBS	Phosphate buffered saline
RBP	Retinol-binding protein
RED	Repeat Expansion Detection
RNA	Ribonucleic acid
SAA	Serum amyloid A
SBMA	Spinal and bulbar muscular atrophy
SCA	Spinocerebellar ataxia
SDS	Sodium dodecyl sulphate
SSA	Senile systemic amyloidosis
STR	Short tandem repeat
T_4	Thyroxine
TBE	Tris buffer EDTA
TBG	Thyroxine-binding globulin
TNR	Trinucleotide repeat
TRE	Trinucleotide repeat expansion
TTR	Transthyretin
UTR	Untranslated region
UV	Ultra violet

Chapter I

Introduction

Introduction

1.1. GENERAL INTRODUCTION

Familial Amyloidotic Polyneuropathy (FAP) is a hereditary autosomal dominant disorder characterized by the deposition of a mutant form of the serum protein transthyretin (TTR) as fibrillar material in several tissues and organs leading to death in 10 to 15 years. First described by Andrade in 1952, FAP has its major focus in Northern Portugal with spread to Japan, Sweden, Spain, and other countries.

FAP is one of most common and best studied of the genetically related amyloidoses, a group of disorders characterized by the deposition of extracellular amyloid fibrils. It exhibits a remarkable phenotypic heterogeneity expressed in the clinical symptomatology and the age of onset. The variability of the disease onset in Portuguese kindreds accounts for the phenomenon of genetic anticipation, with clinical symptoms developing at an earlier age in succeeding generations. The molecular basis for anticipation in FAP remains obscure. The only known molecular mechanism underlying anticipation is the expansion of unstable trinucleotide repeats in responsible genes for a number of human hereditary dominant disorders. The identification of such mechanism has provided a satisfactory explanation for anticipation at the level of genomic DNA, and has opened the possibility to the speculate about the existence of similar repeat instability in the responsible or accessory genes of other familial diseases with obvious anticipation phenomena such as FAP.

The first section of this chapter will refer to the general aspects of the amyloidoses, with particular incidence on TTR-related amyloidoses and more specifically, FAP. For a better understanding of FAP pathology and the disease-involved mechanisms, the biochemistry and molecular biology of transthyretin will be described. The phenotypic heterogeneity with special reference to the age of onset of the disease and the phenomenon of genetic anticipation will also be discussed.

The second section will introduce the characteristics of a recently emerged mechanism of mutation - the unstable expansion of DNA-trinucleotide repeats. Some aspects of triplet repeat expansion-associated disorders will be described in detail to illustrate the connection of human gene mutation with pathology at the cellular level.

1.2. AMYLOID AND TRANSTHYRETIN-RELATED AMYLOIDOSIS

Amyloidoses are diseases characterized by the aggregation of overproduced or abnormal proteins into stable, insoluble fibrils. The component of the amyloid deposits, initially thought to be a single substance, revealed an unexpected chemical diversity when its identification was made possible. Different precursor proteins precipitate to form amyloid fibrils giving rise to a variety of clinical syndromes. The basis for the classification of the amyloidoses is presently the chemical nature of the deposited protein fibrils (Table I.).

A combination of physicochemical properties define all types of amyloid fibrils, regardless the precursor protein involved. The fibrillar substance can be identified by Congo red staining and exhibits a typical linear parallel organization on electron microscopy (Conhen *et al.*, 1959). It presents a β-pleated sheet structure, as seen by X-ray diffraction, (Eanes *et al.*, 1968) and is highly insoluble under physiological conditions.

The major fibrillar component is always associated with a minor glycoprotein designated by P component which comprise about 5% of all amyloid deposits. Other extracellular components such glicosaminoglycans, proteoglycans, apolipoproteins (E and J) and some serum proteins are also part of the amyloid substance. It is not clear, however, if these molecules play a role in the amyloidogenesis process by inducing conformational changes on the precursor protein prior to its deposition, as suggested by Wisniewski & Frangione (1992), or simply decorate the amyloid fibrils after they are formed.

The transthyretin (TTR) amyloidoses are the most prevalent type of genetically related amyloidosis and are characterized by an autosomal dominant mode of inheritance.

The major biochemical defect underlying these diseases is the extracellular deposition of mutant variants of the serum protein TTR predominantly in the peripheral nerves and in the heart, with neuropathy and cardiomyopathy as major clinical manifestations.

Familial Amyloidotic Polyneuropathy (FAP) was the first disorder of this group to be described and is characterized by the deposition of amyloid fibrils mainly in the peripheral nervous system, starting by the nerves of the lower limbs (Andrade, C., 1952).

Since the description of the first cases, the number of identified kindreds with transthyretin amyloidosis has steadily increased leading to the identification of different clinical patterns of organ involvement. FAP was thus divided in four clinical types (I to IV) and the

TABLE 1. Amyloidoses

Amyloid protein	*Precursor protein*	*Clinical syndrome*
Aβ	β-protein precursor	Alzeimer's disease
		Down syndrome
		Hereditary cerebral hemorrhage with amyloidosis - Dutch type
		Sporadic cerebral angiopathy
Aβ2M	β2-microglobulin	Dialysis-associated amyloid
AA	apo-SAA	Familial Mediterranean fever
		Musckle-Wells syndrome
		Reactive amyloidosis
AANF	Atrial natriuretic factor	Isolated atrial amyloidosis
AApoAI	Apolipoprotein AI variants	Familial amyloidotic polyneuropathy - Iowa type
		Familial nephropathic amyloidosis
ACal	Calcitonin	Medulary carcinoma of thyroid
ACys	Cystatin C variant	Hereditary cerebral hemorrhage with amyloidosis - Iceland type
AFibA	α-chain fribrinogen variants	Hereditary renal amyloidosis
AGel	Gelsolin variants	Familial amyloidosis - Finish type
AH	Immunoglobulin G1	Heavy chain-associated amyloidosis
AIAPP	Islet amyloid polypeptide	Diabetes mellitus type II
		Insulinoma
AL	Immunoglobulin L-chain	Idiopathic amyloidosis
		Myeloma-associated amyloidosis
		Macroglobulinemia-associated amyloidosis
ALys	Lysozyme variants	Hereditary non-neuropathic amyloidosis
APrP	Cellular prion protein	Creutzfeldt-Jacob disease (sporadic)
	Prion variants	Kuru
		Creutzfeldt-Jacob disease (familial)
		Gertsmann-Strussler-Scheinker syndrome
ATTR	TTR	Senile systemic amyloidosis
	TTR variants	Familial amyloidotic polyneuropathy
		Familial amyloidotic cardiomyopathy
		Vitreous amyloidosis
		Meningocerebrovascular amyloidosis
ATub	Tubulin fragments	Familial cerebral amyloid angiopathy-British

term Familial Amyloidotic Cardiomyopathy (FAC) was introduced to describe a Danish kindred with extensive cardiac TTR deposition and very little nerve involvement (Nordlie, 1988). Another type of cardiac TTR-related amyloidosis is Senile Systemic Amyloidosis (SSA), distinguished from the other disorders by the deposition of normal TTR, rather than mutant forms, as amyloid fibrils in the heart. This condition is diagnosed in about 25% of individuals over age 80 (Cornwell, 1983).

The primary cause of TTR-amyloid formation is believed to be the presence of point mutations within the TTR gene originating single amino acid substitutions in the mature protein. An increasing number of TTR point mutations associated with TTR-amyloidoses has been reported, accounting to a certain extent for the proliferation of FAP and FAC clinical subtypes. Nonetheless, it appears that a single pathogenic process underlies the clinical and molecular variations presented by the various forms of TTR-related amyloidoses.

1.2.1. TTR: BIOCHEMISTRY AND MOLECULAR BIOLOGY

The serum protein transthyretin was first isolated from human plasma in 1956 by Shultze *et al.* (1956). It was identified as a band migrating ahead of serum albumin on electrophoresis of whole plasma, and was originally named prealbumin. TTR interactions with circulating thyroid hormones, retinol-binding protein and other physiologically important ligands were progressively discovered. The innumerous studies on transthyretin make it one of the best studied plasma proteins reflecting the physiological importance attributed this molecule.

1.2.1.1. TTR STRUCTURE AND PHYSIOLOGICAL FUNCTIONS

TTR is a soluble tetrameric protein synthesized predominantly by the liver. It is composed of four identical subunits of 127 amino acids (Kanda *et al.*, 1974) with a molecular weight of 13,745 dalton. The total mass of the circulating tetramer is 54, 980 dalton (Smith *et al.*, 1979).

X-ray diffraction analysis has revealed the three dimensional structure of TTR as a globular protein with substantial β-pleated sheet content and a small amount of α helix (Blake *et al.*, 1971). The secondary structure of the polypeptide chains presents 8 β-strands (identified as A to H) aligned in antiparallel fashion, forming two β-sheets composed of strands DAGH and CBEF of each monomer. The β-sheets are linked by seven loops and a single α helix segment is located at the end of β-strand E (Figure 1).

The strong interactions between strands F and H of each monomer make the TTR dimer, which is the basic unit of the protein structure.

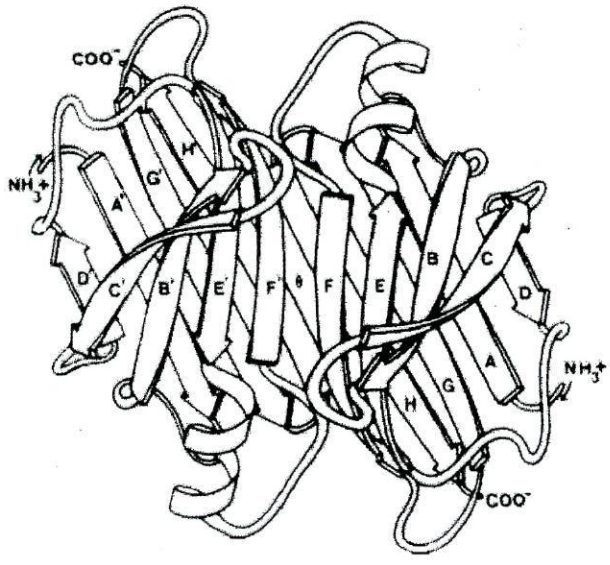

Figure 1. Schematic representation of the TTR dimer.

The main physiological role of transthyretin is the transport of the thyroid hormone thyroxine (T_4) (Fergunson, *et al.*, 1975), and retinol (vitamin A alcohol) through the formation of a protein complex to retinol-binding protein (RBP) (Kanai *at al.*, 1968).

TTR has two structurally identical binding sites for T_4 (Blake *et al.*, 1977) and is responsible in humans, together with thyroxine binding globulin (TBG) and albumin, for the distribution of the hormone throughout the body. Although in a lower concentration in plasma than transthyretin, TBG carries about 70% of the circulating T_4 due to a higher binding affinity to the molecule. In turn, TTR is believed to play a particularly important role as a T_4 carrier in the central nervous system (CNS) since it is synthesized in high rate by the epithelial cells of the choroid plexus, and is present in high concentration in the cerebrospinal fluid. (Dickson *et al.*, 1986). The synthesis of TTR in the choroid plexus early in development suggests a very important function of the protein in the CNS presumably as a thyroxine hormone carrier. Recent studies with TTR knockout mice have shown, however, that T_4 transport to the brain is not impaired by the absence of transthyretin (Palha *et al.*, 1997).

Transthyretin is an important component of the retinol transport complex. It has four potential binding sites for RBP (van Jaarsveld *et al*, 1973) yet only two molecules of RBP bind to the TTR tetramer (Monaco *et al.*, 1995).

The association with RBP protects the retinol transporter from renal catabolism and glomerular filtration (Raz *et al.*, 1970) and assures the delivery of retinol to the target cells. It has been proposed that TTR controls the levels of circulating RBP thus regulating the distribution of retinol to the tissues (Sivaprasadarao & Findlay, 1988). Nonetheless, as demonstrated by studies with TTR null mouse strains, it is not the only mechanism for retinol transport and delivery: both serum RBP and retinol levels were very low but the mice presented a normal phenotype without any signs suggestive of retinol deprivation. (Episkopou *et al.*, 1993).

In addition to RBP and T_4, TTR interacts with diverse compounds including retinoic acid (Smith *et al.*, 1994), noradrenaline oxidation products (Boomsma *et al.*, 1991), hemin and hemoglobin (Martone and Herbert, 1993), pterins (Ernstrom *et al.*, 1995), amyloid β-peptide (Schwarzman *et al*, 1994), among others. The meaning of most of these interactions is still very obscure.

1.2.1.2. STRUCTURE, EXPRESSION AND REGULATION OF TTR GENE

The gene encoding human TTR is a single copy gene located on the long arm of chromosome 18 (Whitehead *et al.*, 1984). Assigned to region 18q11.2-q12.1 (Wallace *et al.*, 1985), TTR gene contains four exons with approximately 200 bases each within about 7.0 kilobases. The first exon encodes a signal peptide of 20 amino acid residues and the first 3 amino acids of the mature protein; exon 2 encodes residues 4-47; exon 3, residues 48-92; and exon 4, the last 35 residues, from 93 to 127. The introns, designated A, B and C, span 934 bp, 2090 bp and 3308 bp, respectively. Introns B and C contain two *Alu* sequences with opposite polarity suggesting a hairpin formation in the precursor mRNA. The first and third introns contain two open reading frames with respective regulating sequences. The meaning of these sequences is not known but it has been suggested that they might encode gene expression regulating proteins (Tsuzuki *et al.*, 1985). Upstream the transcription initiation site, consensus sequences include a TATA box at position -30, a G+C rich region of about 20 bp, a CAAT box at position -101, and further up, at positions -224 and -212, two sequences homologous to glucocorticoid responsive elements. The $(CG)_n$ dinucleotide is the only known repeated motif in the entire TTR gene, including regulatory regions. Downstream the coding sequence, a

polyadenilation site has been located at position 123 of the 3' untranslated region (Sasaki *et al.*, 1985) (Figure 2).

Circulating TTR is synthesized predominantly by the adult liver. Normal plasma concentration is 20-40 mg/dl (Gitlin and Gitlin, 1975) but a significant depression of this level occurs when the liver is participating in the acute phase response to injury or in malnutrition (Dickson *et al.*, 1985; Fung *et al.*, 1988; Murakami *et al.*, 1988).

TTR is also synthesized by the choroid plexus of the brain, as demonstrated by studies on gene expression in rat and human (Soprano *et al.*, 1985; Herbert *et al*, 1986), the synthesis beginning very early in development at the eighth week of gestation (Jacobsson, 1989a). This premature synthesis, together with a high transcriptional activity, imply a particularly important role for TTR in the brain. The protein is also present in the pigmented epithelium of the retina (Dwork *et al.*, 1990) and the pineal gland (Martone, *et al.*, 1993) in mammals.

The human, mouse and rat TTR genes demonstrate 80% sequence homology . Amino acid sequence homology is even higher, of about 91% (Costa *et al.*, 1986) with nearly complete conservation of sequence in the regions involved in ligand binding (Blake *et al.*, 1978; Wakasugui *et al.*, 1985). Studies on regulation and expression of mouse TTR gene were performed in human hepatoma cells - HepG2 - (Costa *et al.*, 1986; Costa *et al.*, 1989) leading to the identification of regulatory sequences in the human gene by comparative analysis.

The promoter region of the human gene contains elements for hepatocyte-specific expression, including binding sites for HNF-1, C/EBP, HNF-3 and HNF-4. Comparative analysis of human and mouse sequences showed that the binding sites for HNF-3 and HNF-4 are well conserved in the human TTR gene, but not those for C/EBP (Sakaki *et al.*, 1989) (Figure 2). Two other liver-specific nuclear protein potential binding sites were identified in the human gene at positions -216 ~ -221 and -199 ~ -204. The motif (TGG/AA/CCC/T) is common to factors Tf-LF1, Tf-LF2 and LF-A1.

A region homologous to a tissue-specific enhancer of the mouse gene was found at - 3,4 kb, containing a binding site for factor HNF-4. This region seems to be important for TTR gene expression in the choroid plexus. Another regulating segment, located at approximately 6 kb of the cap site, includes a sequence with homology to binding sites for nuclear factor APF1 (Sakaki *et al.*, 1989).

TTR gene expression in transgenic mice has been shown to be dependent on the

presence of about 600 bp upstream the transcription initiation site for hepatic and yolk sac expression, and 6 kb for choroid plexus expression (Yan *et al.*, 1990; Nagata *et al.*, 1995).

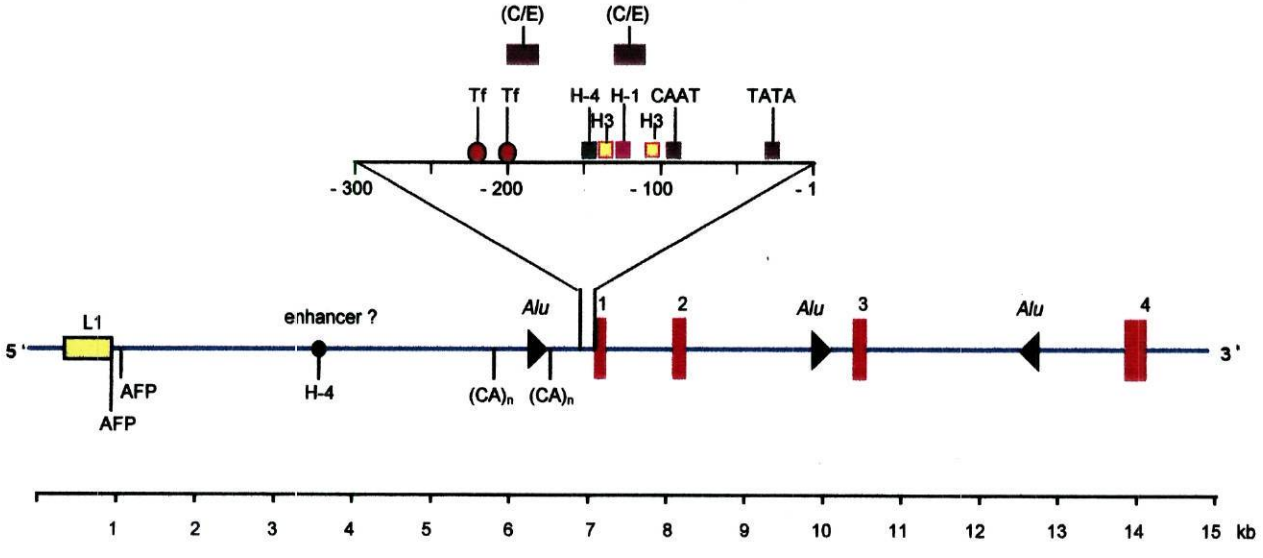

Figure 2. Schematic representation of the structure of the human TTR gene and localization of the respective regulation regions. The exons (red boxes) are numbered from 1 to 4. *Alu*, L1 and (CA)n: respective repetitive sequences. TATA: TATA box; CAAT: CAAT box. H1, C/E, H3, H4: binding sites of hepatocyte nuclear factors HNF-1, C/EBP, HNF-3 and HNF-4, respectively. Tf: common motif to Tf-LF-1, Tf-LF-2, LF-A1. Enhancer: a region highly homologous to a tissue-specific enhancer of the mouse TTR gene. AFP: binding site for AFP-1 factor (from Sakaki *et al.*, 1989).

1.2.1.3. MOLECULAR TTR VARIANTS

Following the description of the first transthyretin variant in 1984 (TTR Met30) (Saraiva *et al.*, 1984), an increasing number of mutant forms were steadily identified. As previously referred to, the vast majority is associated with neuropathic and cardiopathic hereditary amyloidoses, however a number of non-amyloidogenic molecular variants have also been recognized (Tables 2, 3, 4, and 5). Amyloidogenic TTR Met30 is the most frequent TTR variant and it will be discussed later in detail. Other amyloidogenic variants occur with high frequency like the cardiopathic variant TTR Ile122 (0,02), as determined in the Afro-American population by Jacobson (1992). Amyloidogenic mutant alleles are found almost exclusively in heterozygosity but homozygotic individuals have been reported, namely for TTR Met30 (Holmgren *et al.*, 1988), TTR His58 (Jacobson *et al.*, 1994b), and TTR Ile122 (Jacobson *at al.*, 1991).

Non-amyloidogenic TTR variants are either associated with euthyroid hyperthyroxinemia, like TTR Thr109, and Val109, or do not seem to have any pathological effects, as observed for TTR Ser6, Asn90 and Met119, rather frequent in healthy carriers of the normal population (Jacobson *et al.*, 1995; Alves *et al.*, 1997). TTR Ser6 is a common polymorphism with an allele frequency of 0,06 in Caucasians (Jacobson *et al.*, 1995) and the absence of any association with amyloidosis suggests that it is a neutral polymorphism. In screening studies of TTR variants in the Portuguese population (Alves *et al.*, 1997) TTR Met119 was found to be the most frequent variant in the analyzed 5,000 individuals, the frequency (0,007) being very similar to that observed in other populations (Ii *et al.*, 1992). Initially thought to be associated with euthyroid hyperthyroxinemia, this variant was shown not to have any pathogenic consequences in the Portuguese carriers. In fact, in addition to the referred study, several other reports suggest that TTR Met119 carriers are not hyperthyroxinemic. TTR Asn90, the second most frequent variant observed in the referred study, occurred with a frequency of 0,0024 and does not seem to be pathogenic, as well.

Since the mentioned variants occur with high frequency, carriers of two different mutant alleles have been detected (Saraiva *et al.*, 1991; Izumoto *et al.*, 1993; Alves *et al.*, 1993; Jacobson *et al.*, 1994b and Booth *et al.*, 1994). Compound heterozygosity in the Portuguese population has been reported in individuals carrying both Met30/Met119, Asn90/Met119 (Alves *et al.*, 1993), and Met30/Asn90 (Saraiva *et al.*, 1991).

TABLE 2. Neuropathic TTR variants

Position	*Normal residue*	*Mutant residue*	*Phenotype*	*References*
10	Arg	Cys	N, CTS, C,VO,NPH	Uemichi *et al.*, 1992; Benson and Uemichi, 1996
18	Glu	Asp	N	Booth *et al.*, 1996
24	Ser	Pro	N, CTS, C	Uemichi *et al.*, 1995
30	Met	Val	N, C, VO	Saraiva *et al.*, 1983; Tawara *et al.*, 1983
30	Leu	Val	N	Nakazato *et al.*, 1992; Murakami *et al.*, 1992a
30	Ala	Val	N	Jones *et al.*, 1992
33	Ile	Phe	N, VO	Nakazato *et al.*, 1984
33	Leu	Phe	N	Hardling *et al.*, 1991; Ii *et al.*, 1992
33	Val	Phe	N	Booth *et al.*, 1996
34	Thr	Arg	N, C	Patrosso *et al.*, 1996
35	Asn	Lys	N	Reilly *et al.*, 1995
36	Pro	Ala	N, VO	Jones *et al.*, 1991
42	Gly	Glu	N	Ueno *et al.*, 1990a
47	Arg	Gly	N	Murakami *et al.*, 1992b
47	Val	Gly	N, CTS, C	Booth *et al.*, 1994
47	Ala	Gly	N, C	Ferlini *et al.*, 1994
49	Ala	Thr	N, C, VO	Almeida *et al.*, 1992
50	Arg	Ser	N, C	Ueno *et al.*, 1990a; Takahashi *et al.*, 1992
50	Ile	Ser	N	Saeki *et al*, 1992
52	Pro	Ser	N, C, NPH	Booth *et al.*, 1994
54	Gly	Glu	N	Booth *et al.*, 1994
55	Pro	Leu	N, C, VO	Jacobson *et al.*, 1992
58	His	Leu	N, CTS, C	Nichols *et al.*, 1989; Benson and Uemichi, 1996
58	Arg	Leu	N, CTS, VO	Saeki *et al.*, 1991
60	Ala	Thr	N, CTS, C	Wallace *et al.*, 1986; Benson and Uemichi, 1996
61	Lys	Glu	N	Shiomi *et al.*, 1993
64	Leu	Phe	N, C	Ii *et al.*, 1991
70	Asn	Lys	N, CTS, C	Izumoto *et al.*, 1992
71	Ala	Val	N, CTS, C, VO	Almeida *et al.*, 1993; Benson II *et al.*, 1993
77	Tyr	Ser	N	Wallace *et al.*, 1988
77	Phe	Ser	N	Bordeneuve *et al.*, 1996
84	Ser	Ile	N, CTS, C, VO	Dwulet and Benson, 1986
89	Gln	Glu	CTS, N, C	Almeida *et al.*, 1992
91	Phe	Ser	N	Bordeneuve *et al.*, 1996
97	Gly	Ala	N, C	Yasuda *et al.*, 1994; Nakazato *et al.*, 1994
107	Val	Ile	N, CTS	Jacobson *et al.*, 1994a; Uemichi *et al.*, 1994a
112	Ile	Ser	N, C	DeLucia *et al.*, 1993
114	His	Tyr	CTS	Murakami *et al.*, 1994
114	Cys	Tyr	N, VO	Ueno *et al.*, 1990b
116	Tyr	Ser	N	Bordeneuve *et al.*, 1996
122	-	Val	N, C	Uemichi *et al.*, 1996

N – Neuropathy; CTS – Carpal Tunnel syndrome; C – Cardiomyopathy;
VO – Vitreous opacities; NPH – Nephropathy

TABLE 3. Cardiopathic TTR variants

Position	*Normal residue*	*Mutant residue*	*Phenotype*	*References*
20	Ile	Val	C	Jenne *et al.*, 1996; Jacobson *et al.*, 1996
45	Thr	Ala	C	Saraiva *et al.*, 1992
45	Asp	Ala	C, N	Jacobson *et al.*, 1994b
50	Ile	Ser	C	Nishi *et al.*, 1992
59	Lys	Thr	C, N	Booth *et al.*, 1995
68	Leu	Ile	C, N	Almeida *et al.*, 1991a
111	Met	Leu	C	Nordlie *et al.*, 1988
122	Ile	Val	C, N	Gorevic *et al.*, 1989

N – Neuropathy; C – Cardiomyopathy

TABLE 4. Other TTR variants

Position	*Normal residue*	*Mutant residue*	*Phenotype*	*References*
12	Pro	Leu	LD, ICH	Booth *et al.*, 1996
18	Gly	Asp	CNSD	Vidal *et al.*, 1996
30	Gly	Val	VO, CNSD	Herbert *et al.*, 1994
69	His	Tyr	VO	Zeldenrust *et al.*, 1994
84	Asn	Ile	VO, C, CTS	Skinner *et al.*, 1992

LD – Liver disfunction; ICH – Intracerebral hemorrhage; CTS – Carpal Tunnel syndrome;
C – Cardiomyopathy; VO – Vitreous opacities; CNSD – Central nervous system disfunction

TABLE 5. Non-amyloidogenic TTR variants

Position	*Normal residue*	*Mutant residue*	*Phenotype*	*References*
6	Ser	Gly	N	Fitch *et al.*, 1991
74	His	Asp	N	Uemichi *et al.*, 1994b
90	Asn	His	N	Saraiva *et al*, 1991
102	Arg	Pro	N	Almeida *et al.*, 1991b
104	Cys	Arg	Neur	Torres *et al.*, 1996
109	Thr	Ala	EH	Moses *et al.*, 1990
109	Val	Ala	EH	Refetoff *et al.*, 1996
119	Met	Thr	N / EH	Harrison *et al.*, 1991

N – Normal; Neur – Neuropathy; EH – Euthyroid hyperthyroxinemia

In some cases, as in TTR Met30/Asn90 carriers, the presence of a second mutation does not seem to affect the pathogenicity of Met30 (Saraiva *et al.*, 1991). In others, as in TTR Met30/Met119 carriers, the Met119 mutation appears to have a protective effect on the clinical evolution of the disease, particularly referring to the age of onset which is later than for heterozygous Met30 patients (Coelho *et al.*, 1996).

The detection of double mutations in the same allele in two individuals - Ser6-Ile33 (Jacobson *et al.*, 1994b) and Asn90-Gly42 (Skare *et al.*, 1994) - has broaden even more the known molecular variability of TTR. Very recently, Palha *et al.* (1996) have reported the first silent mutation in transthyretin gene resulting from a C for T substitution at codon 108.

The mutations that originate a number of TTR variants, including TTR Met30, Ile122, Thr109 and Met119, occur in a CpG dinucleotide, known as a sequence with high frequency of mutation (Cooper and Youssoufian, 1988). The analysis of the human TTR gene demonstrates the existence of many more CpG hot spots that could originate different amino acid substitutions.

Polymorphisms in the non-coding region of the TTR gene have also been shown by the existence of 5 different haplotypes defined by 7 intronic polymorphic substitutions (Yoshioka *et al.*, 1989). Analysis of these substitutions in several individuals carriers and non-carriers of TTR Met30 mutation have demonstrated that none of them is related with FAP.

1.2.2. TTR MET30 AND FAMILIAL AMYLOIDOTIC POLYNEUROPATHY

In 1952, Corino de Andrade described for the first time a peculiar type of neuropathy. The new clinical entity was characterized as a sensory-motor neuropathy starting in the lower extremities, having associated a variety of symptoms in several organs which were attributed to an autonomic neuropathy, rather than to primary disease of the organs themselves (Andrade, C., 1952). The syndrome was latter designated by Familial Amyloidotic Polyneuropathy reflecting the major clinical manifestation and the hereditary nature of the disease. The first symptoms appear typically in the third decade of life with a slow progression to death in 10 to 15 years.

Following the discovery of transthyretin as the major component of the amyloid fibril deposits by Costa *et al.* (1978), the biochemical mechanisms involved in FAP pathology begun to be unraveled. The deposited TTR was reported to be a variant of the normal protein with a

substitution of a methionine for a valine residue at position 30 of the polypetide chain (Saraiva et al. 1983) and the associated mutation at the DNA level - a single base substitution - was soon revealed after TTR gene cloning (Mita *et al.*, 1984; Tsuzuki *et al.*, 1985). TTR Met 30 was shown to be a biochemical marker for FAP in the Portuguese population due to the complete agreement between genotype and phenotype analysis in diseased individuals (Saraiva *et al.*, 1985).

TTR Met30 is the most frequent mutation associated with FAP and accounts for the most common type of the disease, called type I or Portuguese. To date more than 500 kindreds with FAP have been reported in Northern Portugal, the largest focus in the world, with an estimated prevalence rate in 1991 of $105x10^{-5}$ (Sousa *et al.*, 1995) and a frequency of gene carriers calculated as 1 in 625. The Met30 variant is also present in other foci in Sweden, Japan, Spain, Italy and Brazil. Some kindreds of French, English, Cypriot, Dutch and Turkish origins also have this mutation (Benson *et al.*, 1986; Saraiva *et al.*, 1986; Holt *et al.*, 1989; Munar-Qués *et al.*, 1990; Salvi *et al.*, 1990; Ferlini *et al*; 1988; Hazenberg *et al.*, 1990; Skinner *et al.*, 1990). Recently, FAP type I has been associated with other TTR mutations (Saraiva *at al.*, 1993), however, in Portugal, Brazil and Sweden, no pathogenic TTR mutations other then TTR Met30 have been described so far.

Work by Yoshioka *et al.* (1989) in Japanese FAP kindreds has shown that the Met30 mutation occurs in a CpG hot spot and was found associated with 3 different haplotypes (defined by a set of 7 intronic polymorphic substitutions and classified as I, II and III), as previously referred to. This observation suggested that the Val→Met mutation has probably recurred in the human population to generate FAP families of independent origin. Almeida *et al.* (1995) have also shown two different haplotypes associated with the mutation in European kindreds, substantiating the mutation independent origin hypothesis. Nevertheless, the possibility of the one founder hypothesis in Portugal and Sweden cannot be exclude, since only the haplotype I has been found in those areas.

1.2.2.1. PHENOTYPIC VARIABILITY IN TTR Met30-ASSOCIATED FAP

The clinical phenotype first described by Andrade and thought to be "constant, repetitive and monomorphic" was latter revealed as being very heterogeneous in respect to the patterns of organ involvement and age of onset of the disease.

A) Clinical Presentations

Variability in clinical manifestations secondary to variation in the sites of amyloid deposition have been observed in TTR Met30 associated FAP. The classical site of amyloid deposition, as described by Andrade is the peripheral nervous system, giving rise to sensory and motor neuropathy (the majority of cases in Portugal). Amyloid deposits are also frequently found in the autonomic nervous system, in some cases producing the first symptoms of the disease. Coutinho and coworkers (1980) reviewed 483 cases of FAP type I diseased individuals showing a substantial variation of the first symptoms.

Deposition of amyloid fibrils can also take place in the vitreous of the eye and in the kidney, producing vitreous opacities and renal disease, respectively. This phenotypes can appear either associated with neuropathy, or as single pathological manifestations. Although such clinical features are not frequently observed among Portuguese patients carrying the Met30 mutation, they have been found to arise in some families, and in some but not all branches of the same family.

B) Age of Onset

The most striking feature of phenotypic heterogeneity in Portuguese FAP is by far the age of onset of the disease. The onset of TTR Met30 amyloidosis ranges from an average of 33,5 years in the Portuguese population to an average of 56,6 years in the Swedish population (Sousa *et al*, 1995). Japanese and Majorcan populations present intermediate mean ages-at-onset of 34,2 and 49 years, respectively (Ikegawa *et al*., 1991; Munar-Qués *et al*., 1996).

In the Portuguese population, the age of onset varies from 17 to 78 years, with an average of 33,5 and a standard deviation of 9,5 years. Eighty one percent of the patients exhibit

the first symptoms before the age of 40 and 92% before the age of 50 years. The mean age of onset is significantly higher in females (35,6 years, SD 9,0) than in males (31,9 years, SD 9,6) (Sousa, A., 1995). It has also been shown that carriers of the TTR Met30 mutation could remain disease free up to 93 years of age (Lobato *et al.*, 1988; Sousa *et al.*, 1988; Coelho *et al.*, 1994). Furthermore, sporadic cases have been reported. In a recent revision of 1,233 Portuguese FAP cases, neither parents of 159 individuals had shown symptoms (Coelho *et al.*, 1994). They form a distinct group with an age of onset higher than the average (mean 45,1 years, SD 12,0), and belong to families with a geographical origin slightly different from the areas of FAP major prevalence. Nevertheless, this group of individuals is not significantly different from the general group of patients in clinical presentation at onset and severity of symptoms.

Homozygosity of the Met30 allele has been reported in several individuals of Swedish kindreds but neither the clinical symptoms nor the age of onset of the disease differ significantly from the heterozygous individuals. Curiously, homozygous sporadic cases (in which none of the progenitors had shown any symptoms) have been described in the Swedish population. Interestingly, one of the homozygous individuals was an asymptomatic carrier aged 62 (Holmgren *et al.*, 1988). It is clear, therefore, that gene dosage is not the responsible mechanism for the differences in the age of onset of the disease. Moreover, since the absence of normal alleles does not cause the disease by itself, as observed in the case of the asymptomatic homozygous Met30 carrier, the effect of isoalleles is also excluded as an explanation for the disease late onset in FAP.

Several hypothesis have been raised to explain the existing variability of the age-at-onset but the underlying mechanism of such phenomenon remains obscure. No evidence has ever been found for the existence of a different mutation in the TTR gene segregating either with late- or typical onset diseased individuals. If the late-onset FAP cases were derived from a TTR mutation different from that found in the typical forms of the disease, such mutation would have to occur in the noncoding region of the TTR gene, in addition to the point mutation responsible for the Val → Met substitution at residue 30. Studies on TTR gene expression and structure in late versus typical onset FAP Met30 pedigrees (Saraiva *et al.*, 1986) have not revealed any differences in TTR levels or in haplotype association. No correlation between levels of TTR Met30 and age or duration of clinical symptoms has been found in those kindreds.

Although the role of environmental factors cannot be excluded, the existence of modifier genes involved in the TTR amyloidogenesis process is still the most attractive hypothesis to

explain the phenotypic variability in FAP.

1.2.3. GENETIC ANTICIPATION IN FAP

Genetic anticipation is a phenomenon characterized by a progressive reduction in the age of onset and by an increase in severity of the disease in successive generations. The finding of genetic anticipation was for many years attributed to ascertainment biases postulated by Penrose in 1948. According to Penrose, anticipation was an artifact produced by the selection of pedigrees, rather than a phenomenon with biological meaning. The biases were attributed to 1) the preferential detection of progenitors with late-onset; 2) favored selection of descendants with early onset (presenting more severe phenotypes); and 3) preferential detection of progenitors and descendants with simultaneous onset. The arguments favoring the idea of a statistical illusion begun to be questioned with the observation of different expressions of anticipation depending on the parental transmission of the disease-causing allele (Ridley *et al.*, 1988; Höweler *et al.*, 1989). A few years ago the phenomenon of anticipation was dismissed as a statistical artifact and a molecular mechanism explaining the perplexing features of genetic inheritance displayed by a number of dominant disorders was identified.

Genetic anticipation regarding the age of onset has been for long recognized in FAP Met30 kindreds of the Portuguese population (Coutinho *et al*, 1980; Bastos Lima A, Martins da Silva A, 1980). Initially taken as the result of statistical artifacts, genetic anticipation in Portuguese FAP was latter on proved to be a real phenomenon (Sousa *et al*, 1990a, 1991). Anticipation of the age-at-onset was shown not to be the result of a simple effect of regression to the mean (Sousa, A., 1995). Moreover the existence of the "complementary pairs" postulated by Penrose (1948), i.e., generational pairs in which the progenitors exhibit early onset and the descendants late onset, has never been found in Portuguese FAP kindreds. Contrarily, the age of onset was reported to be earlier in the second generation in the majority of cases, with a mean anticipation of 6,6 years, in a study of 227 generational pairs of the Portuguese population (Sousa, A., 1995). According to the same study, the anticipation range depends on the gender of the diseased individual and the affected progenitor, as male descendants of affected mothers have an earlier onset than female descendants of affected fathers. Sousa (1995) has also reported a few cases of negative anticipation, i.e., descendants with an earlier onset than the progenitors,

in average. These cases were mostly observed in descendants of male progenitors with early onset (before 30 years).

The responsible mechanism for the phenomenon of anticipation in FAP is still unknown. Dynamic mutation is an attractive model as the molecular underlying cause of this perplexing mode of inheritance since it has been associated with other disorders exhibiting genetic anticipation of the age-at-onset. Presently, at least a dozen human genetic diseases have been shown to be caused by trinucleotide repeat expansions (TREs). Although there are 10 possible trinucleotide repeat motifs, only 3 [$(CAG)_n$ / $(CTG)_n$, $(CGG)_n$, and $(AAG)_n$] have been associated with disease. The search for TREs in FAP kindreds exhibiting anticipation becomes the first step to identify modifier genes, suggested to underlie phenotypic variability in FAP.

2. Dynamic mutations: trinucleotide repeat expansions (TREs)

2.1. SIMPLE TANDEM REPEATS AND DYNAMIC MUTATION

The human genome contains many nucleotide sequences that occur repeatedly. The complexity of these repeated sequences vary from complete genes (such as the ribosomal RNA genes) to simple sequences of one or a few base pairs. Dinucleotide repeats are among the simplest and most common repeats, but other frequently occurring simple tandem repeats (STRs) involve mono-, tri-, tetra, and pentanucleotide repeating units. The extensive characterization of simple repeat sequences in the human genome came about as a consequence of their use as highly informative genetic markers. Since their copy number is polymorphic in the human population and their distribution is relatively uniform throughout the genome, simple tandem repeats have been used for the construction of genetic linkage maps, diagnosis by linkage analysis and several other purposes. The general function of the STRs (if any) is still unknown although sequence-specific DNA-binding proteins have been identified for di- and trinucleotide repeats (Richards *et al*, 1993), and one of the repeats can act as a preferential site of nucleosome assembly *in vitro* (Wang *et al*., 1994).

A general characteristic of STRs is the instability of the repeat which appears to be a

function of the information content, i.e. the number of perfect repeating units. The greater the repeat copy number without interruptions, the higher instability it reveals. Such behavior is very evident in trinucleotide STRs, a feature of which is their ability to undergo dynamic mutation. Dynamic mutation is a process that can occur over several generations and is distinguished from conventional mutations by a number of properties: 1) the product of a dynamic mutation has a different risk of undergoing further change than the original DNA sequence; 2) the probability of mutation of a STR is a function of the number of perfect repeating units; 3) the mutation of a given repeated sequence from a harmless copy-number polymorphism to a disease causing unstable DNA sequence is not a single event, but a process involving multiple (sometimes small) changes.

The unstable expansion of DNA-triplet repeats has recently emerged as a completely new mechanism of mutation. A number of disorders displaying perplexing features of genetic inheritance have been in recent years associated with expansion and intergenerational instability of stretches of identical trinucleotides that occur as normal shorter stable elements throughout the human genome. Genetic anticipation, an intriguing phenomenon observed in a significant number of human disorders exhibiting a dominant mode of inheritance has now been attributed to the expansion of unstable trinucleotide repeats. The identification of a satisfactory explanation for anticipation at the level of genomic DNA has led to a new series of challenges.

2.2. PATHOLOGICAL PHENOTYPES

Two different groups of conditions associated with triplet repeat expansion can be considered. One group of disorders is characterized by a progressive neuronal loss. Designated type I (Reddy and Housman, 1997) this group includes Huntington's disease (The Huntington's Disease Collaborative Research Group, 1993), spinal and bulbar muscular atrophy (SBMA) (La Spada *et al.*, 1991), dentatorubral-pallidolusyan atrophy (DRPLA) (Koide *et al.*, 1994; Nagafuchi *et al.*, 1994), Machado-Joseph disease (MJD) (Kawaguchi *et al.*, 1994) and the spinocerebellar ataxias (SCAs) (Orr *et al.*, 1993; Imbert *et al.*, 1996; Pulst *et al.*, 1996; Sanpei *et al.*, 1996; and Zhuchenko *et al.*, 1997). The pattern of neuronal loss is different for all theses neurodegenerative disorders but it is, in all cases, a function of age and the size of the triplet repeat expansion (Table 6).

The other group of triplet repeat disorders includes myotonic dystrophy (DM) (Brook *et al.*, 1992), fragile X syndrome (Kremer *et al.*, 1991; Verkerk *et al.*, 1991; Gu *et al.*, 1996) and Friedreich's ataxia (Campuzano *et al.*, 1996), designated type II diseases and involving a more variable pathology (Table 7).

2.2.1. TYPE I DISORDERS

Type I diseases are caused by moderate expansions of CAG repeats within a coding sequence, producing an extended stretch of polyglutamine in the corresponding protein. Several of this disorders have been reported to display genetic anticipation, and CAG repeat length of disease alleles typically correlates in an inverse manner with age of onset of the symptoms. Surprisingly, and in contrast with type II disorders, an increase of copy number by as little of 5% above a normal level can result in disease (Reddy and Housman, 1997). The number of triplets never exceeds 150 which probably explains why anticipation in these diseases is rather limited, although longer expansions in general result in earlier onset and more severe disease. In both type I and type II disorders, the transmission of a triplet repeats in the range of 40 to 100 repeats to the next generation, through either the male of female germline, exhibits mutation to a new repeat number with a frequency approaching 100 %. This instability contrasts with the transmission of repeat lengths in the order of 5-20 units were variations in size is almost never observed.

Parental transmission of unstable repeats in the 40-100 repeat size range has been observed to present a rather different instability pattern depending on the transmitting parent (Brunner *et al.*, 1993; Trottier *et al.*, 1994). Paternal transmission gives rise to a distribution of repeat sizes among the offspring with a much higher median value than the repeat length of the father, whereas in maternal transmission the maternal mean in children remains normally distributed. This phenomenon already referred to as "paternal anticipation" seems to be the result of a significant increase in sperm TNR lengths compared to the repeat lengths of the father, as observed in studies of individual sperm of males with HD (Leeflang *et al.*, 1995). These studies suggest that either the replication of DNA in primary male germ cells favors repeat expansion, or there is a selection for germ cells or mature sperm with longer TREs.

While the disorder-related genes are widely expressed, the pathologic expression of the diseases is late onset and limited to specific neurological tissue of the brain. They are

characterized by ataxia, chorea, dementia, and in some cases psychosis.

Table 6. Type I trinucleotide repeat diseases (from Reddy and Housman, 1997)

Disease	Repeat	Clinical manifestation	Sites of neuropathology	Repeat Number		Location of the repeats*	Gene product	Gene function	Change in gene function
				Normal	Disease				
Huntington's disease	CAG	Chorea, impairment of cognitive function, emotional disturbances	Primarily in corpus striatum, also in cortex in late stage	6-34	36-121	ORF	Huntingtin	Unknown	Gain
Dentatorubral-pallidoluysian atrophy (Smith's disease)	CAG	Choreanthetosis, ataxia, dementia, myoclonus and epilepsy	Globus pallidus, dentatorubral and subthalamic nucleus	7-25	49-88	ORF	Atrophin	Unknown	Gain
Spinobulbar muscular atrophy (Kennedy's disease)	CAG	Muscle weakness, atrophy and fasciculations	Degeneration of anterior horn cells, bulbar neurons and dorsal root ganglia	11-34	40-62	ORF	Androgen receptor	Transcription factor	Gain
Spinocerebellar ataxia type 1	CAG	Ataxia, dysarthria, dysmetria and decreased vibration sense	Cerebellar cortex, dentate nucleus and brainstem	6-39	41-81	ORF	Ataxin-1	Unknown	Gain
Spinocerebellar ataxia type 2	CAG	Ataxia, dysarthria	Cerebellum, pontine nuclei, substantia nigra	15-29	35-59	ORF	Ataxin-2	Unknown	Gain
Spinocerebellar ataxia type 3 (Machado-Joseph disease)	CAG	Ataxia, dystonia and ophtalmoplegia	Substantia nigra, globus pallidus, pontine nucleus, cerebellar cortex	13-36	68-79	ORF	Ataxin-3	Unknown	Gain
Spinocerebellar ataxia type 6	CAG	Ataxia, dysarthria, nystagmus and vibratory sensory loss	Cerebellar and mild brainstem atrophy	4-16	21-27	ORF	Ataxin-6	Encodes calcium channel subunit (α_{1A})	Gain

* ORF, open-reading frame

The physiological function of CAG repeats translated into polyglutamine tracts is unknown. Amplified CAG repeats might impair this activity (loss-of-function), or modify it resulting in disease (gain-of-function). The finding of normal mRNA or protein levels in these diseases (Strong *et al.*, 1993; Hoogeveen *et al.*, 1993; Banfi *et al.*, 1994) and the absence of SBMA and HD phenotype in patients with deletion of the androgen receptor (Quigley *et al.*, 1992)

or the HD locus (The HD Collaborative Research Group, 1993), respectively, argue against the loss-of-function effect of the CAG triplet repeats. Also, the observation that diseases such as HD are truly dominant with a similar phenotype in homozygotes for the disease allele (Wexler *et al.*, 1987), suggest a gain of function.

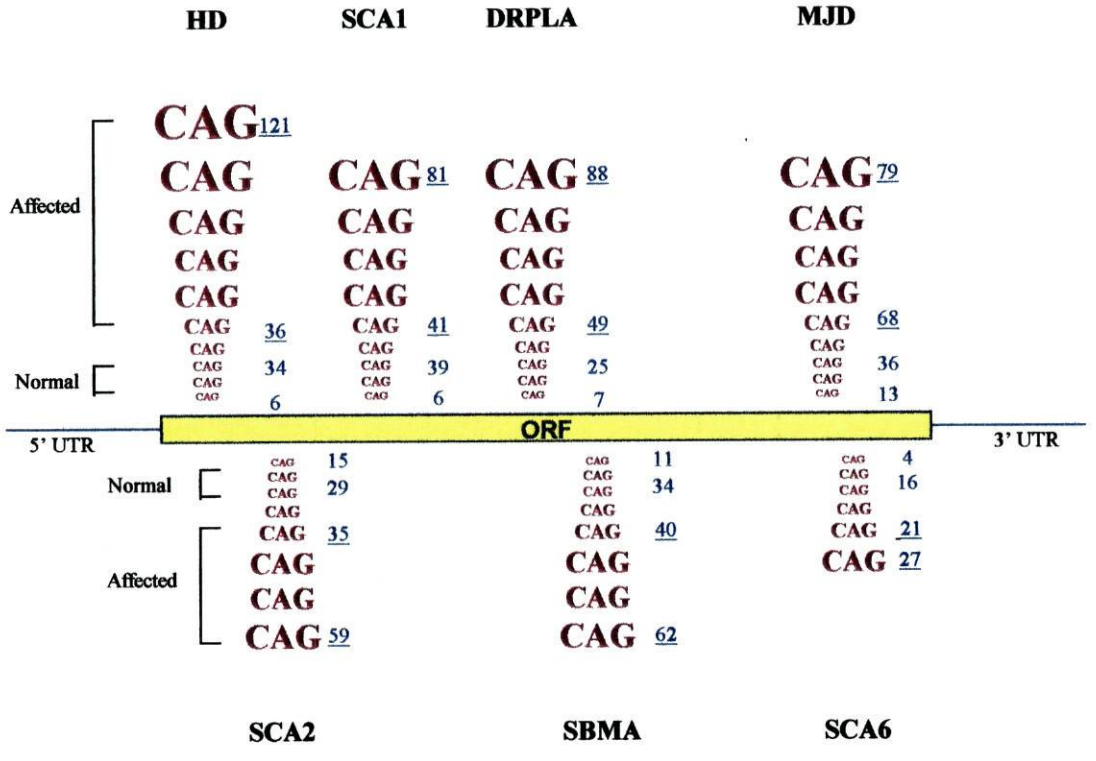

Figure 3. Diagrammatic representation of type I trinucleotide repeat diseaes, showing the number and location of the expanded repeats within a model gene. The repeats are represented by CAG, with increasing font size representing expanding number of repeats. The number of repeats are shown on the right of the trinucleotides. The number of repeats causing disease phenotype are underlined. The repeat expansions in each disease gene are shown at an approximate location within the model gene. ORF, open-reading frame; UTR, untranslated region (from Reddy and Housman, 1997).

2.2.2. TYPE II DISORDERS

Type II disorders exhibit a much larger range of repeat expansion lengths which correlates very clearly with the severity of the disease. Moreover, unlike $(CAG)_n$-related diseases, triplet repeat expansions are not found in coding regions of genes but in flanking regions of coding sequences. Since the pathological phenotypes of fragile X syndrome, myotonic dystrophy and Friedreich's ataxia are very different, and the disease-associated repeated motifs are not a common feature as observed in type I disorders, these diseases will be discussed separately.

TABLE 7. Type II trinucleotide repeat diseases (from Reddy and Housman, 1997).

Disease	Repeat	Clinical Manifestation	Repeat Number			Location of the repeats	Gene product	Gene function	Change in gene function
			Normal	Premutation	Mutation				
Myotonic dystrophy	CTG	Muscle weakness, wasting myotonia, cataracts, mental retardation	5-37	~50-180	~200->2000	3' UTR	Myotonin	Encodes serine/threonine protein kinase	?
Fragile X syndrome (FRAXA)	CGG	Mental retardation	6-52	~60-200	~200->2000	5' UTR	FMR-1	Encodes RNA-binding protein	Loss
Fragile X syndrome (FRAXE)	GCC	Mental retardation	6-25	43-200	>200	?	?	?	?
Friedreich's ataxia	GAA	Progressive gait, limb ataxia, dysarthria	7-22	-	200->900	Intron	Frataxin	Unknown	Loss

The question mark indicates an unknown location, gene product, gene function or change in gene function, and the dash denotes that the premutation is not seen in Friedreich's ataxia.

2.2.2.1. FRAGILE X SYNDROME

Fragile X syndrome was the first genetic disease to be associated with the dynamic mutation of trinucleotide STRs (Yu *et al.*, 1991; Oberlé *et al.*, 1991). It is the most common form of mental retardation and is associated with the presence of a folate-sensitive fragile site (*FRAXA*) at chromossome Xq27.3 (Nelson D.L.,1995). Affected males have the fragile site on their single X chromosome and present moderate to severe mental retardation, developmental delay and autistic behavior. Female carriers have only mild cognitive defects and are more difficult to identify by cytogenetics.

The pattern of inheritance of fragile X shows a marked form of anticipation (Sherman *et al.*, 1984). The disorder is caused by an expanded CGG trinucleotide repeat located in the 5' untranslated region of the *FMR1* gene, which encodes a widely expressed product that has been characterized as a RNA binding protein (Verkerk *et al.*, 1991; Ashley *et al.*, 1993). This repeat is polymorphic in normal X chromosomes, having 6 to ~ 50 copies, typically interrupted by AGG triplets (Kunst, *et al.*, 1994; Eichler *et al.*, 1994). These chromosomes are inherited according to Mendelian patterns of inheritance, without expansion. At a length above ~ 50 copies, the repeat begins to display a certain degree of instability and can increase progressively each time with a higher probability. Up to ~ 200 repeating units, the expansion-containing allele is considered in the premutation range, since it is not associated with an abnormal phenotype, although very unstable in intergenerational transmission. The lengths of normal and premutation alleles may overlap but the different stability reflects internal differences in the presence or location of AGG interruptions, the instability being a function of the length of the longest perfect CGG stretch (Eichler *et al*, 1994). The full mutation comprises up to thousands of copies of the repeated triplet originating the clinical expression of fragile X syndrome. Curiously, male transmission of the premutation alleles invariably remains within the premutation size range, while female transmitted alleles have an increasing probability of greater expansion into full mutation.

Pieretti *et al.*, 1991 have demonstrated that expansion of the CGG repeat up to hundreds of copies causes loss of the *FMR1* mRNA. The mechanism by which the transcription ceases is the methylation of the CpG residues within the repeat (Oberlé *et al.*, 1991;Wang *et al.*, 1996). Thus the mode of action of *FRAXA* mutation is to functionally inactivate the *FMR1* gene. FMR1 protein seems to interact with other RNA-binding proteins, FXR1 and FXR2, and this complex presumably binds to the 60S ribosomal subunit, suggesting a role of FMR1 in modulating translation (Siomi *et al.*, 1996). Whatever the role is played be this protein in the cell machinery it must be an important one since the absence of FMR1 protein alone leads to clinical pathology (De Boulle *et al.*, 1993; Lugenbeel *et al.*, 1995).

Two more fragile sites (FRAXE and FRAXF) have been revealed in families displaying fragile X cytogenetic features but without CGG expansions (Nelson D.L., 1995). Both are located distal to FRAXA and exhibit GCC triplet expansions behaving similarly to the CGG repeat at FRAXA. FRAXE expanded alleles are associated with mild mental retardation while FRAXF does not have any abnormal phenotype.

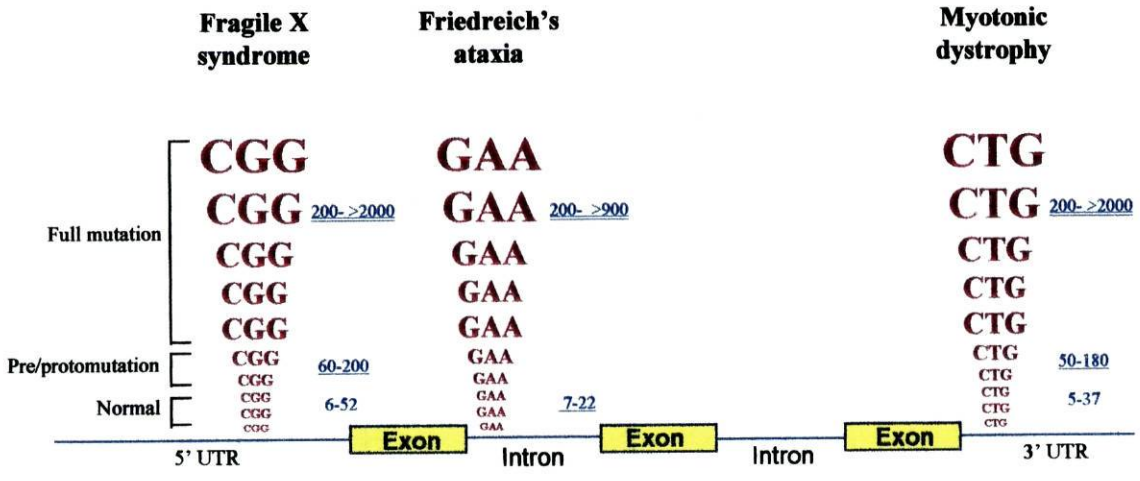

Figure 4. Diagrammatic representation of type II trinucleotide repeat diseases, showing the number and location of the expanded repeats within a model gene. The repeats are represented by CTG, CGG and GAA, with increasing font size representing expanding number of repeats. The number of repeats are shown on the right of the trinucleotides. The number of repeats for pre/protomutation and full mutation are single and double underlined, respectively. The approximate location of the repeats in each disease gene is also shown in the diagram. UTR, untranslated region (from Reddy and Housman, 1997).

2.2.2.2. MYOTONIC DYSTROPHY

It was the finding of a dynamic mutation in myotonic dystrophy that offered an explanation (and legitimization) of the phenomenon of anticipation by providing a clear biological mechanism to explain the perplexing inheritance pattern observed in this disorder (Suthertland *et al*, 1991). Myotonic dystrophy is an autosomal dominant disease characterized by myotonia and muscle weakness. It exhibits anticipation of severity within an affected family increasing over three or four generations. The mutation carriers in the earlier generation may have no discernible phenotypic abnormalities, or merely simple cataracts, but in succeeding generations there can be progressively earlier onset of muscle disease, culminating in children with congenital myotonic dystrophy (Harper, P.S., 1989). The length of the expanded DM allele is correlated with severity of the disorder, unlike in Fragile X where the length of the expanded trinucleotide repeat simply determines the presence or absence of symptoms.

The affected gene in myotonic dystrophy is located in 19q13.3 and contains an expanded CTG repeat within the 3' untranslated region. It is believed to encode, by sequence homology, a cyclic adenosine monophosphate (cAMP)-dependent serine-threonine protein kinase designated by myotonic dystrophy protein kinase (DMPK), but its *in vivo* substrate remains unknown (Mahadevan *et al.*, 1992; Fu *et al.*, 1992; Brook *et al.*, 1992).

The expanded CTG stretch is highly polymorphic in the normal population ranging from 5 to 30 repeats. The trinucleotide repeat can undergo either expansions or contractions, with a strong bias in favor of expansion up to many hundred repeats. In females, the size of the repeat can increase without apparent restrain from generation to generation reaching the point of genetic lethality. On the other hand, in males the repeat reaches a maximum size of ~1000 copies and then appears to decrease in size upon transmission (Lavedan *et al.*, 1993; Mulley *et al.*, 1993). Although the smaller DM alleles are more unstable in male transmissions, the very large alleles that transmit the most severe form of the disease are almost always transmitted by a female. The different meiotic behavior of the repeat in the two sexes is also observed in fragile X syndrome, where a female meiosis in an absolute requirement for the expression of the disease. This behavior contrasts with the transmission pattern observed in type I disorders that show much greater increases in size during paternal transmission - "the paternal anticipation" already referred to.

The mechanism by which increased CTG copy number results in disease is not clear. Reduction of DMPK mRNA levels has been reported (Fu *et al.*, 1993), but the absence of point mutations in the gene giving rise to a phenotype similar to DM, together with the dominant nature of the disease, argue against the hypothesis of DMPK gene loss of function to be the cause of DM pathology. Alternatively, Boucher *et al.* (1995) have suggested that the CTG repeat expansion has an effect on the neighboring genes, contributing in such manner to myotonic dystrophy pathology.

2.2.2.3. FRIEDREICH'S ATAXIA

Friedreich's ataxia is the most recent disorder found to be associated with trinucleotide repeat expansions (Campuzano *et al.*, 1996). It has joined the type II disease group exhibiting the expansion of a new disease-related triplet: GAA. As an autosomal recessive disease with

little evidence for genetic anticipation, FA does not fit the mold of a dynamic mutation. Although the age of onset may vary, most patients exhibit the symptoms in adolescence and the onset in middle age or later is mostly uncommon, unlike the other triplet diseases with anticipation.

The GAA repeat is located within the first intron of frataxin gene which encodes a protein of unknown function. The normal alleles include 10 to 21 copies of the repeat whereas nearly 95% of FA alleles contain from ~ 200 to 900 copies - similar to the premutation alleles found in FRAXA and DM. Recently, premutation alleles of around 40-60 GAAs have been reported in FA (Cossée *et al.*, in press). The disease-causing expansion appears in heterozygosity in about 90% of the patients, but half of the remaining are compound heterozygotes carrying the repeat expansion in one allele and point mutations in the other.

The expanded GAA repeat in intron 1 appears to interfere in frataxin nuclear RNA processing resulting in the absence of a mature message in the cytoplasm (Campuzano *et al.*, 1996). Although such hypothesis has not been proved, the massive number of AG splice acceptor sites formed by the expanded GAA repeating units, is likely to underlie the mechanism by which the transcript levels are reduced in FA. Moreover, the GAA repeat cannot form hydrogen-bonded hairpins, thus being unable to form the non-conventional DNA structures characteristic of CG-rich repeats, which may be involved in DNA transcription impairment in other TREs-associated diseases.

2.2.3. OTHER DISORDERS

Recent studies have shown that the expansion of a 12-bp repeat is responsible for a monogenic form of epilepsy known has progressive myoclonus epilepsy (EPM1) (Lalioti *et al.* 1997; Lafrenière *et al.*, 1997; Virtaneva *et al.*, 1997). The gene encoding cystatin B, a proteinase inhibitor, contains a $(CCCCGCCCCGCG)_3$ in the 5' flanking region within the promoter sequence which expands up to 600-900 bp in diseased alleles. EPM1 is the second autosomal dominant disorder joining the growing number of genetic diseases that are associated with unstable repeat expansions.

Chapter II

Experimental Research

Experimental Research

1. Objectives

The molecular mechanisms underlying genetic anticipation in FAP remain one of the major unsolved dilemmas concerning the phenotypic variability presented by this disorder. As a neurological disease with an autosomal dominant mode of inheritance and evident anticipation, FAP is a strong candidate for the involvement of trinucleotide repeat instability in the decreasing age of onset over successive generations. The pathology of FAP is produced by point mutations in the TTR gene, rather than unstable trinucleotide repeat expansions. Indeed, TTR gene structure does not include repeated triplets neither within the coding regions nor in the untranslated regulatory sequences (Sakaki *et al.*, 1989). Since TTR amyloidogenesis is a multistep process, modifier gene(s) are very likely to be involved in phenotypic variability manifested as genetic anticipation. We have questioned whether anticipation in FAP might be related to unstable trinucleotide repeat expansions in a modifier gene (or genes), rather than TTR gene itself.

The main purposes of this project were 1) the determination of maximal trinucleotide repeat lengths in generational pairs of Portuguese FAP kindreds, and 2) the detection of intergenerational instability of any of the ten trinucleotide repeats in the same kindreds.

We have performed a complete genome scanning of trinucleotide repeat lengths in 8 generational pairs of FAP Portuguese families with a difference in age of onset greater than 12 years, using the Repeat Expansion Detection (RED) method. This technique does not require flanking sequence information and allows a genome-wide search for potentially pathological repeat expansions.

2. Materials and Methods

2.1. GENERATIONAL PAIRS AND CONTROLS

Eight generational pairs of Portuguese kindreds with FAP were scanned for trinucleotide repeat expansions using the RED method. In seven of the tested pairs the differences in the age of onset were greater than 12 years. One pair with identical ages of onset was used as a control. The gender and age-at-onset of the individuals of each pair are described in the following table.

Pair	*Individual*	*Onset*	*Age*
I	Mother	32	Deceased
	Daughter	32	43
II	Mother	74	76
	Daughter	30	40
III	Mother	68	73
	Daughter	40	52
IV	Father	57	53
	Son	31	36
V	Uncle	62	69
	Niece	50	54
VI	Mother	Asymptomatic	69
	Daughter	39	44
VII	Mother	63	64
	Daughter	33	34
VIII	Mother	Asymptomatic	76
	Son	44	52

All affected individuals were confirmed carriers of the TTR Met30 mutation, and were diagnosed as FAP patients based on the presence of characteristic clinical features of the disorder. The two asymptomatic individuals did not exhibit any pathological symptoms, although confirmed carriers of the TTR Met30 allele. None of the above individuals had the TTR Met119 mutation.

As negative controls, nineteen unrelated individuals of the Portuguese population without known genetic disease (11 females, 6 males and 2 individuals of unknown gender) were used.

Blood samples were collected using vacutainer tubes with ACD (Becton Dickinson) for lymphocyte immortalization and DNA extraction.

2.1.1. LYMPHOCYTE IMMORTALIZATION

A 3.0 ml whole blood aliquot was carefully laid onto 3.0 ml of Histopaque-1077 (Sigma) (previously brought to room temperature), on a 15 ml centrifuge Falcon tube. This mixture was centrifuged at 1800 rpm, for 30 minutes, at room temperature.

After centrifugation, the upper layer to within 0.5 cm of the opaque interface, was carefully aspirated using a Pasteur pipette and discarded. The opaque interface, containing mononuclear cells, was then transferred with a Pasteur pipette into a clean centrifuge Falcon tube. Ten ml of RPMI-1640 Medium (Sigma) were then added to the tube, and the contents were mixed by gentle aspiration.

After a centrifugation at 1400 rpm, for 10 minutes, the supernatant was discarded and the cell pellet was resuspended in 5.0 ml of RPMI-1640 medium. Another centrifugation followed, at 1400 rpm, for 10 minutes, the supernatant was discarded and the cell pellet was once again resuspended in RPMI-1640 medium and then centrifuged one last time at 1400 rpm, for 10 minutes.

Using a Pasteur pipette, the resulting cell pellet was resuspended in 2.0 ml of RPMI-1640 medium and the transformation step followed.

TRANSFORMATION BY EPSTEIN-BARR VIRUS (EBV)

A 2.0 ml volume of Transformation Medium was added to 1.0 ml of resuspended cell pellet obtained by the procedure described above, and 1.5 ml aliquots of the mixture were transferred to a couple of 25 cm^3 culture flasks. 1.5 ml of EBV suspension (previously incubated at 37°C for 3-4 hours) was added to each flask.

After 24 hours, 2.0 ml of Transformation Medium were added to the culture, and this procedure was continued in each two days, for a week, after which the medium was transferred to a clean centrifuge Falcon tube followed by a centrifugation at 1200 rpm, for 10 minutes. Half of the supernatant volume was discarded and replaced with the same volume of Complete Medium.

In each two days that followed the previous step, a given volume of Complete medium (depending on the evolution of the culture) was added. After a week or two, the cells

were transformed and could by frozen for storage.

FREEZING OF LYMPHOCYTES

A 1.0 ml volume of Freezing Medium (Gibco), previous placed on ice, was added to 1.0 ml of resuspended cell pellet, very slowly and using a Pasteur pipette. During this step, the tube containing the cells was kept on melting ice and was continuously shaken.

The mixture was then transferred to a Nunc Vial freezing tube and stored at –70°C for at least 4 hours up to 24 hours, after which the tube was conserved in liquid nitrogen.

CULTURE OF FROZEN LYMPHOCYTES

One ampoule containing the frozen cells, was removed from the liquid nitrogen and immediately thawed in a shaking water bath at 37°C. Before the disappearance of the last ice crystals, the tube was placed on ice and the cell suspension was very carefully transferred to a centrifuge Falcon tube, containing 5.0 ml of base Medium supplemented with 20% FCS, at 4°C.

After centrifugation at 1200 rpm, for 10 minutes, the supernatant was discarded, and the cell pellet resuspended in 2.0 ml of Complete Medium (supplemented with 20% FCS). The suspension was then transferred to a 25 cm^2 flask and, following an incubation at 37°C, for 24 hours, 2.0 – 6.0 ml of Complete Medium were added.

Depending on the evolution of the culture, each two days, a given volume of Complete medium was added, until the culture reached the desired point.

MEDIA

Base Medium

This medium contains 500.0 ml of RPMI-1640 with L-glutamine and Hepes (Gibco), 2.0 ml of penicillin/streptomycin (Gibco), 2.0 ml of kanamycin (25 mg/ml) and 5.0 ml of fungizone (250µg/ml). After preparation was stored at 4°C.

Complete Medium

This medium contains 100.0 ml of Base Medium; 5.0 ml of foetal calf serum (FCS), previously inactivated at 56°C, for 30 minutes and 1.5 ml Ultroser HY hydrated (Gibco). After preparation this medium was stored at 4°C.

Transformation Medium

This medium contains 100.0 ml of Base Medium, 20.0 ml of FCS (previously inactivated at 56°C, for 30 minutes) and 0.15 ml of ciclosporin (1 mg/ml in ethanol).

Freezing Medium

This medium contains 0.8 ml of FCS (previously inactivated at 56°C for 30 minutes) and 0.2 ml of sterile DMSO. After preparation was stored at –20°C.

2.1.2. GENOMIC DNA EXTRACTION

Twelve ml of total blood were centrifuged at 2,000 rpm for 10 minutes. The plasma layer was gently removed and the buffy coat - the leukocyte layer - was carefully placed in eppendorf tubes. 1-2 volumes of a solution containing 72.0 mg of ammonium bicarbonate and 7.0 g of ammonium chloride in a liter of dd H_2O were added to the cells for erythrocyte lysis. The tubes were left on ice for 10 minutes, centrifuged at 10,000 rpm for 5 minutes, and the supernatant discarded. This procedure was repeated a number of times in order to obtain a clear white cell pellet.

A 5.0 ml volume of proteinase K buffer (Tris 0,05M pH 7,5; NaCl 1M; EDTA 0,001M; and SDS 0,5%) was added to the cell pellet followed by 50.0 µl of proteinase K and incubated overnight at 55 °C. Following the leukocyte lysis, 2.0 ml of an NaCl saturated solution and 2.0 ml of chloroform were added to the pellet and vortexed for 30 seconds. After a centrifugation at 9,000 rpm for 10 minutes, the supernatant was discarded and 2 volumes of 100% ethanol were added to the tube which was inverted until DNA visualization. The precipitated DNA was remove carefully and resuspended in an appropriate volume of TE buffer.

2.2. RED METHOD

Repeat Expansion Detection (RED) method is a generally applicable method which provides a direct path to identify potentially pathological repeat expansions (Schalling *et al*, 1993). Single copies of large trinucleotide repeats can be detected based on the use genomic DNA as a template for the annealing and ligation of repeat-specific oligonucleotides. Consequently, it does not require flanking sequence information, or single copy probes. A thermostable ligase is used in a cycling procedure which generates multimers of the oligonucleotide used.

This method constitutes a unique way of studying triplet repeat expansion-associated inherited disorders, particularly those characterized by anticipation.

2.2.1. GENERAL PRINCIPLE

The genomic DNA containing a long segment of repeated trinucleotides serves as a template for annealing of oligonucleotides composed of several copies of the complementary triplet (Figure 5). As two or more oligonucleotide molecules anneal to the genomic template in adjacent positions, they are ligated by a thermostable ligase, forming a longer single stranded molecule. This step is repeated several hundred times in a two-step cycling process using adequate denaturation and annealing temperatures. The presence of long trinucleotide repeats anywhere in the genome will give rise to the formation of large multimers of the original oligonucleotide. The single stranded multimers are separated by size in denaturing conditions by polyacrylamide gel electrophoresis, transferred to a nylon membrane and detected by hybridization to a ^{32}P-labeled complementary oligonucleotide. The radiolabeled probe maximizes the detection of the RED reaction products.

2.2.2. OLIGONUCLEOTIDE PHOSPHORYLATION

For 20 RED reactions 100 µl of T4 polynucleotide kinase (Epicentre) mixture was prepare by combining 73.0 µl of ddH_2O; 10.0 µl of ATP (10 mM); 10.0 µl of 10x T4 polynucleotide kinase buffer; 1.0 µl of the testing oligonucleotide (1 or 2 µg/µl depending on

the length of the oligo, being the higher concentration for oligonucleotides with about 17 repeated units); and 6.0 µl of T4 polynucleotide kinase (PNK). The reaction mix was incubated at 37 °C for 50 minutes and used in the RED reaction immediately. Alternatively, the mixture can be heated at 65 °C to inactivate PNK and stored at -80 °C for posterior use.

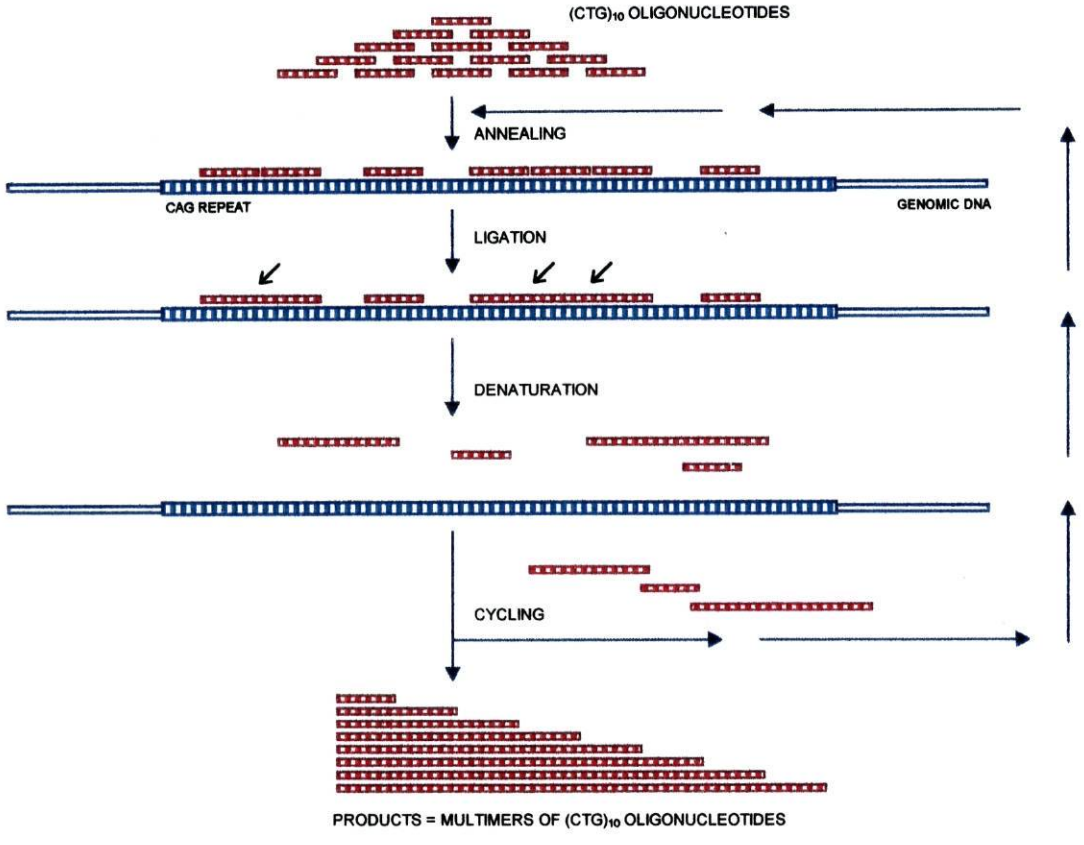

Figure 5. Diagram depicting amplification and detection of DNA-trinucleotide repeat using RED. Since only one strand of oligonucleotide multimer is produced per target molecule per cycle, a large amount of DNA is needed (10 µg). Products vary in length as multimers of the oligonucleotide used, and depend upon size of the target (from Schalling *et al.*, 1993).

2.2.3. REACTION CONDITIONS

Following the addition of 5.0 µl of phosphorylated oligonucleotide to ~10.0 µl of DNA template (~ 1 µg/µl), the reaction mixture was covered with 20.0 µl of mineral oil in eppendorf

tubes, heated at 98 °C for 10 minutes, to denature the DNA, and placed on ice (for CGG oligonucleotide, 10.0 µl of 5M Betaine were added to the mixture). While keeping the samples on ice, 10.0 µl of ligation reaction mix A containing: 2.0 µl of 10x Ampligase reaction buffer; 7.0 µl of ddH_20; and 1.0 µl of Ampligase DNA ligase (5 Units) (Epicentre). The reaction mixtures were quickly centrifuged for 30 seconds, and the tubes were placed into the thermocycler (Perkin Elmer DNA Thermal Cycler) for cycling according to the following parameters:

For $(CTG)_{10/17}$-, $(CGG)_{11}$-, $(TGG)_{12}$-, and $(CGT)_{14}$-oligonucleotides - 100 cycles at 70 °C for 30 seconds and 95 °C for 10 seconds; for $(CCT)_{15}$-, $(AAG)_{10}$-, $(GTT)_{15}$,- $(AGT)_{14}$-, and $(ATG)_{12}$- oligonucleotides - 100 cycles at 60 °C for 30 second and 95 °C for 10 seconds; for $(TAA)_{11}$ - 100 cycles at 50 °C for 30 seconds and 95 °C for 10 seconds.

After the first 100 cycles, the samples were placed on ice and 5.0 µl of ligation reaction mix B containing: 3.5 µl of ddH_2O; 0.5 µl of 10x Ampligase reaction buffer; and 1.0 µl of Ampligase DNA ligase (5 U), were added. The samples were quickly centrifuged for 30 seconds and the cycling was restarted for 300 additional cycles at the same conditions described above. The RED reaction products were immediately used for electrophoretic separation or stored at 4 °C.

2.3. ELECTROPHORESIS

The running gel consisting of 50.0 ml of a pre-warmed 6% acrylamide / 6M urea solution, 500.0 µl of APS and 35.0 µl of TEMED was poured into a SequiGen II electrophoresis apparatus (0.5 mm thick) (Biorad) and pre-run at 50 watts for 30 minutes. Meanwhile 20.0 µl of the RED reaction products were mixed with 15 µl of gel loading dye, denatured for 5 minutes at 95 °C and placed on ice.

Following the gel pre-run (30 minutes), the wells were cleaned with a Pasteur pipette to prevent urea deposits, the samples were loaded, and the gel was run in 1x TBE for approximately 1 hour and 10 minutes at 50 watts (the xylenocyanol dye should migrate to middle of the plates). The intact gel apparatus was then put under cold tap water for 10 minutes.

2.4. MEMBRANE TRANSFER

A Hybond N+ membrane (Amersham) was quickly soaked in 1x TBE and placed in direct contact with the gel. The membrane was then covered with 3 layers of 3M paper and the glass plate was placed on top. The RED products were left transferring by capillary action overnight.

The membrane was removed, rinsed for 2 minutes in 2x SSC, dried at room temperature for 1 hour and exposed to UV light to cross-link the DNA to the membrane. Following this procedure, the membrane could be immediately hybridized or stored at room temperature.

2.5. LABELING OF THE PROBE

The probe was radiolabeled by combining 1.5 µl of the probe (200 ng/µl); 6.5 µl of ddH_2O; 4.0 µl of 5x terminal transferase buffer; 2.0 µl of $CoCl_2$; 5.0 µl of P^{32}-dCTP; and 1.0 µl of terminal deoxynucleotidyl transferase (Boehringer Mannheim) in an Eppendorf tube. Following the incubation for 60 minutes at 37 °C, 4.0 µl of glycogen solution (20 µg/µl) and 50.0 µl of 100% ethanol were added, followed by a centrifugation for 30 seconds. The ethanol was carefully removed and the precipitated DNA was dried in the speedvac for at least 60 minutes. The radiolabeled DNA was finally resuspended in 100.0 µl of ddH2O.

2.6. HYBRIDIZATION

The dried membranes were pre-hybridized in Rapid-Hyb buffer (Amersham) for at least 1 hour at the following temperatures: for $(CTG)_{10/17}$-, $(CGG)_{11}$-, $(TGG)_{12}$-, and $(CGT)_{14}$-oligonucleotides - at 65 °C; for $(CCT)_{15}$-, $(AAG)_{10}$-, $(GTT)_{15}$,- $(AGT)_{14}$-, and $(ATG)_{12}$-oligonucleotides - at 60 °C; for $(TAA)_{11}$ - at 55 °C. The hybridization with the radiolabeled probe was done overnight in the same buffer at the same temperature. After 2 washes in 2x SSC (20 minutes each) at room temperature, followed by 2 washes in 1x SSC (30 minutes each) at 5 °C below hybridization temperature, the membrane was exposed to Kodak X-AR

or Kodak Biomax MS films at -80 °C, using intensifying screens, for 1-15 days.

3. Results and Discussion

3.1. CTG/CAG TRINUCLEOTIDE

Eight inherited neurodegenerative disorders are known to involve expansions of CAG trinucleotide repeats within susceptibility genes, resulting in an abnormally long stretch of polyglutamine within the encoded proteins. CTG repeat expansions are associated with severe phenotypes of congenital myotonic dystrophy as long segments of the repeated triplet in the 3' untranslated region of the myotonin gene result in disease. These inherited diseases, in which the length of the CTG or CAG repeat region is expanded, exhibit genetic anticipation.

We have tested the genomic DNA from 9 FAP kindred pairs and 8 negative controls using $(CTG)_{10}$ and $(CTG)_{17}$ oligonucleotides to assess trinucleotide repeat lengths and to detect the existence of possible expansions eventually responsible for genetic anticipation in FAP. Some generational pair and control DNAs have been tested in duplicate or triplicate to confirm the results obtained for this particular trinucleotide, and to define the reproducibility of RED in our laboratory. As a positive control, the DNA of an individual exhibiting a very long stretch of the repeated motif was used in all reactions. The RED reaction results are shown in the autoradiographs of Figures 6, 7, 8, and 9.

All samples of seven of the eight pairs exhibited a maximal repeat size of 120 bp, corresponding to 40 repeated triplets. Only pair IV has broken the consistency of the repeat length distribution in FAP gene carriers by exhibiting a fragment of 210 bp corresponding to 70 copies of the trinucleotide (Fig. 6, lanes 11 and 12; Fig. 8, lanes 4 and 5; Fig. 9, lanes 8 and 9). No differences in maximal repeat size have been found within the tested pairs with the exception of an extra pair used only in CTG/CAG repeat detection experiments - pair Y. The descendant, diseased with an onset at 40, presents a slightly longer repeat than the progenitor, asymptomatic carrier, corresponding to the ligation of one more $(CTG)_{10}$ oligonucleotide referring to a 30 bp (10 copies) difference (Fig. 6, lanes 1 and 2). The control samples have shown repeat lengths ranging between 60 bp (20 repeats) and 270 bp (90 repeats). CTG/CAG repeat sizes, as determined by RED, are compared in Figure I.

CTG/CAG repeats are highly polymorphic in length in the human genome but their number usually does not exceed 40 repeats in normal individuals. Hofferbert *et al.* (1997) have

reported, however, repeat sizes ranging from 120 bp to 270 bp as determined by RED in 74 unrelated control individuals, that is, a triplet copy number of 40 to 90 repeats. The existence of such unexpected high triplet copy number in normal individuals has been confirmed in a study on 75 normal Japanese individuals and 30 randomly chosen healthy Caucasians (Nakamoto *et al.*, 1997). According to this report, the length of the polymorphic CAG/CTG repeats ranges from 10 to 92 in the Japanese and Caucasian individuals sample, the frequency being much higher for repeat copy number between 10 and 30 repeats, as expected. Our results fall in the expected range of the reported size spectrum, not exceeding 70 repeats in the FAP kindred individuals and 90 repeated units in the unrelated healthy controls.

Pairs I and IV were also tested using a longer oligonucleotide - $(CTG)_{17}$. The repeat lengths have shown good correlation with both oligonucleotide substrates, presenting identical sizes (Figure 6 and 7). The samples scanned in duplicate or triplicate (pairs I, II, IV, and controls N_3, N_4) also show identical triplet sizes in different reactions, demonstrating the reproducibility of the RED assay.

Some samples exhibit CTG/CAG repeat segments with sizes corresponding to CTG repeat lengths of myotonic dystrophy premutation alleles, and to CAG repeat lengths of disease-causing alleles of neurological disorders like HD, DRPLA, Kennedy's disease and SCA3. Nonetheless, our results clearly exclude the existence of unstable CTG/CAG repeat expansions from one generation to another in FAP-diseased individuals exhibiting genetic anticipation, making TREs an unlikely explanation.

3.2. AAG / CTT TRINUCLEOTIDE

The autoradiographs shown in Figures 10, 11 and 12 represent RED reaction products of seven generational pairs tested for the presence of AAG/CCT trinucleotide repeat expansions. The oligonucleotide used in the cycling reaction was an $(AGG)_{10}$.

Trinucleotide repeat lengths range from 110 repeated units (330 bp) up to 200 triplets (600 bp) in the tested pairs. The controls vary between 70 (210 bp) and 170 repeats (510 bp) (Figure II). No significant differences in repeat size are observed within 6 of the 7 pairs, the major difference being the ligation of two more $(CTG)_{10}$-mers in the progenitor of pair I that has the same age of onset as the daughter, representing a putative contraction of the repeated motif.

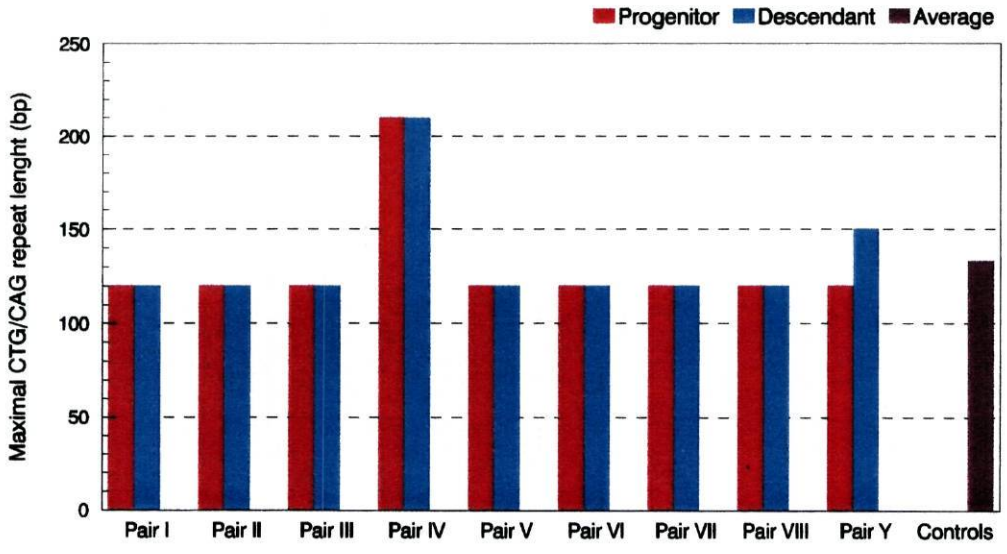

Figure I. Maximal sizes of CTG/CAG repeat multimers in FAP kindred pairs and unrelated controls as determined by RED.

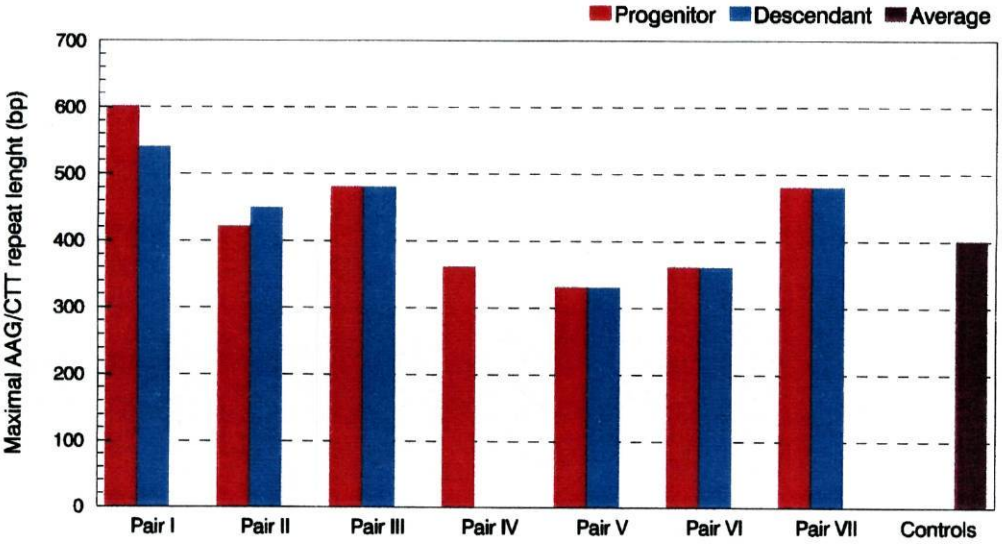

Figure II. Maximal sizes of AAG/CTT repeat multimers in FAP kindred pairs and unrelated controls as determined by RED.

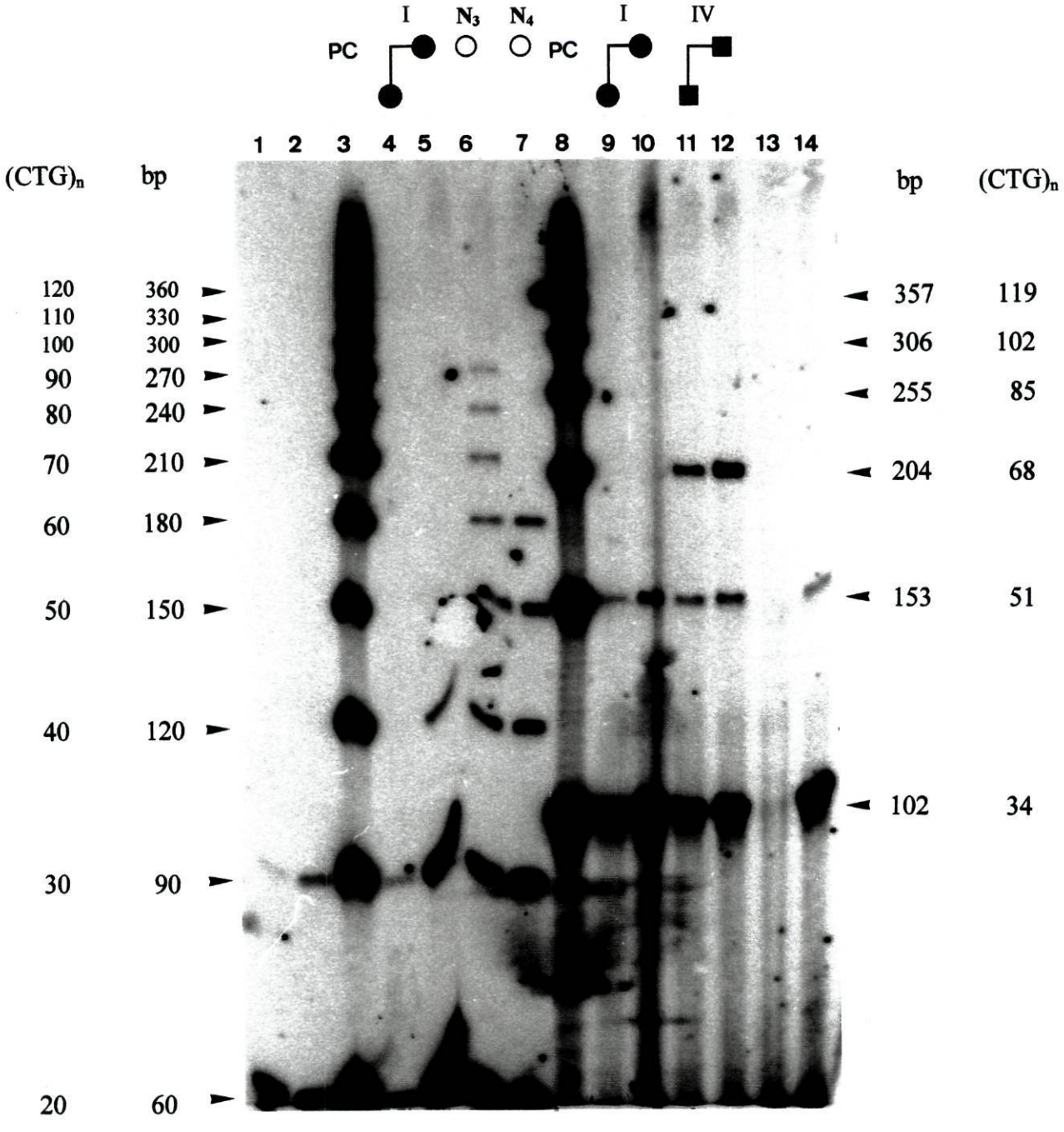

Figure 6. Autoradiograph of the RED reaction products using $(CTG)_{10}$ and $(CTG)_{17}$ oligonucleotides (lanes 1-7 and 8-14, respectively). PC: positive control; lanes 4, 5 and 9-12: FAP generational pairs; lanes 6 and 7: control individuals. The remainder lanes refer to samples excluded from our study. Note the correlation of repeat sizes obtained with both oligonucleotide substrates. 10 μg of genomic DNA template were used in each reaction. Sizes of $(CTG)_n$ multimers are indicated in bp.

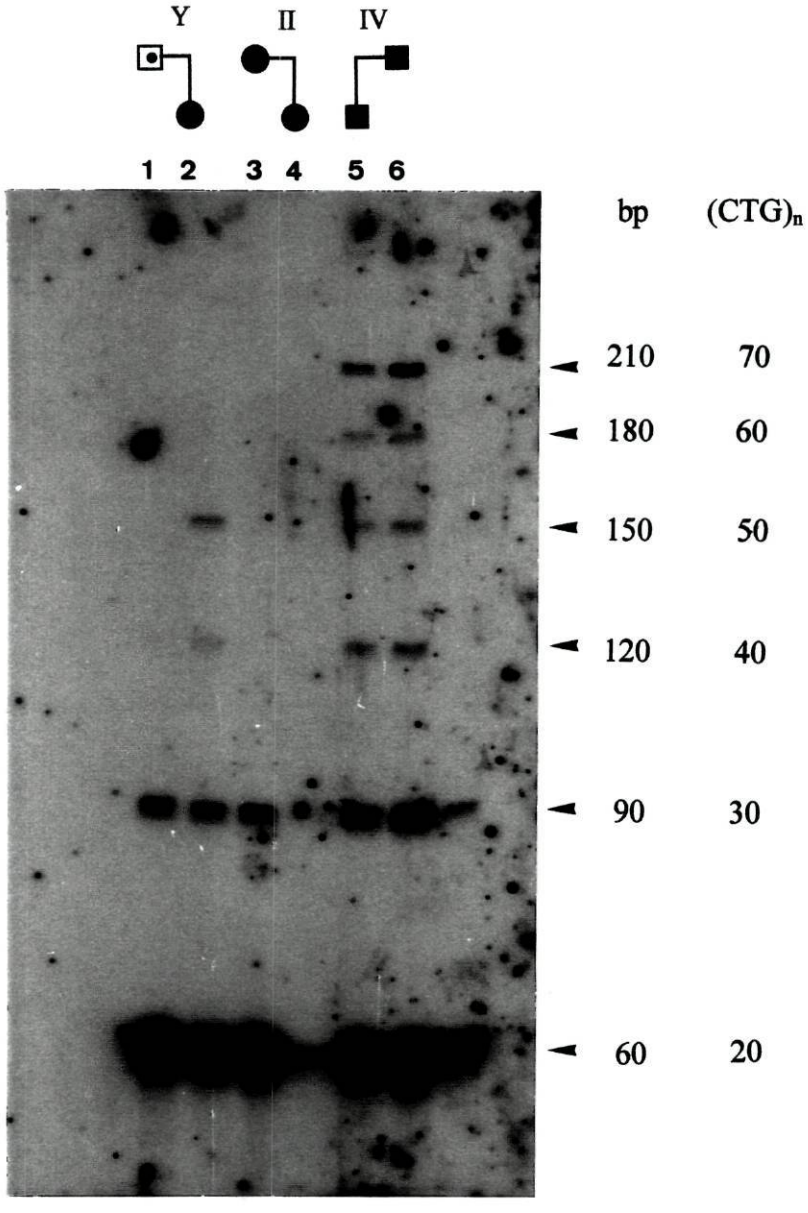

Figure 7. Autoradiograph of the RED reaction products using $(CTG)_{10}$. Lanes 1-6: FAP generational pairs. 10 μg of genomic DNA template were used in each reaction. Sizes of $(CTG)_n$ multimers are indicated in bp.

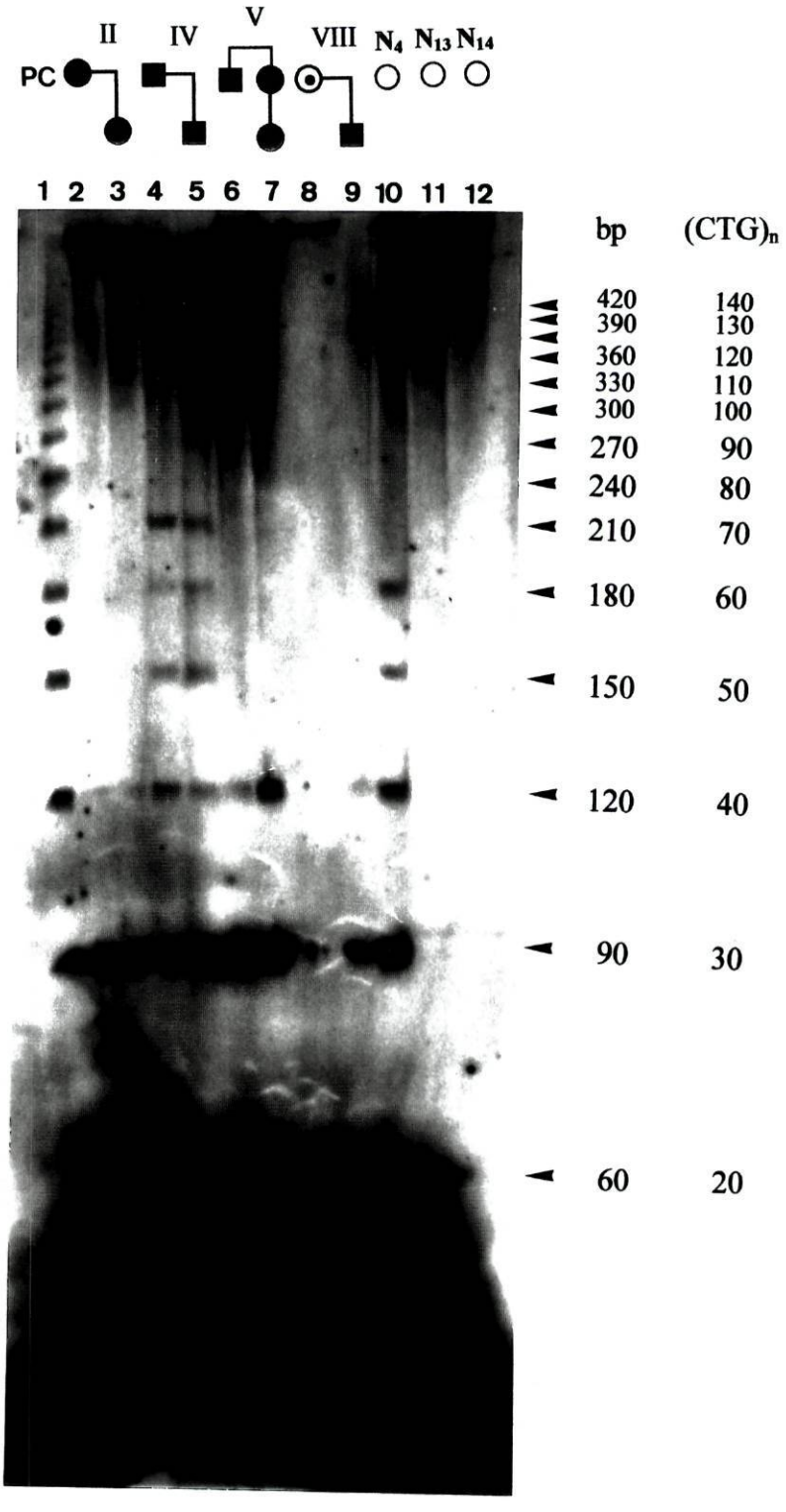

Figure 8. Autoradiograph of the RED reaction products using $(CTG)_{10}$. Lanes 2-9: FAP generational pairs; lanes 10-12: control individuals; PC: positive control. 10 μg of genomic DNA template were used in each reaction. Sizes of $(CTG)_n$ multimers are indicated in bp.

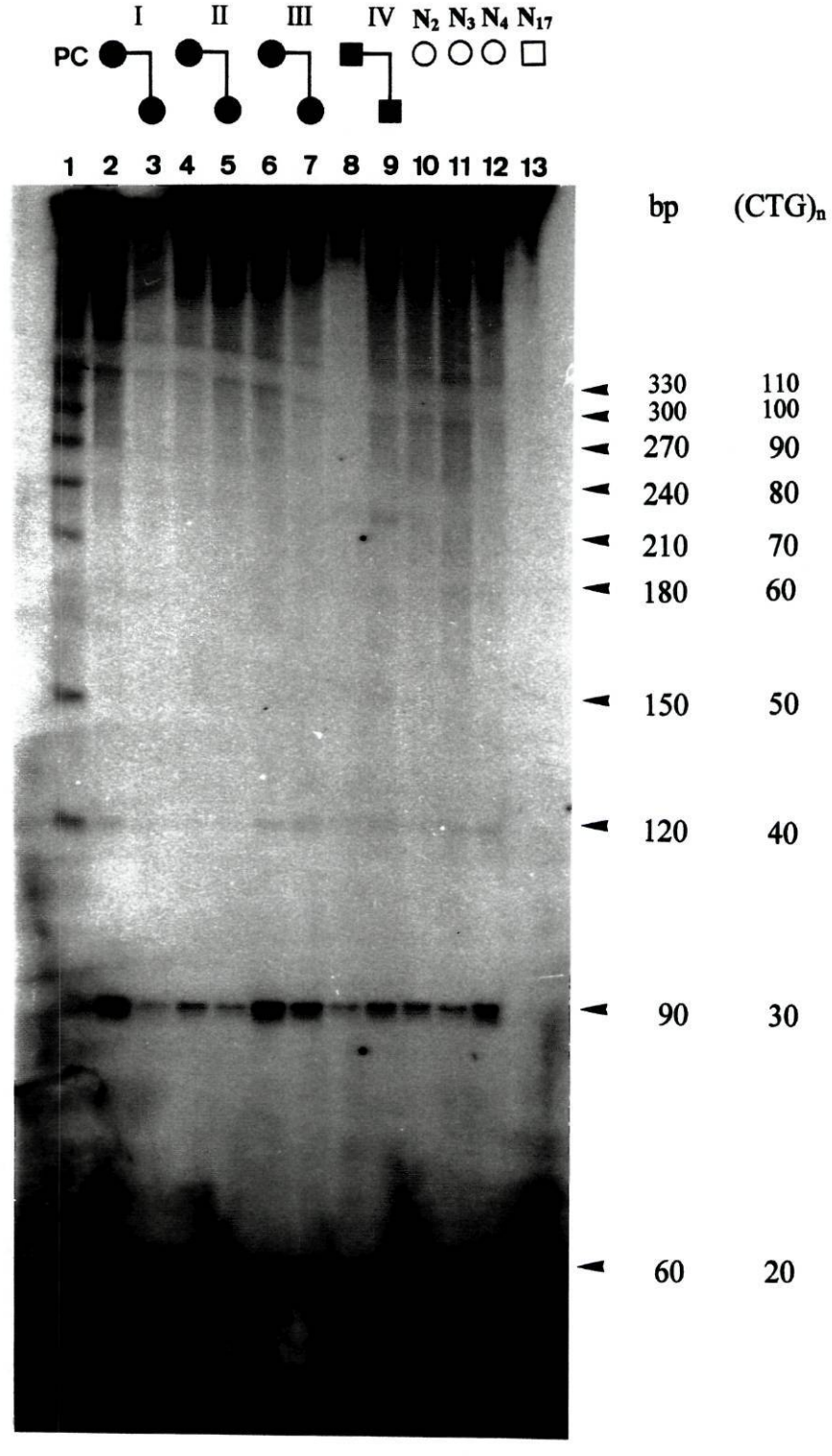

Figure 9. Autoradiograph of the RED reaction products using $(CTG)_{10}$. Lanes 2-9: FAP generational pairs; lanes 10-13: control individuals; PC: positive control. 10 μg of genomic DNA template were used in each reaction. Sizes of $(CTG)_n$ multimers are indicated in bp.

A difference in one ligation is also observed in pair II (Fig. 12, lanes 2 and 3). The accuracy of band reading at the upper portion of the gel is slightly diminished by a progressive loss of resolution which might explain the small differences found in pairs I and II.

The length of the AGG repeat could not be determined for the descendant of pair IV (Fig. 12, lane 10) and for some controls (Fig. 11, lanes 4, 5; and Fig. 12, lanes 11, 13) due to a weak cycling reaction which could not produce the necessary amount of $(AAG)_{10}$ multimers for exact size detection.

Our results show the absence of AAG/CTT repeat expansions in the second generation elements of the tested pairs, thus excluding the involvement of such mechanism as the molecular basis of genetic anticipation in these pedigrees. The maximal number of copies is consistent with previously reported studies in unrelated Caucasians (Hofferbert *et al.*, 1997) where it ranges from 252 bp to 540 bp.

AAG trinucleotide repeat expansions are associated with Friedreich's ataxia. Long stretches of the AAG repeated motif are located within an intronic sequence of the frataxin gene leading to the loss of function of the protein. The mutation-causing repeat number ranges between 200 and > 900 triplets, while the results show a triplet repeat with similar length in one patient - the progenitor of pair I - containing 200 copies, with a phenotype typical of FAP classical onset. The length of the repeat is highly polymorphic and such high copy number is not uncommon in normal individuals.

3.3. TTA / TAA TRINUCLEOTIDE

The products of the RED reaction of TTA/TAA triplet are shown in Figure 13. All samples show a ligation product of 66 base pairs corresponding to a dimer of the $(TTA)_{11}$ substrate. The samples refer to three FAP generational pairs (I, II and III) and 7 control individuals. RED reaction has produced low and unequal amounts of oligonucleotide dimers but the single band is clearly visible for all samples in which the reaction has worked. No larger multimers have been formed which is in accordance with the study by Hofferbert *et al.* (1997) where a single ligation corresponding to 72 bp was expected as the maximal repeat length determined for the 52 tested unrelated individuals. The discrepancy of size in base pairs is due to the triplet copy number of the oligonucleotide used in both RED studies.

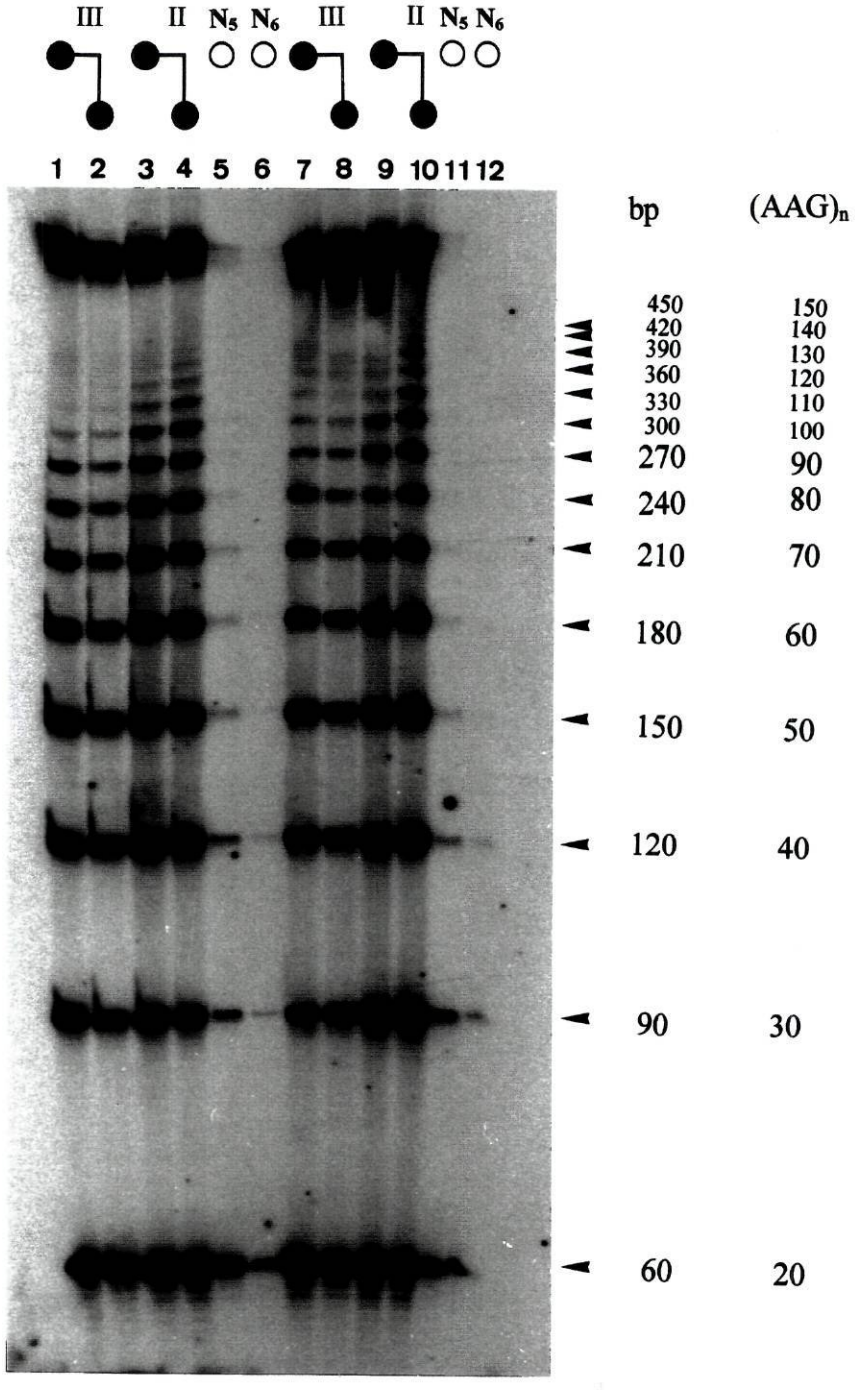

Figure 10. Autoradiograph of the RED reaction products using $(AAG)_{10}$. Lanes 1-4: FAP generational pairs; lanes 5 and 6: control individuals. The samples loaded on lanes 7 to 12 are the same but resulting from a different cycling reaction. 10 μg of genomic DNA template were used in each reaction. Sizes of $(AAG)_n$ multimers are indicated in bp. Note the reproducibility of the RED assay.

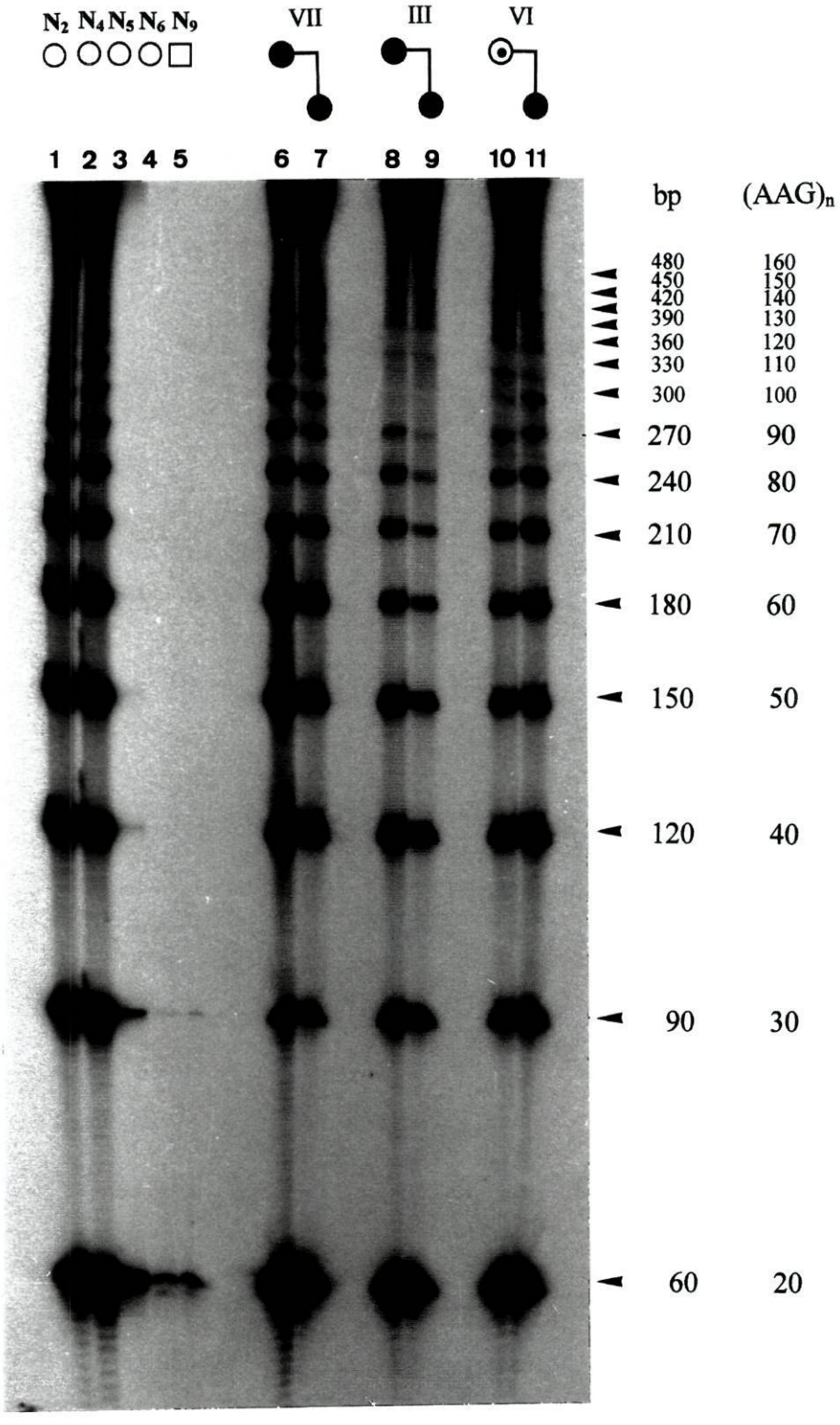

Figure 11. Autoradiograph of the RED reaction products using $(AAG)_{10}$. Lanes 1-5: control individuals; lanes 6-11: FAP generational pairs. 10 μg of genomic DNA were used in each reaction. Sizes of $(AAG)_n$ multimers are indicated in bp.

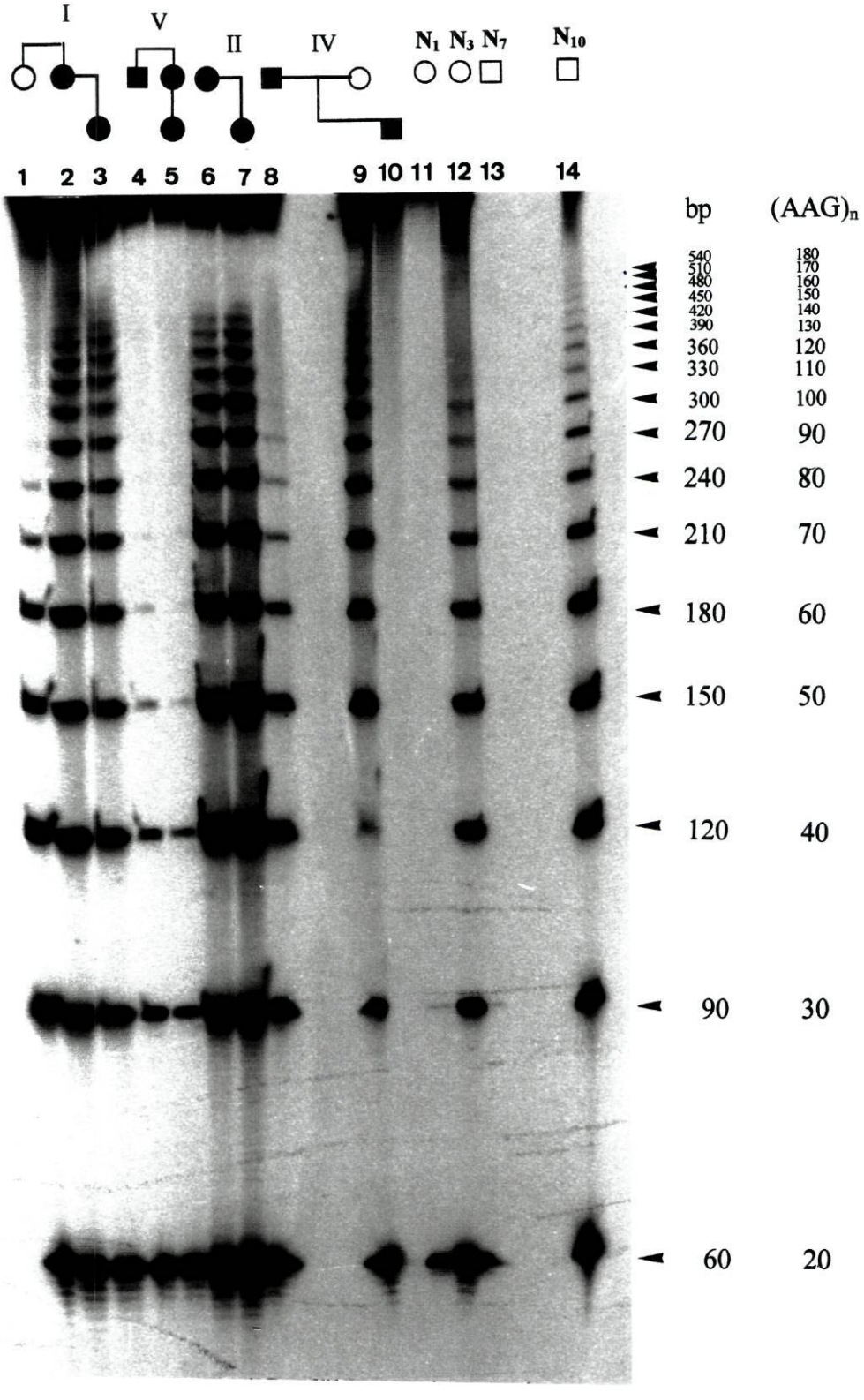

Figure 12. Autoradiograph of the RED reaction products using $(AAG)_{10}$. Lanes 1-10: FAP generational pairs; lanes 11 and 14: control individuals. 10 µg of genomic DNA template were used in each reaction. Sizes of $(AAG)_n$ multimers are indicated in bp.

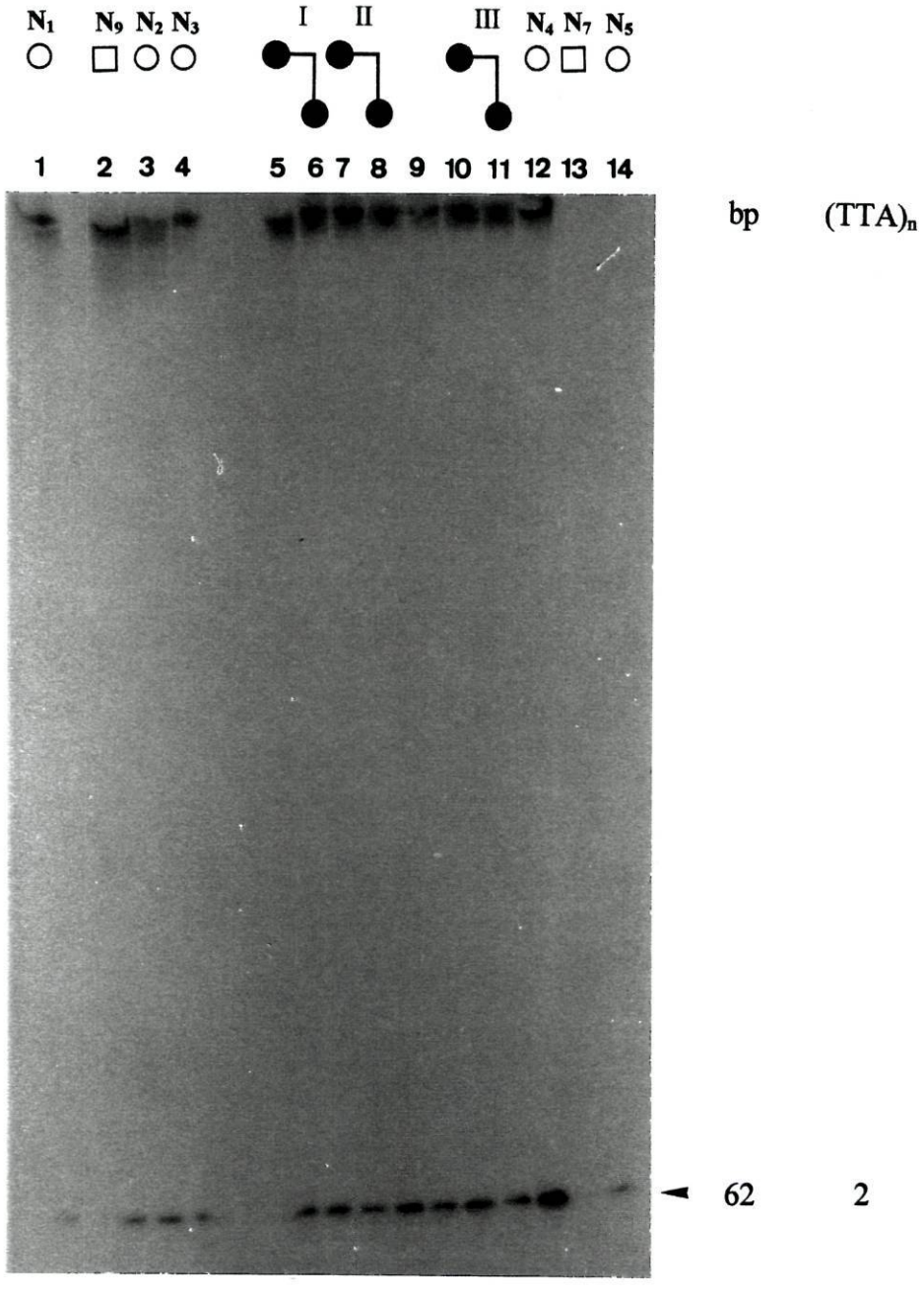

Figure 13. Autoradiograph of the RED reaction products using $(TTA)_{11}$. Lanes 1-4 and 12-14: control individuals; lanes 5-8: FAP generational pairs. 10 μg of genomic DNA template were used in each reaction. A single ligation product is seen in all samples. Sizes of $(TTA)_n$ multimers are indicated in bp.

3.4. AGT / ACT TRINUCLEOTIDE

Genomic DNA of all FAP kindred pairs and 12 negative controls were used to determine AGT/ACT repeat lengths. Autoradiographs of AGT triplet RED reaction products are shown in Figures 14 and 15. The $(AGT)_{14}$ oligonucleotide was ligated only once in every sample, giving rise to a single band of 84 bp (28 repeats). The resulting products are clearly visible except in 2 control samples (Figure 14, lane 15; and Figure 15, lane 12) presenting faint bands. The descendant of pair VII (lane 8 of Figure 14) exhibits a different pattern of migration, observed in almost every reaction, characterized by a slower mobility throughout the gel which would accelerate further down to end ahead of all other sample bands. This phenomenon is probably due to the high viscosity of that particular DNA sample. The irregular bands observed in the film of Figure 15 reflect irregularities of the polyacrylamide gel when the nylon membrane was laid for DNA transfer, rather than different mobility patterns of the reaction products.

AGT/ACT trinucleotide repeats have not been associated with any triplet repeat expansion disorder, and seem not to be polymorphic in size as observed in 57 unrelated Caucasian individuals, with a constant size of about 72 bp (one single ligation), using the RED method (Hofferbert *et al.*, 1997). Our results support this view, although the size of the repeated motif has been determined to be slightly different due to the oligonucleotide number of repeated triplets.

3.5. CGT / ACG TRINUCLEOTIDE

The CGT/ACG repeat, tested with a $(CGT)_{14}$ oligonucleotide, was difficult to detect by the RED method. Figures 16 and 17 illustrate the RED reaction results in seven FAP generational pairs and 8 unrelated negative controls. The single oligonucleotide species appears at the bottom of the gel as large blots overlapping with several bands corresponding to ligation products of oligonucleotide fragments. The existence of such small products is indicative of oligonucleotide impurity which may have been responsible for the formation of very few ligated molecules during the cycling reaction. All samples show a faint band resulting from a single ligation and corresponding to 28 repeated triplets (84 bp). Hofferbert *et al.* (1997) have shown

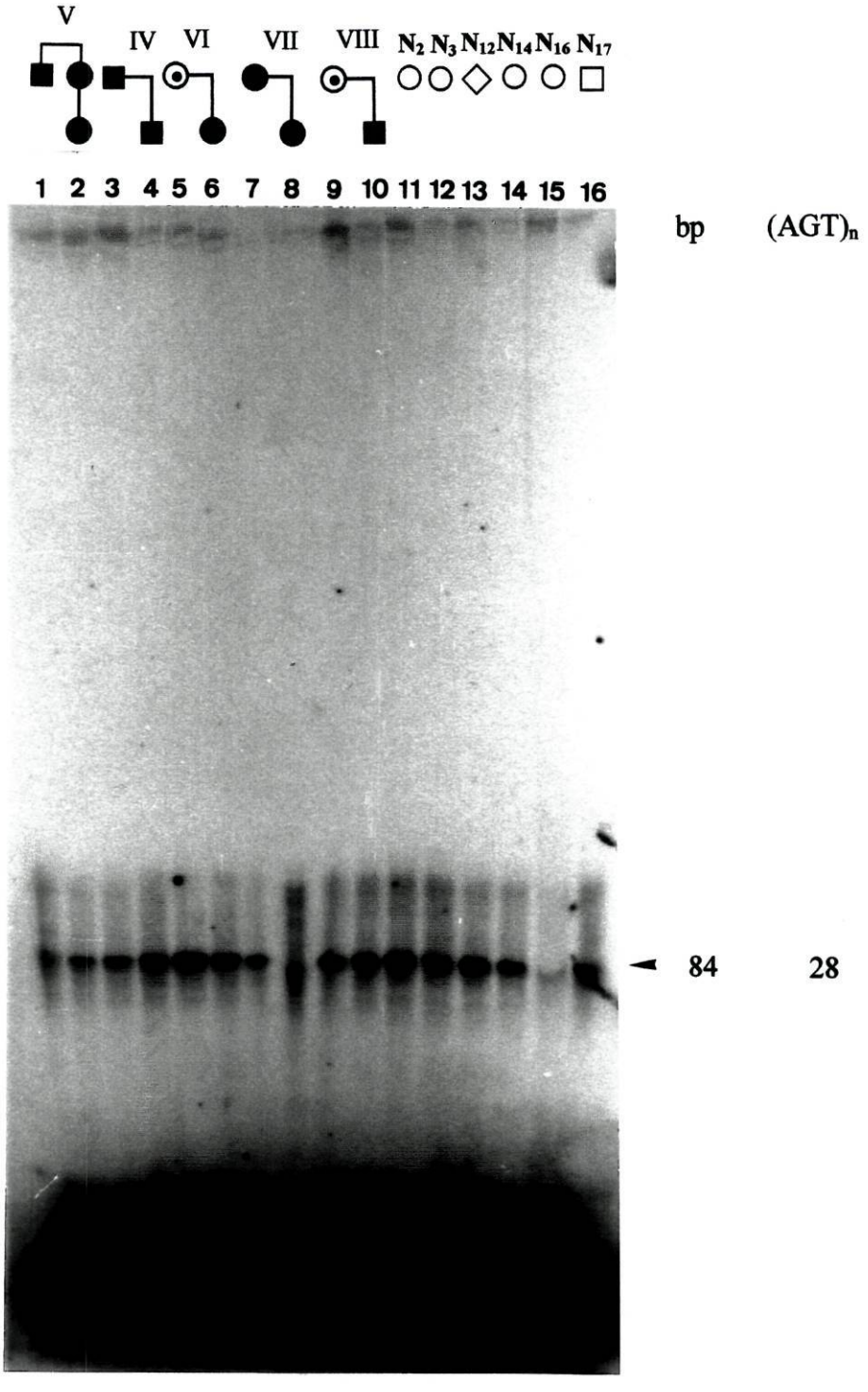

Figure 14. Autoradiograph of the RED reaction products using $(AGT)_{14}$. Lanes 1-10: FAP generational pairs; lanes 11 and 16: control individuals. 10 μg of genomic DNA template were used in each reaction. A single ligation product is seen in all samples. Sizes of $(AGT)_n$ multimers are indicated in bp.

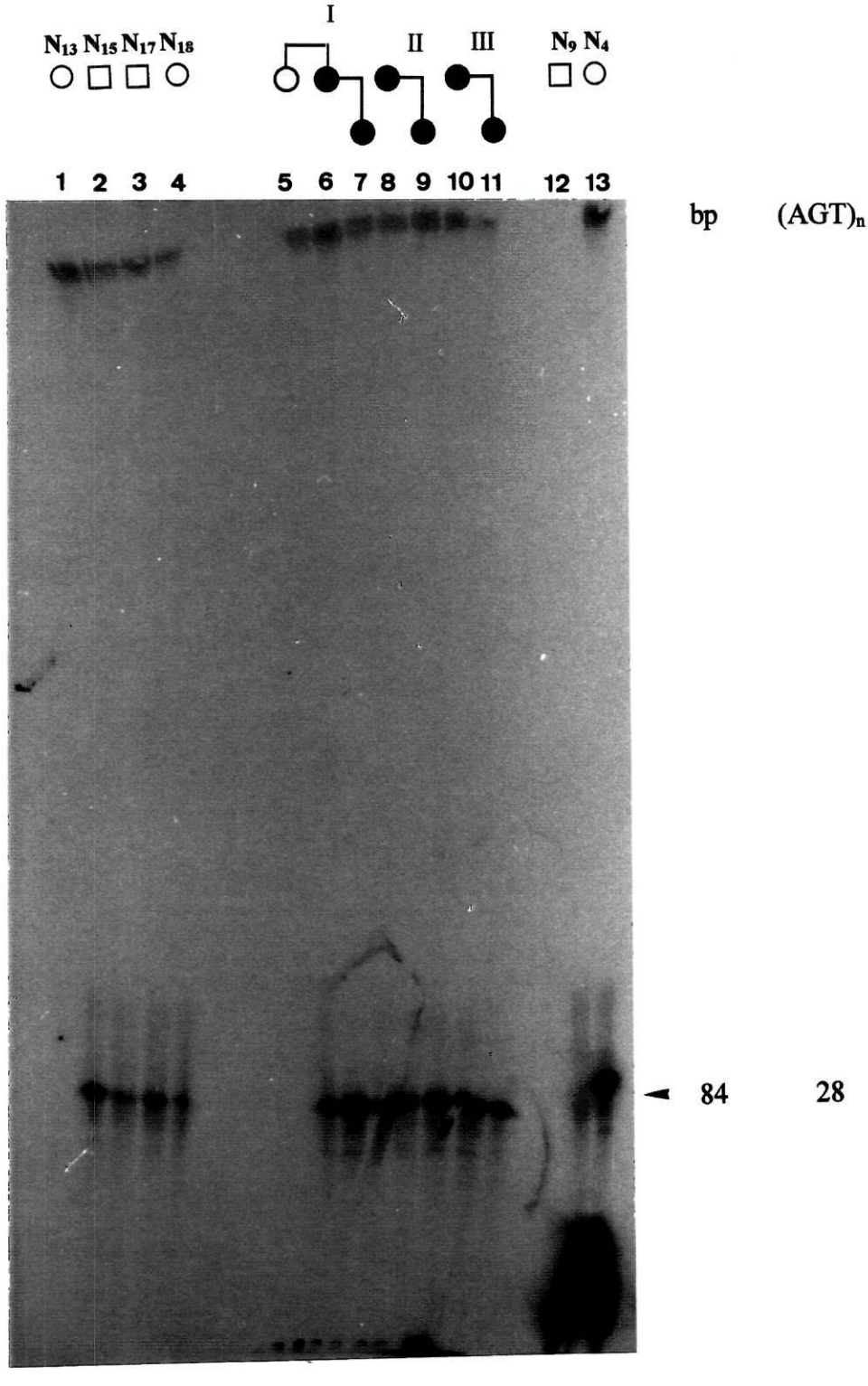

Figure 15. Autoradiograph of the RED reaction products using $(AGT)_{14}$. Lanes 1-4 and 12, 13: control individuals; lanes 5-11: FAP generational pairs. 10 μg of genomic DNA template were used in each reaction. A single ligation product is seen in all samples. Sizes of $(AGT)_n$ multimers are indicated in bp.

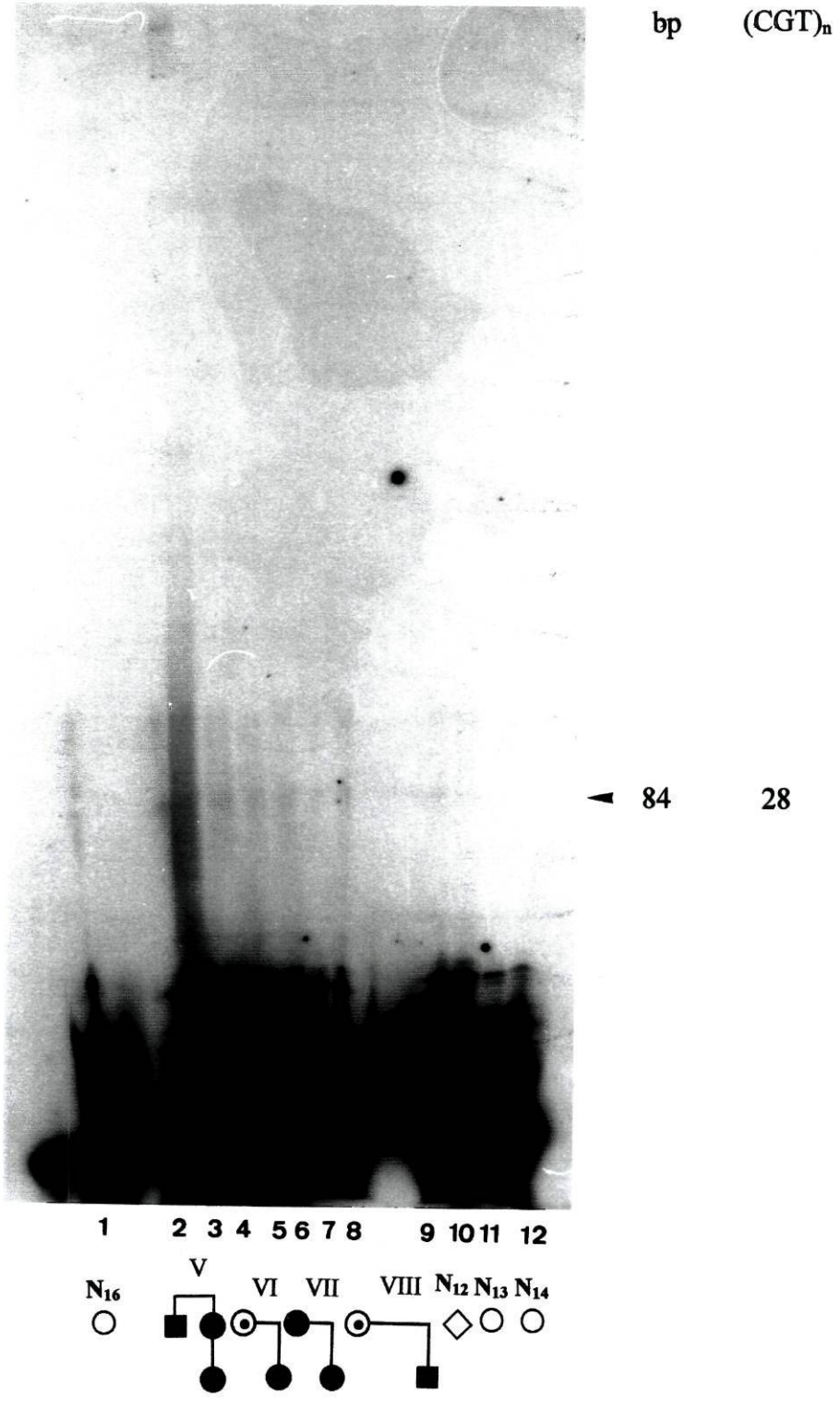

Figure 16. Autoradiograph of the RED reaction products using $(CGT)_{14}$. Lanes 2-9: FAP generational pairs; lanes 1 and 9-12: control individuals. 10 μg of genomic DNA template were used in each reaction. A faint band corresponding to a single ligation product is visible in all samples and is indicated by the arrow. The other bands result from the ligation of oligonucleotide fragments. Sizes of $(CGT)_n$ multimers are indicated in bp.

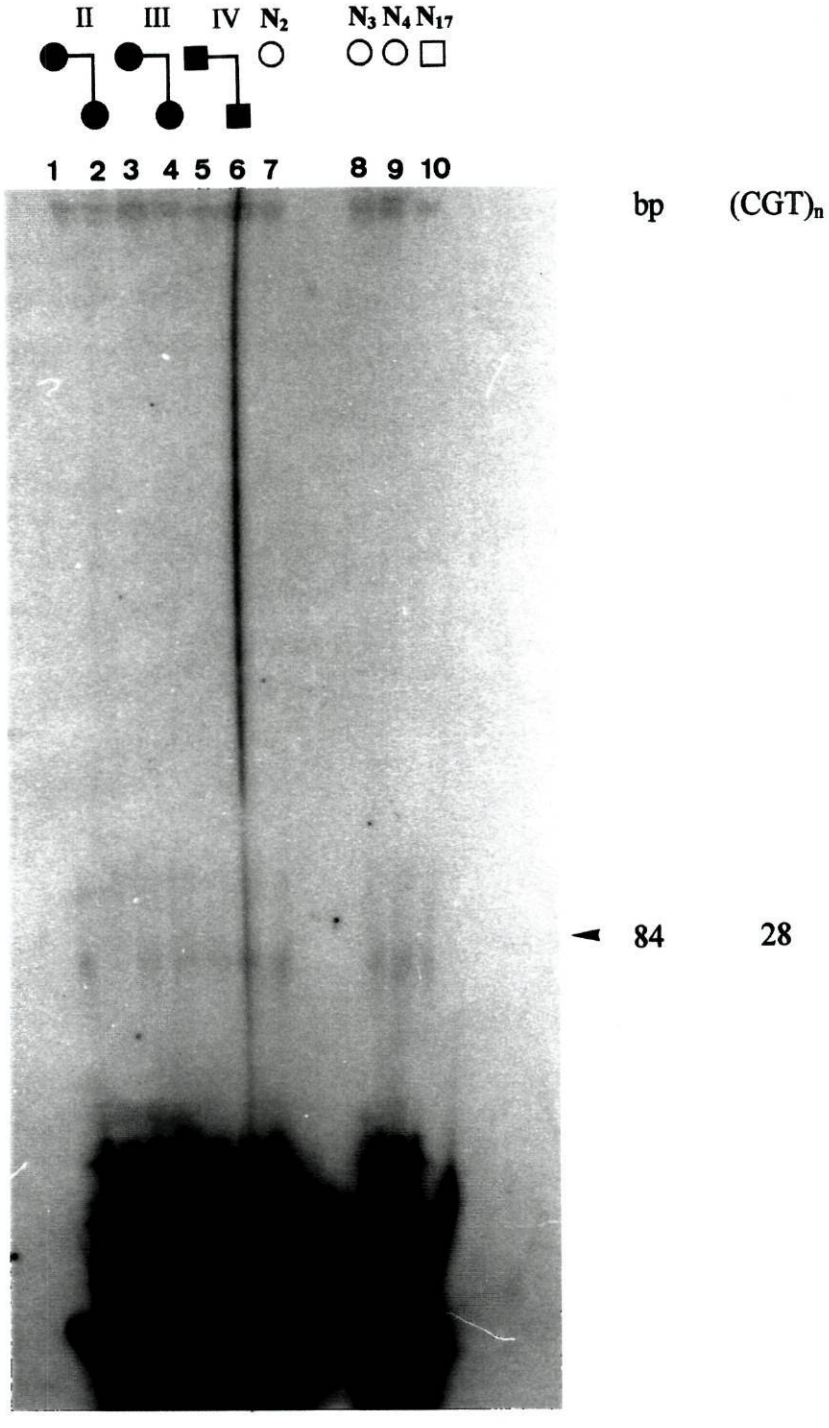

Figure 17. Autoradiograph of the RED reaction products using $(CGT)_{14}$. Lanes 1-6: FAP generational pairs; lanes 7-10: control individuals. 10 μg of genomic DNA template were used in each reaction. A faint band corresponding to a single ligation product is visible in all samples and is indicated by the arrow. The other bands result from the ligation of oligonucleotide fragments. Sizes of $(CGT)_n$ multimers are indicated in bp.

a maximal size for CGT trinucleotide repeat of approximately 60 bp in 54 unrelated individuals, again the result of a single ligation of the oligonucleotide substrate [$(CGT)_{10}$]. Our results cannot exclude the existence of longer segments of repeated CGT triplets in the analyzed DNA samples, but the existing literature reports repeat lengths of less than 90 bp - 30 copies - of the repeated motif (Lindblad *et al.*, 1994; Hofferbert *et al.*, 1997) . Moreover, only one band resulting from a dimer formation has been consistently observed in all our experiments with this particular oligonucleotide.

3.6. CGG / CCG TRINUCLEOTIDE

Expansion of CGG triplets is associated with the fragile X syndrome leading to the loss of function of FMR1 protein (Pieretti *et al.*, 1991). Disease-causing trinucleotide repeat lengths range from 200 triplets up to thousands of copies of the repeated motif. Premutation alleles, i.e., unstable triplet repeat segment-containing alleles exhibit a range of ~ 50 to 200 trinucleotide copies.

The DNA from eight generational pairs and 8 normal individuals has been analysed for the presence of CGG repeat expansions. The results presented in Figures 18, 19 and 20 show the presence of a single band corresponding to a segment of 22 repeats (66 bp) in almost every sample. The RED reaction has not produced detectable amounts of substrate oligonucleotide dimers in 4 control individuals (Fig. 18, lane 12; Fig. 19, lanes 11, 12 and 13), in the second generation element of pair V and the progenitor of pair VIII (Fig. 20, lanes 3 and 8). Some of these samples exhibit a "smirr" throughout the gel, suggesting migration irregularities in addition to the weak repeat detection reaction.

The absence of larger multimers of the $(CGG)_{11}$ oligonucleotide substrate is shown in every film, excluding the presence of another of the "potential candidates" in the tested FAP kindreds. Previous reports on CGG trinucleotide repeat lengths have revealed distinct fragments of 60 bp in 36 unrelated Caucasians and putative larger fragments of 90 to 120 bp in some of them (Hofferbert *et al.*, 1997). This particular trinucleotide is difficult to detect by the RED method presumably due to secondary structure formation (Pearson and Sindren, 1996) or other processes that inhibit hybridization of the oligonucleotides. In order to resolve the former problem, we have added betaine to all the reaction mixtures to prevent impairment of oligonucleotide annealing resulting from hairpin or other complex DNA conformations.

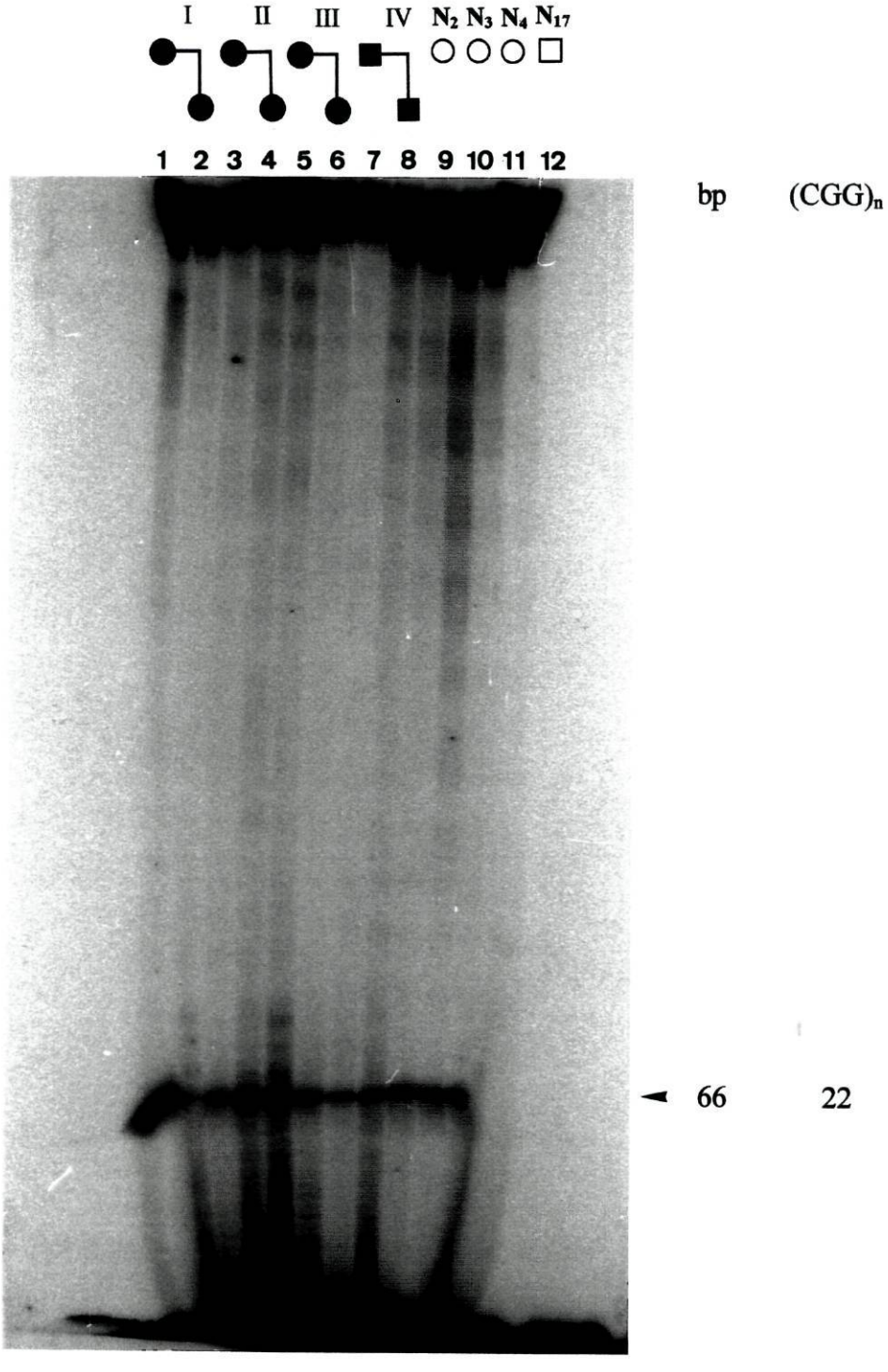

Figure 18. Autoradiograph of the RED reaction products using $(CGG)_{11}$. Lanes 1-8: FAP generational pairs; lanes 9-12: control individuals. 10 µg of genomic DNA template were used in each reaction. A single ligation product is seen in almost every sample. Sizes of $(CGG)_n$ multimers are indicated in bp.

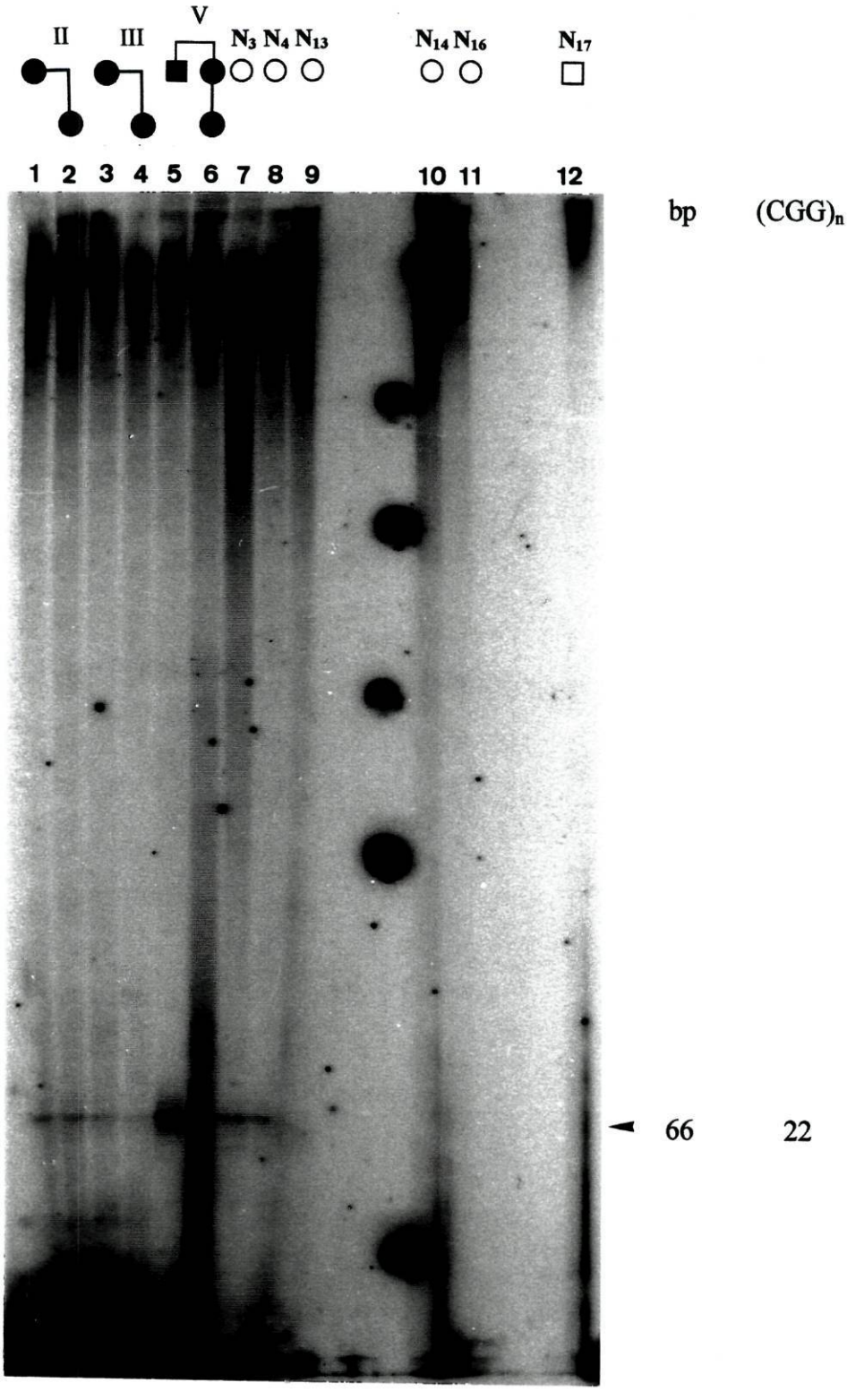

Figure 19. Autoradiograph of the RED reaction products using $(CGG)_{11}$. Lanes 1-6: FAP generational pairs; lanes 7-12: control individuals. 10 μg of genomic DNA template were used in each reaction. A single ligation product is seen in lanes 1-6 and 7; the remaining reactions did not work. Sizes of $(CGG)_n$ multimers are indicated in bp.

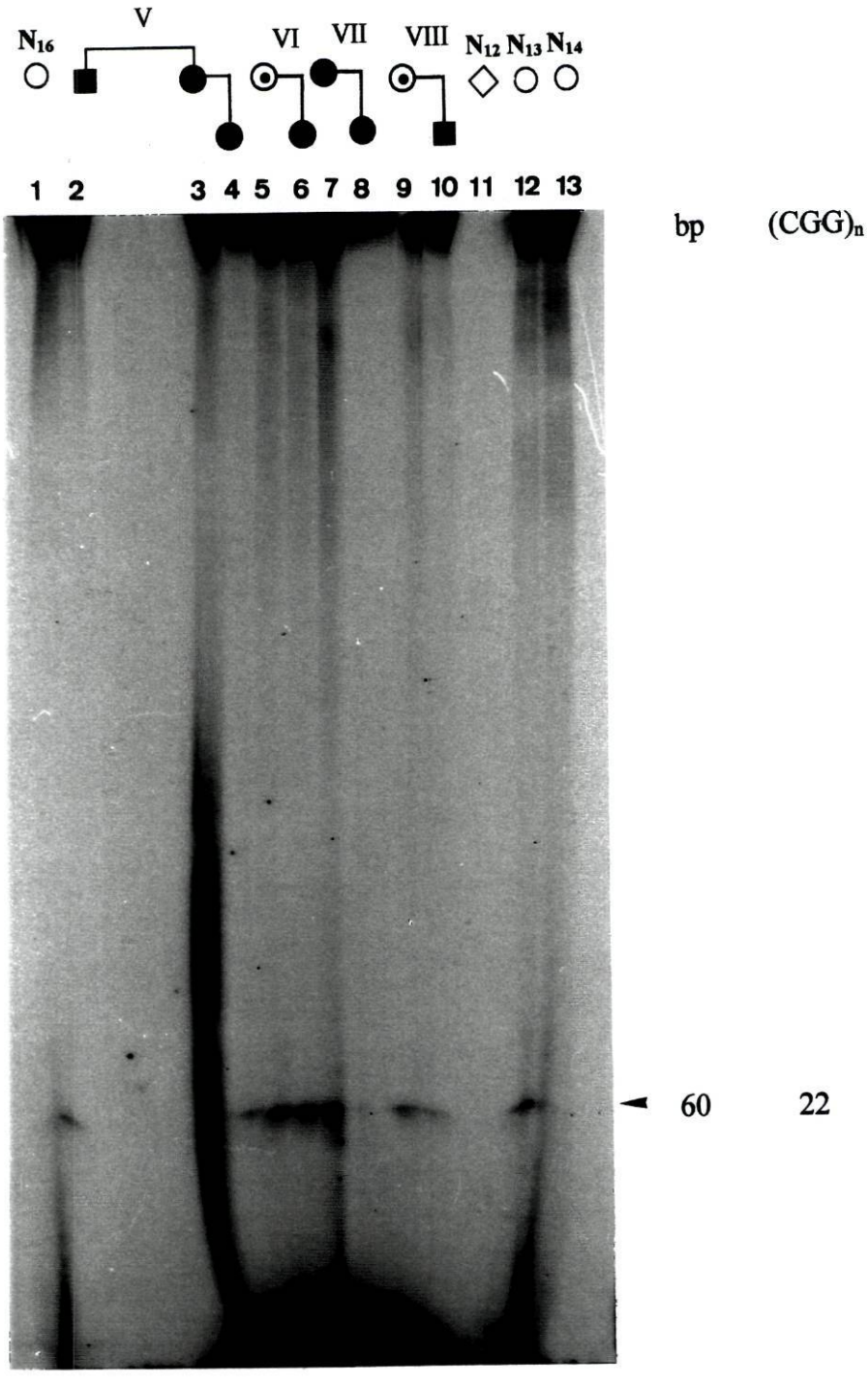

Figure 20. Autoradiograph of the RED reaction products using $(CGG)_{11}$. Lanes 1 and 11-13: control individuals; lanes 2-10: FAP generational pairs. 10 μg of genomic DNA template were used in each reaction. A single ligation product is seen in all samples except in lane 3. Sizes of $(CGG)_n$ multimers are indicated in bp.

3.7. TGG / CCA TRINUCLEOTIDE

The maximal repeat size obtained for TGG/CAA trinucleotide ranged between 72 bp (24 repeats) and 144 bp (48 repeats) corresponding to the formation of dimers to tetramers of the oligonucleotide substrate, respectively (Figures 21 and 22). The exact repeat sizes were difficult to assess in all samples since the RED reactions have generated weak signals.

All generational pairs and eleven control individuals were used for triplet repeat size detection. Distinct fragments of 72 bp (24 repeated units) could be detected in all samples. In pairs I to III, fragments of 108 bp and 144 bp could also be seen (Figure 21, lanes 2-7). Pair IV also presented the 108 bp band and a putative larger fragment of 144 bp (48 repeated triplets) (lanes 7 and 8 of Figure 22). All the other pairs exhibit a weak signal and the presence of larger multimers of $(TGG)_{12}$ cannot be excluded (data not shown). The control DNA samples were found to have repeat lengths of 72 and 108 bp resulting from the ligation of 2 or 3 oligos, respectively. The faint bands observed for the control samples and for those of pairs V to VIII are suggestive of a low yield reaction, perhaps producing an incorrect estimate of the genomic trinucleotide repeat length. In any case, our results do not show any difference in repeat size within the FAP generational pairs, thus excluding the presence of TGG trinucleotide unstable expansions from one generation to the other. Moreover, the estimated sizes ranges are in accordance with previous studies where the repeat lengths vary between 120 and 210 bp (Lindblad *et al.*, 1995; Hofferbert *et al.*, 1997).

3.8. ATG / CAT TRINUCLEOTIDE

The results of ATG/CAT trinucleotide repeat size detection are shown in Figures 23 and 24. Seven FAP kindred pairs and 10 control individuals present maximal repeat sizes ranging between 24 repeated triplets (72 bp) and 48 repeats (144 bp). All pairs exhibit 3 bands corresponding to the ligation of four $(ATG)_{12}$ oligonucleotides (144 bp), the third band being very faint in pair VI due to a weaker reaction (Figure 24, lanes 5, 6). Repeat detection was not successful above the 72 bp fragment for pair V and the progenitor of pair VIII, and the 108 bp fragment for the descendent of pair VII (lanes 4, 5, 10 and 8 of Figure 24, respectively). It is clear by comparison with the signals obtained for all the other samples that formation of

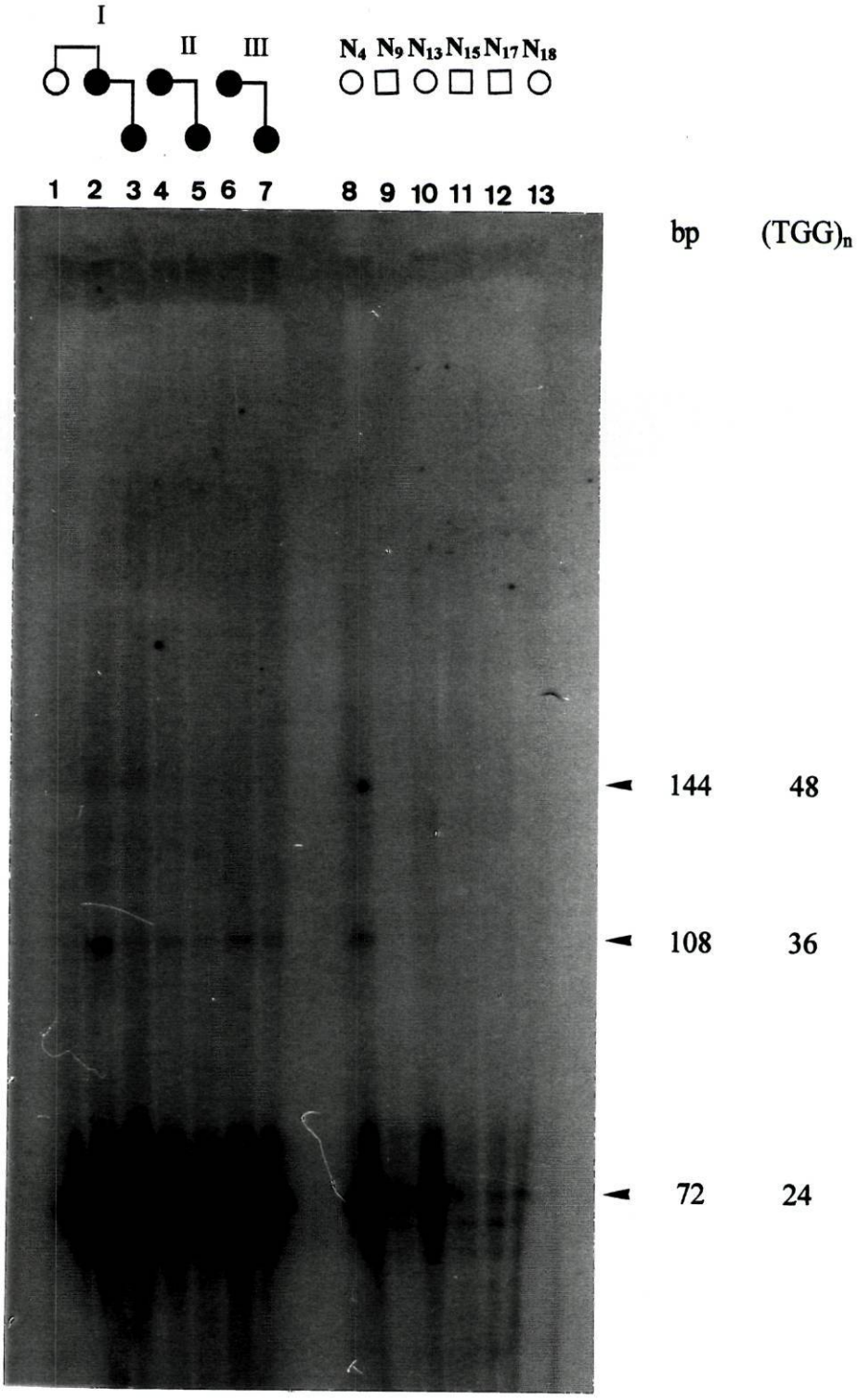

Figure 21. Autoradiograph of the RED reaction products using $(TGG)_{12}$. Lanes 1-7: FAP generational pairs; lanes 8-13: control individuals. 10 μg of genomic DNA template were used in each reaction. Note the reduced $(TGG)_{12}$ multimer formation in lanes 9 and 11-13. Sizes of $(TGG)_n$ multimers are indicated in bp.

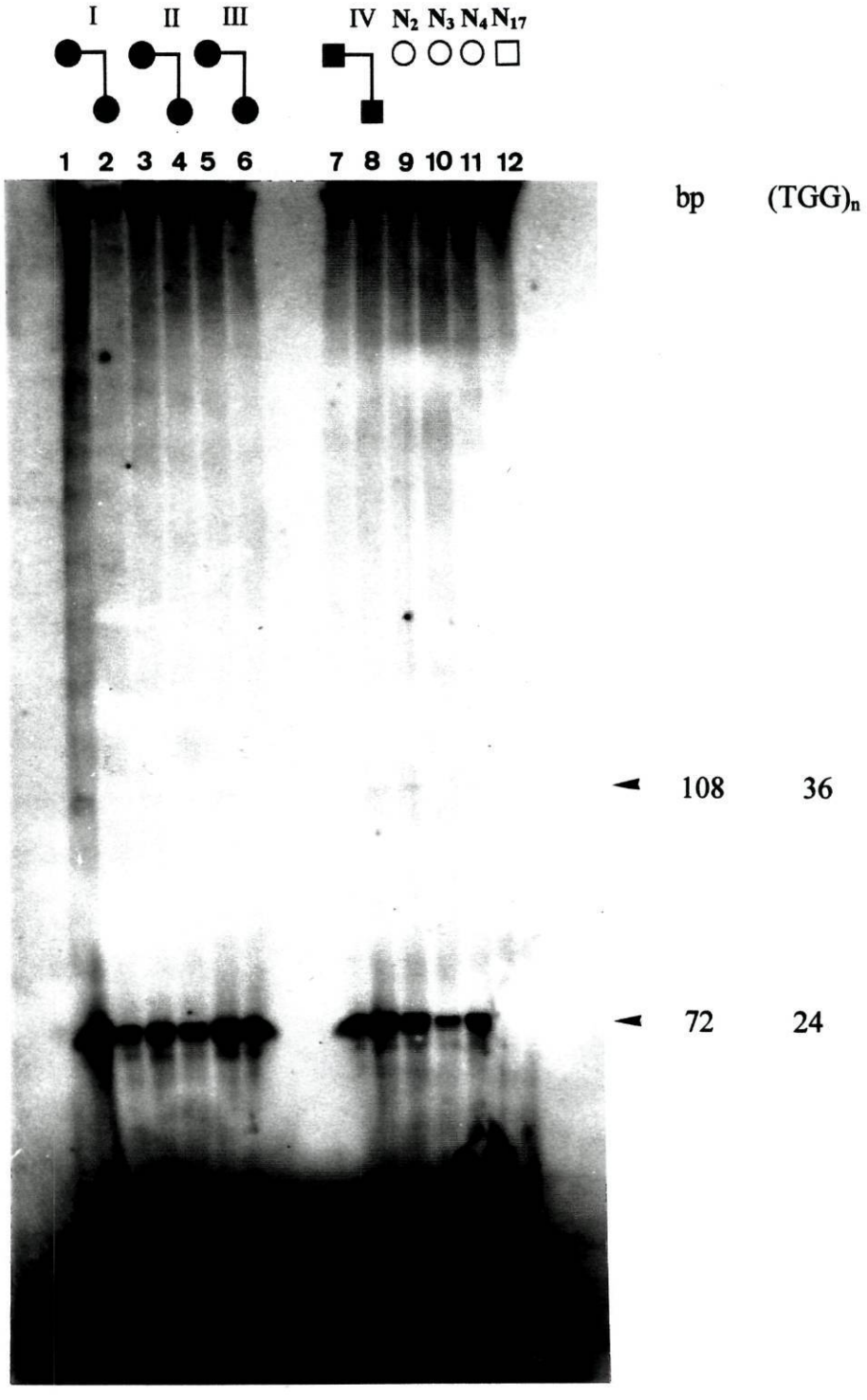

Figure 22. Autoradiograph of the RED reaction products using $(TGG)_{12}$. Lanes 1-8: FAP generational pairs; lanes 9-12: control individuals. 10 μg of genomic DNA template were used in each reaction. Sizes of $(TGG)_n$ multimers are indicated in bp.

oligonucleotide multimers was much lower in the referred individuals, suggesting that the same sizes would be detected if the cycling reaction efficiency had been the same. Identical results were obtained with the control DNA samples where the reaction-generated $(ATG)_{12}$ multimers are clearly insufficient for the correct genomic repeat length detection. Hofferbert *et al.* (1997) have reported a distribution of maximal repeat sizes in 31 unrelated individuals ranging between 60 bp and 240 bp, with a high frequency of 120 bp repeat-containing positives. The repeat lengths obtained in our experiments fall in the medium range of the reported size spectrum.

The triplet repeat lengths are conserved within each tested generational pair arguing against the involvement of an ATG trinucleotide expansion as the responsible mechanism for genetic anticipation in these FAP patients.

3.9. GTT / AAC TRINUCLEOTIDE

The GTT/AAC triplet repeat was difficult to detect by the RED method. The repeat detection reactions have not produced detectable amounts of $(GTT)_{15}$ oligonucleotide multimers in most of the experiments, resulting in the absence of ligation-corresponding bands. Figure 25 represents the only reaction in which a single band was obtained. It is not possible, however, to determine if it corresponds to the oligonucleotide itself or to the product of a single ligation referring to a 90 bp fragment (30 repeats). The shape of the bands more resembles the product of a dimer formation rather than a single oligonucleotide species, which would be in accordance with the reported sizes for this triplet in 54 unrelated Caucasians where the maximal repeat length was 90 bp (Hofferbert *et al.*, 1997). If that is the case, our results shown the absence of any differences in size distribution among the members of four FAP generational pairs (V to VIII) and 5 control individuals, excluding GTT/AAC repeat expansions as the molecular basis for the phenomenon of anticipation.

3.10. CCT / AGG TRINUCLEOTIDE

The detection of CCT/AGG genomic repeat lengths by the RED method was a technical failure. Multimers of $(CCT)_{15}$ oligonucleotide substrate were never formed in any

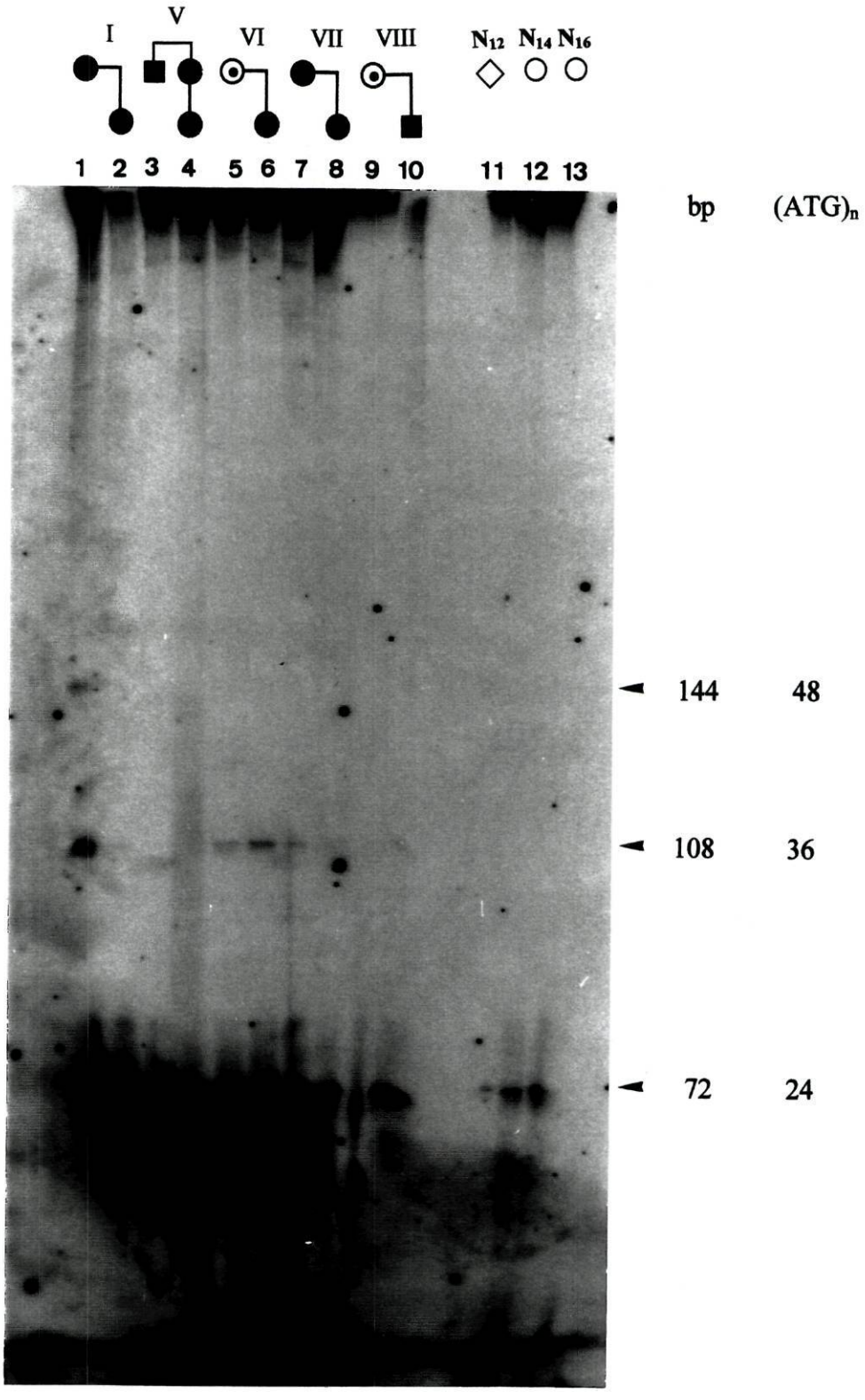

Figure 23. Autoradiograph of the RED reaction products using $(ATG)_{12}$. Lanes 1-10: FAP generational pairs; lanes 11-13: control individuals. 10 μg of genomic DNA template were used in each reaction. Note the reduced $(ATG)_{12}$ multimer formation in lanes 9 and 11-13. Sizes of $(ATG)_n$ multimers are indicated in bp.

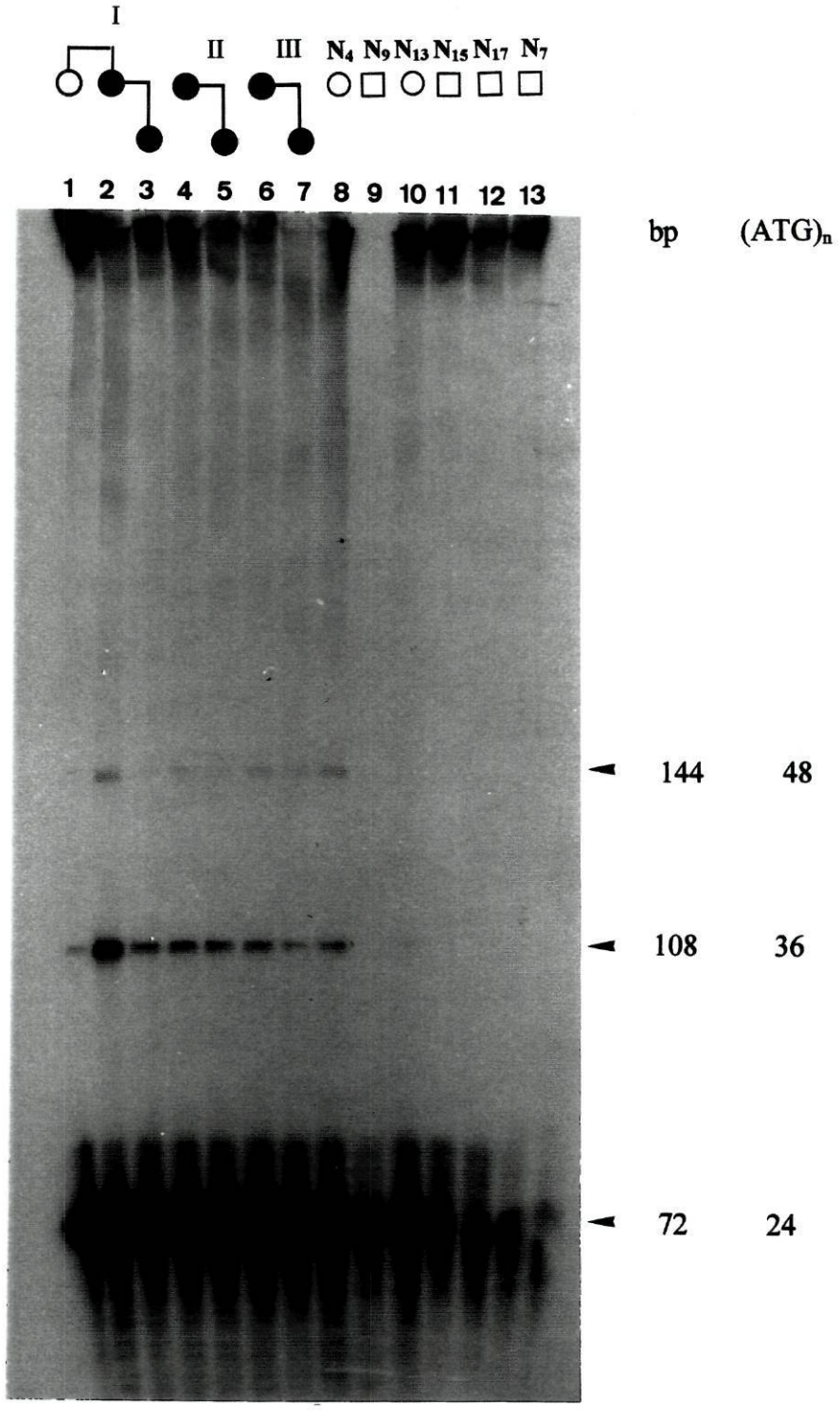

Figure 24. Autoradiograph of the RED reaction products using $(ATG)_{12}$. Lanes 1-7: FAP generational pairs; lanes 8-13: control individuals. 10 µg of genomic DNA template were used in each reaction. Note the reduced $(ATG)_{12}$ multimer formation in lanes 9 and 11-13. Sizes of $(ATG)_n$ multimers are indicated in bp.

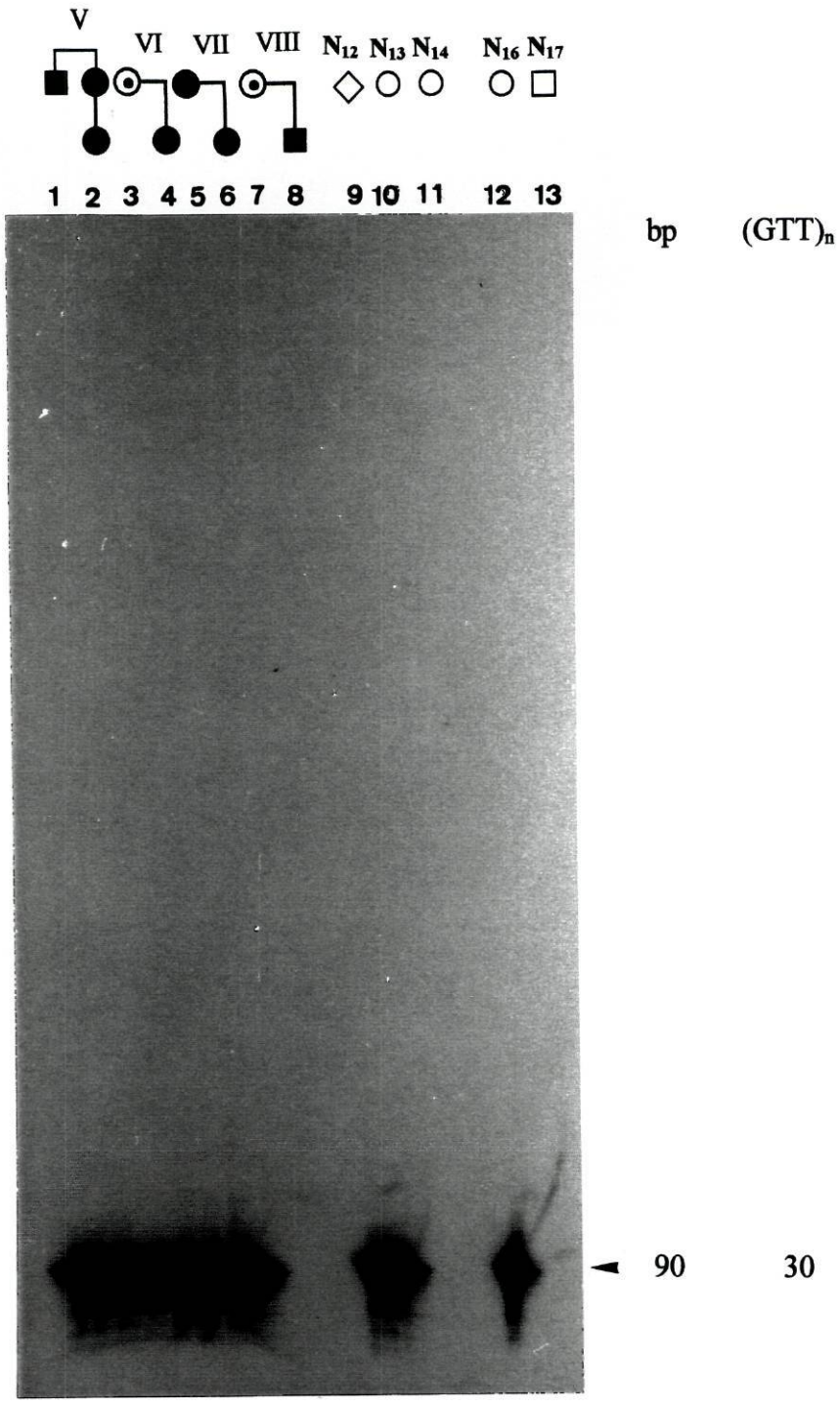

Figure 25. Autoradiograph of the RED reaction products using $(GTT)_{15}$. Lanes 1-8: FAP generational pairs; lanes 9-13: control individuals. 10 μg of genomic DNA template were used in each reaction. The band seen in all samples resembles the product of one ligation, but it can also correspond to the single oligonucleotide species. The size of the putative $(GTT)_{15}$ dimer is indicated in bp.

experiment and only one band corresponding to the single oligonucleotide species was clearly visible in all RED reactions (data not shown), ruling out radiolabeled probe hybridization problems. The reasons why repeat detection has not been possible are not known, however it is most likely that the problem lies on the cycling reaction itself where the oligonucleotide annealing with the genomic DNA might have been impaired. Oligonucleotide impurity, for example, could be the origin for the reaction failure.

The absence of any results prevent us to draw any conclusion about this trinucleotide involvement in genetic anticipation in the tested kindreds, however the expected maximal repeat sizes for this triplet, as determined by RED in 35 unrelated individuals, is 90 bp corresponding to a trimer of the used oligonucleotide substrate (Hofferbert, 1997).

Chapter III

General Discussion and Conclusions

General Discussion and Conclusions

While the initially described disorders caused by trinucleotide repeat expansions (TREs) were limited to CGG/CCG and CTG/CAG repeats, the recent discovery of an intronic GAA repeat expansion as the cause of Friedreich's ataxia has shown that other TREs exist and have disease-causing potential. The genetic instability of the expanded triplets results in increased precocity, severity or penetrance in successive generations, a phenomenon known as genetic anticipation. Indeed, eleven of the twelve triplet repeat-associated disorders exhibit a marked anticipation of the age-at-onset, and/or increased severity of symptoms over generations. The number of trinucleotide repeats appears to be correlated with the age of onset as increased repeat copy number results in phenotypic anticipation. Anticipation cannot be seen in autosomal recessive disorders, because they usually affect only a single generation, which makes FA the sole disorder with little evidence for this phenomenon. Since dynamic mutations are the only known molecular mechanism explaining genetic anticipation, other neurological or neuropsychiatric disorders with dominant inheritance and obvious anticipation are being intensively investigated for associated dynamic mutations.

FAP is a potential candidate for a dynamic mutation-associated disorder, as a neurological disease with autosomal dominant inheritance exhibiting genetic anticipation. However, it is more than likely that such a mutation would occur in a modifier gene (or genes), rather than the TTR gene. The TTR gene does not contain repeated motifs apart from two short $(CA)_n$ stretches in the regulatory sequences of the 5' untranslated region (Sakaki, 1989). Nonetheless, the existence of modifier genes underlying phenotypic heterogeneity in FAP, particularly manifested as anticipation of the age-at-onset, is a possibility. To address this question we have used the Repeat Expansion Detection (RED) technique that allows a genome-wide analysis for TREs (Schalling *et al.*, 1993; Sirugo *et al.*, 1995). The RED assay has been applied to search for triplet expansions in common neuropsychiatric disorders with anticipation (Lindblad *et al.*, 1995; Morris *et al.*, 1995), and to determine triplet repeat size polymorphisms in individuals of several populations (Watkins *et al.*, 1995; Hofferbert *et al.*, 1997; Nakamoto *et al*, 1997). For instance, Carrero-Valenzuela *et al.* (1995) have excluded the association of familial Parkinson's disease with CAG/CTG expansions, using the RED technique.

We have scanned eight generational pairs of Portuguese FAP kindreds exhibiting marked anticipation of the age-at-onset (the differences within the pairs being greater than 12 years), and 19 unrelated healthy Portuguese individuals, for the existence of intergenerational instability of the ten possible triplet repeats.

Our experiments have defined the triplet repeat sizes for 8 out of the 10 trinucleotides. The determined repeat lengths fall in the range of the expected size spectrum when compared with previously reported data (Lindblad *et al.*, 1994 and 1995; Schalling *et al.*, 1993; Hofferbert *et al.*, 1997; Nakamoto *et al.*, 1997). The RED reactions testing CTG, CGG, and AAG triplets - the known disease-causing motifs - have produced large amounts of oligonucleotide multimers allowing good detection of the repeated triplet sizes in all screened individuals. Our analysis of genomic DNA from 9 FAP generational pairs and 8 unrelated control individuals for CTG/CAG triplet repeats (expanded in 8 neurodegenerative disorders), revealed a high frequency of 120 bp repeat-containing positives, the repeat size being exactly the same within the FAP kindred pairs. Only one pair was found to exceed this copy number, albeit without exceeding the expected repeat length for normal alleles or exhibiting intergenerational expansion of the triplet repeat. The use of a positive control DNA sample containing a very long segment of CTG repeats has proved the detectability of expansion of a single copy allele under our experimental conditions.

For the CGG repeat, the cause of fragile X syndrome, a small repeat size corresponding to 22 trinucleotide copies was detected in nearly all screened individuals. Difficulties in detecting repeated CGG triplets were reported by Hofferbert *et al.* (1997) being attributed to secondary structure formation on template DNA with (C+G)-rich repeats (Person and Sinden, 1996). We circumvented that problem by using betaine, a reagent that weakens C-G annealing, preventing the formation of secondary structures that could inhibit oligonucleotide hybridization to the single stranded genomic template. The CGG repeat length determined in our experiments is similar to previously reported data (Hofferbert *et al.*, 1997) and clearly shows the absence of any unstable expansion in the screened FAP individuals.

For the AAG repeat - expanded in FA - large maximal repeat sizes have been detected, particularly in the screened FAP individuals. The $(AAG)_{10}$ oligonucleotide revealed repeat lengths up to 600 bp (200 repeats). The presence of large multimers close to the upper resolution of the gel was carefully assessed in order to determine the exact repeat sizes of the samples. Moreover some samples were scanned in duplicate to ascertain the determined sizes. Although the band readings are relatively difficult in that particular portion of the gel, no

differences in size were observed among the members of each pair, with the exception of pairs I and II, presenting a difference of a single oligonucleotide ligation favoring a contraction and an expansion, respectively. These results allow us to exclude the existence of unstable expansions in second generation individuals of the FAP pairs.

TTA, AGT, and CGT trinucleotides have been shown to present monomorphic repeat lengths, clearly ruling out anticipation-related intergenerational instability in the scanned FAP kindred pairs. RED reaction experiments with the CGT oligonucleotide have produced a very weak signal. The low yielding reactions are most certainly caused by oligonucleotide contaminants, as previously mentioned, expressed by the detection of very small fragments of the oligo substrate either alone or ligated, forming longer single stranded molecule species and appearing as a ladder below the CGT dimer position. The faint band immediately over the 84 bp fragment corresponding to the single ligation also results from the same contamination. As it will be discussed later, RED technique is very sensitive to the purity of the oligonucleotides and genomic DNA templates.

When compared to previously reported data, the frequency of trinucleotide repeats at or above discrete lengths seemed to by similar for TGG and ATG. The determined triplet repeat lengths fall in the polymorphic size range spectrum observed in healthy unrelated Caucasians (Hofferbert *et al.*, 1997), and are identical within each scanned FAP generational pair arguing against the involvement of unstable expansions in these kindreds.

Our analysis of genomic DNA from the FAP kindred pairs and control individuals was unsuccessful for GTT and CCT triplets and did not allow the determination of maximal triplet repeat sizes. A doubt remains regarding GTT trinucleotide RED results since the single band obtained in one experiment might correspond to the ligation of the $(GTT)_{15}$ substrate, rather than to the oligonucleotide monomer.

The power and limitations of the RED assay have been evaluated by Hofferbert *et al.* (1997). A dilution series of cloned trinucleotide repeats with known sizes has defined 10 μg as the required amount of DNA to detected the expansion of a TNR in one allele of a single copy gene. Twenty diagnosed HD probands with previously determined sizes of the expanded HD gene were tested with the RED method. The CTG repeat sizes, as determined by RED, were never smaller than the actual repeat size, matching exactly the repeat lengths in 8 samples and exceeding the previously determined sizes in 12 cases. The results obtained with the control population suggested that those differences were due to CTG/CAG expansions in the genome

unrelated to the HD locus. CTG/CAG repeat lengths in normal alleles have been shown to exceed the copy number expected for non-diseased individuals, i.e., falling in the premutation or mutation size range spectrum of the TRE-associated diseases, suggesting the existence of other expansions unrelated to the loci of those disorders (Nakamoto *et al.*, 1997).

In addition to the sensitivity of the RED technique, evaluated by Hofferbert and co-workers, we have assessed the reproducibility of the RED method, by scanning some samples in duplicate or triplicate for CTG, CGG and AAG triplets. The maximal repeat sizes obtained were identical in all cases. The autoradiograph shown in Figure 10 illustrates well the reproducibility of the RED assay, exhibiting identical products from different cycling reactions using the same samples. Our experiments have also shown that the RED reaction is very sensitive to small variations in the cycling conditions and to the purity of the oligonucleotides and genomic DNA template. The weak signals presented by some samples are consistently observed in most of the RED experiments in which they have been used, suggesting a certain degree if impurity that results in a low formation of oligonucleotide multimers. The hybridization of the oligonucleotide substrate to the genomic template might be impaired resulting in decreased formation of ligated products. The existence of contaminants in the oligonucleotide solution is also suggested by the ladder of bands appearing below the oligo multimer products, as observed, for instance, in the CGT triplet expansion reaction. The presence of contaminants is likely to slow down the annealing and ligation processes, resulting in low multimer production. The radiolabeled probe hybridization to the RED reaction products were not responsible for the faint bands obtained in some autoradiographs since there were always samples with strong signals, arising from a normal labeling procedure. In general, the RED assay is a reliable method for the detection of triplet repeat expansions in the human genome and has been shown to be capable of detecting even small expansions against a background of polymorphic larger repeated sizes as long as the population is homogeneous (Hofferbert *et al*, 1997).

Our results show the absence of any size differences between the elements of the FAP generational pairs, excluding the involvement of intergenerational unstable triplet expansions as the underlying mechanism for genetic anticipation in these kindreds, for the eight successfully tested trinucleotides. No conclusions can be drawn regarding GTT and CCT triplets since the respective RED experiments have been unsuccessful. It should noticed, however, that expansion of any of these two trinucleotides has not been associated with any prior instance of anticipation.

A limitation of our experiments was the absence of positive controls which would prove

the detectability of a single copy allele expansion under our experimental conditions. We have used a positive control only for CTG/CAG trinucleotide repeat detection, showing that the reaction conditions were perfectly adequate for genomic CTG repeat detection. Although all other RED assays were performed without positive controls, the determined repeat lengths were in accordance with previously reports, suggesting that the experimental conditions were suitable for the exact repeat size detection.

The number of scanned FAP kindred generational pairs constitute a relatively small sample of the Portuguese kindreds exhibiting anticipation. In a study with 227 generational pairs of Portuguese FAP kindreds, Sousa (1995) has reported anticipation of the age-at-onset in approximately 60% of the pairs. Considering that the Portuguese population is homogeneous and relatively constrained in reproductive terms, we would expect to find an unstable trinucleotide expansion in at least one pair if such mutation would indeed be responsible for phenotypic anticipation in FAP. A statistical analysis of our results suggests that no more than 11% [(95% CI (0-37%)] of the Portuguese FAP kindreds exhibiting anticipation is likely to have TREs as the underlying mechanism. The distribution of maximal triplet repeat sizes both for the FAP individuals and the unrelated controls, as determined by RED, is rather uniform, implying that the Portuguese population is genetically homogeneous with respect to genomic triplet repeat sequence lengths, although our sample size is too small to make a definitive statement.

Our results are suggestive - within the sensitivity and limitations of the RED method - that trinucleotide repeat expansions are unlikely to be responsible for genetic anticipation in FAP kindreds in the Portuguese population. To further substantiate our conclusions, additional pairs should be scanned for triplet repeat expansions.

This study has not excluded dynamic mutation as a potential molecular mechanism responsible for genetic anticipation in FAP. Several other simple tandem repeats are known to be unstable in the human genome and the cause of expansion-related disorders (Lalioti *et al.* 1997; Lafrenière *et al.*, 1997). The role of environmental factors in phenotypic anticipation in FAP cannot be excluded but is very unlikely. The involvement of multiple genes in TTR amyloidogenesis is strongly suggested, justifying a genome-wide scanning for modifier genes by genetic linkage analysis and positional cloning. The detection of TREs systematically in a significant proportion of FAP kindreds, would suggest that the gene bearing the expansion responsible for anticipation might be one such modifier gene. Thus, the search for TREs in FAP represents the first approach to assess the molecular basis for genetic anticipation and could

provide insight into the mechanisms of pathology at the cellular level, with immediate applications on genetic counseling. Furthermore, the identification of protein products of the TREs in amyloidogenesis, might help to elucidate the mechanism of amyloid deposition with important consequences at the therapeutic level.

Chapter IV

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Chapter V

Appendices

Appendices

1. Abbreviations for Amino acids

AMINO ACIDS	THREE-LETTER ABBREVIATION
Alanine	Ala
Arginine	Arg
Asparagine	Asn
Aspartic acid	Asp
Cysteine	Cys
Glutamine	Gln
Glutamic acid	Glu
Glycine	Gly
Histidine	His
Isoleucine	Ile
Leucine	Leu
Lysine	Lys
Methionine	Met
Phenylalanine	Phe
Proline	Pro
Serine	Ser
Threonine	Thr
Tryptophan	Trp
Tyrosine	Tyr
Valine	Val

2. The Genetic Code

Second position

	U	C	A	G	
U	UUU Phe	UCU Ser	UAU Tyr	UGU Cys	**U**
	UUC Phe	UCC Ser	UAC Tyr	UGC Cys	**C**
	UUA Leu	UCA Ser	UCA Stop	UGA Stop	**A**
	UUG Leu	UCG Ser	UCG Stop	UGG Try	**G**
C	CUU Leu	CCU Pro	CAU His	CGU Arg	**U**
	CUC Leu	CCC Pro	CAC His	CGC Arg	**C**
	CUA Leu	CCA Pro	CAA Gin	CGA Arg	**A**
	CUG Leu	CCG Pro	CAG Gln	CGG Arg	**G**
A	AUU Ile	ACU Thr	AAU Asn	AGU Ser	**U**
	AUC Ile	ACC Thr	AAC Asn	AGC Ser	**C**
	AUA Ile	ACA Thr	AAA Lys	AGA Arg	**A**
	AUG Ile	ACG Thr	AAG Lys	AGG Arg	**G**
G	GUU Val	GCU Ala	GAU Asp	GGU Gly	**U**
	GUC Val	GCC Ala	GAC Asp	GGC Gly	**C**
	GUA Val	GCA Ala	GAA Glu	GGA Gly	**A**
	GUG Val	GCG Ala	GAG Glu	GGG Gly	**G**