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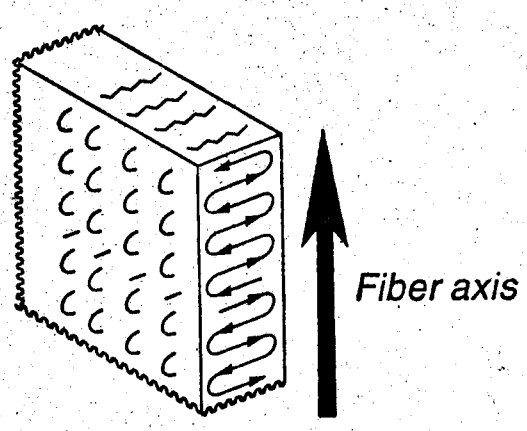

Porto 1996

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Studies on amyloid formation in Familial Amyloidotic Polyneuropathy

Dissertation for the obtention of the *Doctor* degree in Biomedical Sciences, Biochemistry specialty, submitted to the Instituto de Ciências Biomédicas "Abel Salazar", University of Porto. Supervisor: Professor Maria João Mascarenhas Saraiva

Porto 1996

To

Rosa and Manuel, the origins

Paulo and Marta, the present and future

Resumo

Os mecanismos de formação da substância amilóide na polineuropatia amiloidótica familar permanecem desconhecidos, assim como os factores que levam ao seu desenvolvimento. Este estudo procurou contribuir para a elucidação de factores intervenientes na amiloidogénese da ATTR, através do estudo de modelos experimentais *in vitro*, *in vivo* e em cultura celular.

Testamos e optimizamos as condições experimentais de um ensaio fluorométrico com tioflavina T para a quantificação de amilóide, com fibras de amilóide nativas e fibras produzidas a partir de um peptido sintético correspondente a um fragmento de TTR. Foram também produzidas, por acidificação, fibras sintéticas derivadas da molécula de TTR, sendo estudada a dependência do pH e da concentração da proteína, a cinética de formação de amilóide na presença e ausência de nucleos pré-formados, assim como a estabilidade das fibras formadas. A tendência de algumas variantes de TTR para a formação de amilóide (amiloidogenicidade) foi investigada através de ensaios dependentes do pH e de estudos cinéticos. Verificamos que as variantes de TTR estudadas se podiam ordenar numa escala de amiloidogenicidade do seguinte modo: TTR Pro 55 >> TTR Met 30, TTR Met 111 > TTR Ile 122, TTR > TTR Met 119, que se correlaciona com os quadros clinícos associados com estas variantes. Foi também investigada a influência de catiões metálicos e do ião sulfato no processo de formação de amilóide *in vitro*, tendo sido verificado que o ião sulfato não só favorecia a precipitação da proteina em agregados amorfos *vs* polimerização em fibrilhas, como também levava à agregação das fibrilhas formadas. Nenhum dos catiões metálicos afectava o processo de fibrilogénese nas condições estudadas.

O estudo de factores moduladores da amiloidogénese *in vivo* foi efectuado com ratinhos transgénicos portadores do gene humano da TTR Met 30 sob o controle do promotor da metalotionina. Estes animais tinham sido descritos como não possuíndo depósitos de amilóide, contrariamente ao verificado com uma outra estirpe de ratinhos transgénicos portadores de um transgene equivalente. Procuramos compreender os motivos pelos quais não ocorria deposição da proteina humana, e para tal caracterizamos a proteina produzida. Verificamos que a TTR Met 30 existente no soro se encontrava na forma tetramérica e ligava tiroxina; os níveis medidos eram geralmente baixos, sendo o valor máximo encontrado de 1 mg TTR Met 30/100 ml. O estudo da expressão tecidular revelou a presença da proteína humana na pele. Não foram encontrados quaisquer depósitos de amilóide nestes animais, mesmo quando induzidos com zinco por períodos prolongados. Foi então concluído que, ou os níveis de proteina humana não são suficientes para a precipitação da proteina como amilóide, ou existem factores genéticos nestes animais que determinam a ausência de amilóide.

Os modelos experimentais de culturas celulares apresentam algumas vantagens em relação aos modelos *in vitro* e *in vivo*; numa tentativa de desenvolver um modelo de cultura celular para o estudo da amiloidogénese da ATTR, construímos dois vectores retrovirais recombinantes contendo o cDNA para a TTR normal e o cDNA para a TTR-Met30 respectivamente, sob o controle do promotor do virus da leucemia de Moloney. Estes vectores foram introduzidos em células produtoras, e os stocks virais utilizados na infecção de linhas celulares de fibroblastos de ratinho e hepatomas de rato. Verificou-se que os hepatomas infectados secretavam as proteínas humanas em níveis não muito elevados. Foi também verificado que as proteínas humanas se acumulavam no meio de cultura, ao longo do tempo, sem degradação significativa. Procuramos obter formação de amilóide através do envelhecimento da proteína no meio, do aumento da concentração de proteína no meio e através do crescimento das células infectadas em extratos de tecidos e de fibras de amilóide nativas. Nas condições testadas não foi possível a detecção de quaisquer depósitos de amilóide por fluorometria com tioflavina T ou por coloração com vermelho do Congo, no entanto outros factores e matrixes têm ainda de ser testados.

Em resumo, nenhum dos sistemas descritos é suficiente por si para a elucidação dos mecanismos da amiloidogénese, no entanto cada um apresenta vantagens sobre os outros. Estes sistemas experimentais serão importantes no desenvolvimento de estratégias terapêuticas na Polineuropatia Amiloidótica Familiar.

Résumé

Les mécanismes de formation de l'amyloïde ATTR dans la Polyneuropathie Amyloïdotique Familiale (PAF), ainsi que les facteurs responsables par le développement de la pathologie, ne sont pas connus. Nous avons essayé d'éclaircir le rôle d'éventuels facteurs modulateurs de l'amyloïdogenèse dans cette maladie par recours à des modèles experimentaux *in vitro, in vivo,* et de culture cellulaire.

Nous avons essayé et normalisé les conditions d'une détermination fluorimétrique de l'amyloïde, employant la thioflavine T, dans l'étude de préparations d'amyloïde TTR native, et de fibrilles produites à partir de peptides synthétiques. Nous avons alors produit des fibrilles d'amyloïde avec de la TTR, par acidification, et étudié l'effet de la concentration de TTR et la dépendence du pH, la cinétique sans ou en présence de "seeding", et finalement la stabilité des fibrilles. Le penchant relatif de quelques variantes de TTR pour la formation d'amyloïde in vitro a eté evalué par recours à des essays cinétiques et de dépendence du pH. On a constaté que cettes protéines peuvent être ordonnées selon son amyloïdogénicité: TTR Pro 55 >> TTR Met 30, TTR Met 111 > TTR Ile 122, TTR > TTR Met 119, dans une échelle qui s'accorde bien avec le phénotype clinique. Nous avons étudié l'effet des ions métaliques et du sulphate dans le procés de formation d'amyloïde in vitro, ayant constaté que dans les conditions utilisées aucun des ions métaliques a eu un effet mésurable; par contre, le sulphate a favorisé la formation d'un precipité amorphe *versus* polymérisation en fibrilles d'amyloïde, et aussi l'agrégation du produit formé.

Des études *in vivo*, employant une souche transgénique de souris portant le gène de la TTR Met 30 humaine sous le contrôle du promoteur de la metalothionéine, on eté poursuits dans le même but; des dépôts amyloïdes n'ont jamais eté décelés dans cette souche, à l'anvers d'une autre portant un "construct" similaire. Nous nous sommes proposés de comprendre les raisons de l'absence de depôts dans ces souris, et de caractériser la protéine humaine produite. On a démontré que la TTR Met 30 se trouve dans le sérum sous forme tetramérique et fonctionelle, mais à basse concentration, moins de 1 mg/dl. L'analyse tissulaire a permis la démonstration de production du produit transgénique dans la peu. On na jamais observé des dépôts amyloïdotiques dans ces animaux, même a la suite d'une induction prolongée avec du zinc. Nous en concluons que la quantité de protéine humaine presente dans ces animaux est insuffisante pour la formation d'amyloïde, et/ou d'autres facteurs genétiques propres de cette souche empèchent sa déposition.

Les modèles de culture cellulaire on des avantages sur les modèles *in vitro* ou *in vivo*; dans le but de développer un modèle de culture cellulaire pour l'étude de l'amyloïdogenèse de la TTR, nous avons construit des vecteurs recombinants retroviraux possédant le cDNA de TTR ou de TTR Met 30 sous le contrôle du promoteur du virus MoMLV. Ces vecteurs recombinants retroviraux on eté transfectés dans une souche cellulaire ecotropique de "packaging", et les stocks retroviraux obtenus on alors eté utilisés pour infecter des lignes cellulaires d'hépatome murin, ou humaines de fibroblastes. Les hépatomes infectés secrètent efficacément soit la TTR, soit la TTR Met 30, même si à bas niveaux. Des études au cours du temps revélèrent que la protéine humaine accumulait dans le millieux de culture, sans soufrir degradation significative. Nous alons alors recherché la présence d'amyloïde dans des conditions diverses, notament vieillissement de la protéine dans le millieux, différentes concentrations de protéine, et repandir des cultures de cellules produteures de TTR ou TTR Met 30 en contact avec des extraits de tissus normaux ou de fibres d'amyloïde. Dans toutes les conditions essayés nous n'avons jamais decélé de la deposition d'amyloïde, soit par recours à la fluorométrie avec la thioflavine T, soit par la coloration au rouge de Congo. Néamoins, beaucoup d'autres substraits et de conditions doivent être essayés.

En somme, aucun des systèmes décrits s'avera suffisant pour éclaircir les mécanismes de l'amyloïdogenèse; chacun à des avantages envers les autres. Ces systèmes experimentaux pourront s'averer importants dans le développement de stratégies therapeutiques de la PAF.

In order to fulfill legal requisites (Dec Lei 388/70), we declare that in this work were used results published, or in the process of being published, in the following articles:

Bonifácio MJ, Sakaki Y, Saraiva MJM (1996) *In vitro* amyloid fibril formation from transthyretin: the influence of ions and the amyloidogenicity of TTR variants. *Biochimica Biophysica Acta* (in press)

Bonifácio MJ, Sakaki Y, Saraiva MJM (1996) Further characterization of transgenic mice, carrying the human TTR Met 30 gene under the control of mouse metalothionein promoter gene, lacking amyloid deposition. (manuscript in preparation)

Saraiva MJM, Almeida MR, Alves IL, Bonifácio MJ, Damas AM, Palha JA, Goldsteins G, Lundgren E (1996) Modulating conformational factors in transthyretin amyloid. *In:* Goode J ed. *The nature and origin of amyloid fibrils*. (Ciba Foundation Symposium 1995) Wiley, Chichester. 48-57.

Bonifácio MJ, Sakaki Y, Ezzedine D, Breakefield XO, Saraiva MJM (1993) Retrovirus-mediated gene transfer of a mutant transthyretin: potential tool for the study of amyloidogenesis. *Neuromuscular Disorders* **3:** 275-82.

Blaner WS, Bonifácio MJ, Feldman HD, Piantedosi R, Saraiva MJM (1991) Studies on the synthesis and secretion of transthyretin by the human hepatoma cell line Hep G2. *FEBS Letters* **287:** 193-6.

We further state that the execution of the experiments presented here, except where mentioned, their interpretation, discussion and the outlining of this thesis are of our own responsibility.

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Contents

Abreviations

Aβ	Amyloid β
AβPP	Amyloid beta precursor protein
ABTS	2,2'azino-di-(3-ethylbenzothiazoline sulphonate)
AD	Alzheimer's disease
AEF	Amyloid enhancing factor
ANF	Atrial natriuretic factor
Apo AI	Apolipoprotein AI
Apo E	Apolipoprotein E
Apo J	Apolipoprotein J
β2M	β2 microglobulin
BSA	Bovine serum albumin
bp	Base pair
cDNA	complementary DNA
CJD	Creutzfeld-Jacob disease
Da	Dalton
ddH_2O	Double-destilled water
DMEM	Dulbbeccos modified Eagle's medium
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotides triphosphate
DTT	Dithiothreitol
EDTA	Ethelynediaminetetracetic acid
ELISA	Enzyme linked immunoassay
FAC	Familial amyloidotic cardiomyopathy
FAD	Familial Alzheimer's disease
FAP	Familial amyloidotic polyneuropathy
FFI	Familial fatal insomnia
G418	Geneticin
GAG	Glycosaminoglycan
GSS	Gerstmann-Straüssler-Scheinker
HCHWA	Hereditary cerebral hemorrhage with amyloidosis
HDL	High density lipoprotein
HPLC	High performance liquid chromatography
HSPG	Heparan sulphate proteoglycan
IAPP	Islet amyloid polypeptide
IDOX	4'-iodo-4'-deoxydoxorubicin
IEF	Isoelectric focusing
L-chain	Light chain
LTR	Long terminal repeat
MEM	Modified Eagle's medium
MoMLV	Moloney murine leukenia virus
mRNA	Messenger RNA
MT	Metalothionein
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction

PG	Proteoglycan
PHD	Polypeptide hormone derived
PMSF	Phenylmethylsulfonyl fluoride
PrP	Prion protein
PS	Penicillin-Streptomycin
PS-1, PS-2	Presenilins 1 and 2
RBP	Retinol-binding protein
RID	Radial immunodifusion
RNA	Ribonucleic acid
RSV	Rous sarcoma virus
SAA	Serum amyloid A
SAP	Serum amyloid P component
SDS	Sodium dodecyl sulphate
SSA	Senile systemic amyloidosis
T4	Thyroxine
TBS	Tris buffered saline
TCA	Trichloroacetic acid
TTR	Transthyretin
Z3-14	N-tetradecyl-N,N-dimethyl-3-ammonio-1-propanesulfonate

Abstract

The mechanisms of TTR amyloid formation in familial amyloidotic polyneuropathy (FAP) are unknown, as well as what factors determine the development of this pathology. We have tried to elucidate the role of possible modulating factors in TTR amyloidogenesis by using *in vitro*, *in vivo* and cell culture experimental models.

We have tested and standardized the experimental conditions of a thioflavine T based fluorometric assay for amyloid quantification, with native TTR amyloid and amyloid-like fibrils derived from a TTR synthetic peptide. We have then produced amyloid-like fibrils from the whole TTR molecule, by acidification, and studied TTR concentration and pH dependence, the kinetics of the process in the presence or absence of seeding and finally the stability of the amyloid-like fibrils. The relative tendency of some TTR variants towards *in vitro* amyloid formation was evaluated using kinetic and pH dependence assays. It was verified that these proteins could be ordered in terms of decreasing amyloidogenicity as: TTR Pro 55 >> TTR Met 30, TTR Met 111 > TTR Ile 122, TTR > TTR Met 119, which correlates well with the clinical phenotypes associated with these variants. The influence of metal and sulphate ions on the process of TTR amyloid formation *in vitro* was investigated and, under the conditions tested, none of the metal ions significantly affected amyloid formation; sulphate, however, was found to favour the formation of amorphous precipitates *versus* oriented polymerization into amyloid fibrils, and to promote aggregation of the fibrils formed.

In vivo studies of factors modulating TTR amyloidogenesis were carried out with a transgenic mice strain, carrying the human TTR Met 30 gene under the control of the mouse metalothionein promoter; these mice were described not to have amyloid deposits, in opposition to a strain carrying a similar DNA construct. We aimed at understanding the reasons for the absence of amyloid deposition in these animals and characterized the human protein synthesized. TTR Met 30 was found to be present in serum as a functional tetramer, but in a low concentration, up to 1 mg/100 ml. Analysis of additional transgene expression tissues have demonstrated production of the human variant in the skin. No amyloid deposition was observed in these animals, even when induced with zinc for long periods of time. We concluded that the amounts of human protein present in these animals are below a specific critical concentration, needed for amyloid formation, and/or in this mice strain there are genetically determined factors preventing the deposition of the human protein as amyloid.

Cell culture models have some advantages over *in vitro* and *in vivo* models; in order to develop a cell culture model for the study of TTR amyloidogenesis we constructed recombinant retroviral vectors containing the cDNA for either TTR or TTR Met 30 under the control of the MoMLV promoter region. The recombinant retroviral vectors were transfected into an ecotropic packaging cell line and the viral stocks were then used to infect murine hepatoma and fibroblast cell lines. The infected hepatomas were shown to efficiently secrete both the human TTR and TTR Met 30, although not in high levels. Time course experiments revealed that the human protein accumulated in media with no significant uptake and degradation. We then seeked for amyloid formation under several conditions, namely protein aging in media, different media protein concentrations, and growing infected cells, producing TTR or TTR Met 30, in contact with normal tissue extracts and native amyloid fibrils. Under the conditions tested we have not detected amyloid deposition either by thioflavine T fluorometry or Congo red staining; however many other matrixes and conditions need still to be tested.

In summary, none of the described systems *per se* is sufficient for the elucidation of the mechanisms of amyloidogenesis, however each has advantages over the others. These experimental systems, will be important in developing therapeutic strategies for FAP.

Part I

General Introduction

General Introduction

1. Amyloid

The designation *amyloid* was used for the first time by Virchow (1851) to denominate a tissue infiltrating substance with hyaline appearance under the light microscope. Nowadays, amyloid refers to fibrillary protein aggregates, biochemically heterogenous but with distinctive properties, found usually as extracellular deposits, which are characteristic of a complex group of diseases known as *amyloidoses*.

1.1. Biochemical features of amyloid

Amyloid fibrils, although constituted by several unrelated and non homologous proteins in the different amyloidoses, share a number of physical-chemical features that are independent of the primary structure of the amyloid protein precursor.

In tissue sections stained with hematoxylin and eosin, amyloid deposits have a homogenous and amorphous appearance. They exhibit methachromasia when stained with aniline dyes, such as methylviolet and crystalviolet, and yellow green fluorescence upon staining with thioflavin S. If stained with Congo red and analyzed by polarization microscopy, a characteristic apple green birefringence is observed, indicating a highly ordered structure. Congo red staining and polarization microscopy has been the most widely used technique for histopathological diagnosis (Glenner and Page, 1976).

Isolated amyloid fibrils appear in electron microscopy as linear, nonbranched fibrils, with a diameter of 7 to 10 nm and indefinite length; the fibrils appear to be made of two to several parallel filaments, of 2-3 nm each, occasionally twisting around each other (Cohen and Calkins, 1959; Shirama and Cohen, 1967).

X-ray diffraction analyses and infrared spectroscopy of isolated amyloid fibrils led to the proposal of the cross β-fibril model of amyloid; in this model antiparallel polypeptide chains are arranged perpendicular to the direction of fibril growth, forming a β-pleated sheet as represented in figure 1; the strands in each β-sheet interact through hydrogen bonding, and the sheets are stacked in a parallel fashion (Eanes and Glenner, 1968; Glenner *et al.*, 1974).

There are a number of elements universally present in the amyloid deposits, namely: glycosaminoglycans and proteoglycans (Snow *et al.*, 1987), serum amyloid P component (Pepys, 1988), apolipoprotein E (Gallo *et al.*, 1994), and basement membrane components such as fibronectin, laminin and collagen type IV (Husby *et al.*, 1994a). In particular cases, like in

Alzheimer's disease, it is common to find α1-antichymotrypsin (Abraham *et al.*, 1988), apolipoprotein J (Choi-Miura *et al.*, 1992), and complement proteins (Rogers *et al.*, 1992) among other matrix and plasma proteins.

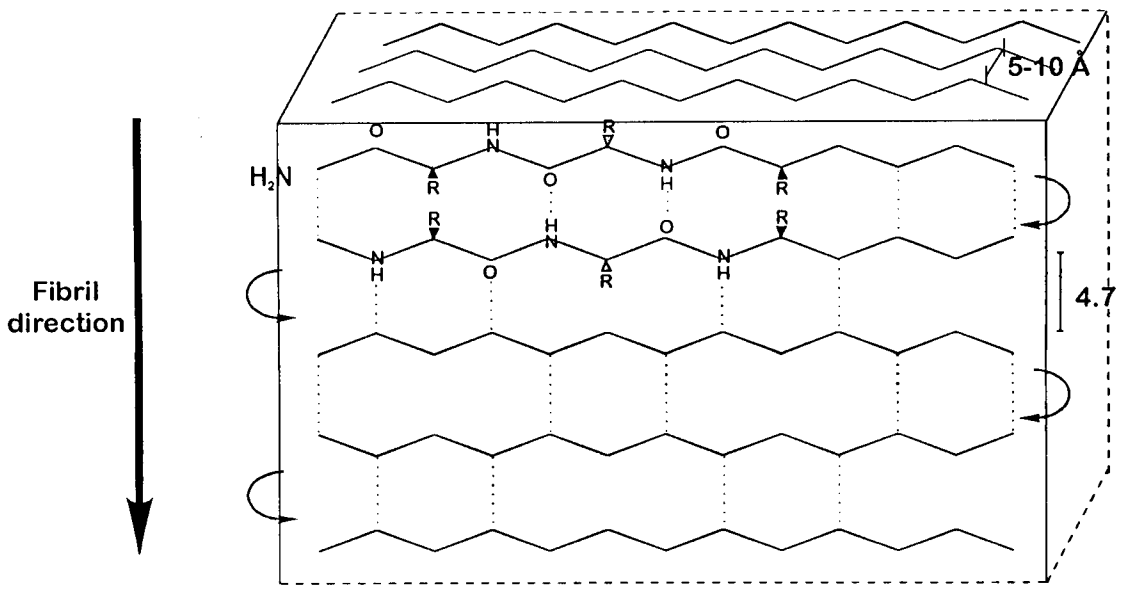


Figure 1 - Diagram of an anti-parallel cross β-fibril. H_2N - amino terminal (adapted from Spencer *et al.*, 1991).

The amyloid fibrils are highly insoluble under physiological conditions and resistant to proteolytic digestion, characteristics responsible for the persistence of the fibril deposits *in vivo*. The enlargement of these deposits as the direct cause of organ dysfunction in the pathology of all forms of amyloidosis (Glenner and Page, 1976), is a matter of controversy.

The classification of the amyloidoses is based on the nature of the amyloid fibril protein component, as discriminated below.

2. Amyloid diseases

The distribution of the amyloid deposits varies with the amyloid precursor; it can be localized to one tissue or organ, such as cerebral and endocrine amyloids, or it can be systemic. In the systemic amyloidosis the amyloid proteins are usually derived from circulating precursors that are either present in excess, abnormal, or both. In table 1 are described the amyloid diseases known to date and their protein precursors.

Table 1 - Amyloid diseases

Amyloid	Protein precursor	Disease
Systemic amyloidosis		
AA	apolipoprotein SAA	Reactive amyloidosis in man and other species
		Familial Mediterranean fever
		Muckle Well's syndrome
Aapo AI	Apolipoprotein AI variants	Familial amyloid polyneuropathy
		Familial nephropathic amyloidosis
Aapo AII	Apolipoprotein ApoAII	Amyloidosis in senescence-accelerated mice
$A\beta_2M$	β2-microglobulin	Long term hemodialysis
ACys	Cystatin C variant	Hereditary cerebral hemorrhage with amyloidosis - Icelandi
AFib A	α-chain fibrinogen variants	Hereditary renal amyloidosis
AGel	Gelsolin variants	Familial amyloidosis - Finnish
AH	Immunoglobulin G1	Heavy chain associated amyloidosis
AL	Immunoglobulin L-chain	Idiopathic amyloidosis
		Myeloma associated
		Macroglobulinemia associated
ALys	Lysozyme variants	Hereditary non-neuropathic systemic amyloidosis
ATTR	TTR	Senile systemic amyloidosis
	TTR variants	Familial amyloid polyneuropathy
		Familial amyloid cardiomyopathy
		Meningocerebrovascular amyloidosis
Localized amyloidosis		
AANF	Atrial natriuretic factor	Atrial amyloidosis
$A\beta$	β amyloid precursor protein	Alzheimer's disease
		Down syndrome
		Sporadic cerebral angiopathy
		Hereditary cerebral hemorrhage with amyloidosis -Dutch
ACal	Calcitonin	Medullary carcinoma of thyroid
AIAPP	Islet amyloid polypeptide	Diabetes mellitus type II
		Insulinoma
AIns	Insulin	Islet amyloid in the *Octodon degu*
APrP	PrPc-cellular prion protein	Creutzfeldt-Jakob disease (sporadic)
		Kuru
		Animal spongiform encephalopathies
	Prion variants	Creutzfeldt-Jakob disease (familial)
		Gerstmann-Straüssler-Scheinker syndrome
ATub	Tubulin fragments ?	Familial cerebral amyloid angiopathy- British

Adapted from Husby *et al.*, 1991.

2.1. Localized amyloidosis

2.1.1. Polypeptide hormone derived (PHD) amyloids

The PHD amyloids are strictly localized, and occur in close vicinity to their producer cells:

A-ANF - Atrial amyloidosis is a condition that occurs with some frequency in aged individuals, associated with congestive heart failure and chronic rheumatic heart disease; it is characterized by the deposition of atrial natriuretic factor (ANF), a 28 amino acid peptide produced by cardiac myocytes, as amyloid along the sarcolemma, beneath the endocardium and in the walls of small vessels (Westermark, 1994).

ACal - Amyloid deposits made of procalcitonin and/or calcitonin are observed in 82% cases of medullary carcinomas of the thyroid (Sletten *et al.*, 1991).

AIAPP - Islet amyloidosis occurs in the majority of individuals with non insulin dependent diabetes, in insulinoma endocrine tumors, and also in more than 50% individuals over 60 years of age, although in the latter affecting just a few islets. The amyloid deposits contain islet amyloid polypeptide (also known as amylin), a 37 amino acid residue hormone synthesized by pancreatic islet β cells as a prepropolypeptide; the mature IAPP is stored with insulin in secretory granules (Westermark, 1992, 1994).

AIns - The islet amyloid in a South American rodent, the *Octodon degu,* was found to be constituted by A and B insulin chains (Hellman *et al.*, 1990). In some rare occasions, the porcine insulin injected in diabetic individuals is converted into subcutaneous amyloid deposits (Dische *et al.*, 1988).

2.1.2. Aβ

Alzheimer's disease (AD) is an age-related neurodegenerative disorder that is the predominant cause of dementia in late life; the majority of Alzheimer's cases are sporadic, however families with an autosomal dominant pattern of inheritance (Familial Alzheimer's disease - FAD) have also been described (Schellenberg, 1995). AD is characterized by the presence of: i) intracellular neurofibrillary tangles - which are composed of paired helical filaments, made of abnormal phosphorylated t proteins; ii) senile or neuritic plaques, which are spherical lesions formed by a central amyloid core, dystrophic neurites, and activated astrocytes and microglial cells; and iii) cerebrovascular amyloidosis - amyloid deposits in small and medium vessels of the leptomeninges and cerebral cortex (Ghiso *et al.*, 1994). The major component of amyloid fibrils is a peptide (Aβ), that in the senile plaques is 42/43 amino acids long and in the vascular amyloid is 39/40 amino acids long, derived from a protein designated as amyloid β protein precursor (AβPP). AβPP is encoded by a gene on chromosome 21, spanning over 190 kb, and has a receptor-like structure, with a large extracellular domain, a membrane spanning region and a short cytoplasmic

tail (Kang *et al.*, 1987). Several isoforms arise from alternative splicing; the major transcript in neurons is $A\beta PP_{695}$ and in peripheral tissues $A\beta PP_{751}$ and $A\beta PP_{770}$; the latter two isoforms contain a Kunitz-type protease inhibitor domain (Schellenberg, 1995). $A\beta PP$ undergoes tyrosine sulfation, N- and O-linked glycosylation and phosphorylation. The $A\beta$ sequence encompasses parts of both the transmembrane and the extracellular domains, thus to be released requires proteolytic cleavage of $A\beta PP$ at both its N- and C-terminals. Several functional roles have been postulated for $A\beta PP$ or its soluble derivatives, namely mediating cell adhesion and neuronal attachment, promoting cellular growth and neurite activity, and some forms might also be involved in the repair process of vascular injury and wound healing (Selkoe, 1994).

Five percent of early onset FAD cases are associated with mutations in the $A\beta PP$ gene, as represented in table 2. The clinical and neuropathological phenotype of AD in these families is the same as in patients without $A\beta PP$ mutations, with the exception of the age of onset (Schellenberg, 1995). Mutations in at least two other genes (PS-1 and PS-2), are responsible for most of the other FAD cases (Van Broeckhoven, 1995).

Table 2 - Pathogenic $A\beta PP$ mutations

$A\beta PP$ variant	Phenotype	References
Asn_{670},Leu_{671}	Early onset-FAD	Mullan *et al.*, 1992
Gly_{692}	Early onset-FAD	Hardy, 1992
Gly_{692}	Dementia or stroke	Hendriks *et al.*, 1992
Gln_{693}	HCHWA-Dutch	Levy *et al.*, 1990
Thr_{713}	Dementia	Carter *et al.*, 1992
Ile_{717}	Early onset-FAD	Goate *et al.*, 1991
Gly_{717}	Early onset-FAD	Chartier-Harlin *et al.*, 1991
Phe_{717}	Early onset-FAD	Murrel *et al.*, 1991

The glutamine for glutamic acid substitution at position 692 of $A\beta PP$, is responsible for the hereditary cerebral hemorrhage with amyloidosis - Dutch type (HCHWA-Dutch, Levy *et al.*, 1990), an autosomal dominant disease characterized by amyloid deposition in the walls of cortical arterioles and small leptomeningeal arteries (Roos *et al.*, 1991). Parenchymal amyloid deposits have also been observed in some patients, and resemble AD immature plaques. Histopathologically and clinically HCHWA-Dutch is similar to HCHWA-Icelandic, however the amyloid fibrils of the first are constituted by $A\beta$ (39 amino acids long).

An alanine for glycine substitution at position 692 of AβPP results in a phenotype which is a variant of both AD and HCHWA-Dutch (Hendriks *et al.*, 1992).

Other disorders related to Aβ amyloid deposition are: Down's syndrome (trisomy 21), where, among other things, patients develop a histopathological phenotype indistinguishable from that of AD; and sporadic cerebral angiopathy, where normal Aβ deposits in the leptomeningeal and cortical vasculature (Ghiso *et al.*, 1994).

2.1.3. ATub

Familial cerebral amyloid angiopathy, British type is characterized by extensive amyloid deposition in small cerebral arteries and arterioles, non-neuritic parenchymal amyloid plaques and ischemic white matter damage. Very recently the amyloid deposits were suggested to be constituted by fragments of α- and β-tubulin, however further confirmation is needed (Baumann *et al.*, 1996).

2.1.4. APrP

The prion diseases include the Gerstmann-Sträussler-Scheinker syndrome (GSS), kuru, Creutzfeldt-Jakob disease (CJD) and fatal familial insomnia (FFI) in humans, scrapie in sheep, mice and hamsters and bovine spongiform encephalopathy. These diseases are characterized by neuronal degeneration with spongiform changes, astrogliosis, microgliosis, and accumulation, sometimes, in the form of amyloid plaques, of an abnormal protease-resistant isoform of a host protein, the prion protein - PrP (Prusiner, 1991). Although considered amyloidoses, amyloid formation is not an obligatory attribute of the prion diseases (Prusiner and DeArmond, 1995); PrP plaques are observed in all GSS cases, but in only 50-70% of kuru cases and in 59% of a CJD group of patients (Wisniewski *et al.*, 1994). Mutations in the PrP gene have been genetically linked to GSS and some cases of CJD (Prusiner, 1994), accounting for 10-15% of the prion diseases; the remaining cases are sporadic, and in less than 1% of the cases iatrogenic (Prusiner and DeArmond, 1995). The physiological role of the cellular isoform of the prion protein is unknown, the protein is found anchored on the external surface of cells by a glycolipid moiety (Stahl *et al.*, 1987).

2.2. Systemic amyloidosis

2.2.1. ACys

Hereditary cerebral hemorrhage with amyloidosis, Icelandic type (HCHWA-Icelandic) is an autosomal dominant disease characterized by the deposition of amyloid in arterial walls throughout the body, but with particular incidence in the cerebral cortex and leptomeninges (Thorsteinsson *et al.*, 1988). The protein depositing as amyloid corresponds to cystatin C, without the ten amino terminal residues, and with a glutamine for leucine substitution at position 68

(Ghiso *et al.*, 1986). Cystatin C, a member of the cystatin superfamily, is a 120 amino acid long basic protein with cysteine protease inhibitory activity (Barret *et al.*, 1984).

2.2.2. AA amyloidosis

Amyloid A (AA) protein is the major component of the amyloid deposits in reactive amyloidosis, a rare complication of chronic infections, and other persistent acute inflammatory processes such as familial Mediterranean fever (FMF), rheumatoid arthritis, and Muckle Well's syndrome; it is also the major fibril protein in spontaneous and experimentally induced amyloidosis in animals (Sipe, 1994; Husby *et al.*, 1994b). Amyloid deposits are found mainly in liver, spleen and kidney. AA protein corresponds to the amino terminal (about 76 amino acids) of serum amyloid A (SAA), an acute phase reactant apolipoprotein (Husebekk *et al.*, 1985). ApoSAA is a 12 kDa amphipathic, highly polymorphic protein. In humans several major isoforms are present in serum, all of hepatic origin, representing the products of three genes (SAA1, SAA2 and SAA4), but other minor polymorphisms have also been described; although these proteins are acute phase reactants, apoSAA4 is constitutively expressed. Most species contain three major acute phase isoforms: two of hepatic origin (SAA1 and SAA2) and one of extrahepatic origin (SAA3) (Husby *et al.*, 1994b). During inflammation the levels of SAA may increase by as much as 1000 fold upon induction by cytokines. The physiological function of SAA remains unclear; it is thought to modulate the role of HDL in reverse cholesterol transport and appears to be involved in the recruitment of leukocytes (Sipe, 1994; Badalato *et al.*, 1993; Xu *et al.*, 1995).

2.2.3. Aapo AI

Familial amyloid neuropathy - Iowa is an autosomal dominant hereditary amyloidosis characterized by peripheral neuropathy, peptic ulcers and nephrotic syndrome, in which the amyloid deposits are composed of the amino terminal fragments of apolipoprotein AI with an arginine for glycine substitution at position 26 (Nichols *et al.*, 1988). The same variant, as well as apo AI Arg 60, are associated with a non neuropathic form of the disease, with predominant kidney involvement, designated as familial nephropathic amyloidosis (Jones *et al.*, 1991a; Soutar *et al.*, 1992). Apo AI is a 28 kDa nonglycosylated protein that constitutes the major apolipoprotein of HDL during homeostasis (Zannis *et al.*, 1993).

2.2.4. Aapo AII

Several strains of mice (SAM-P1, 2, 7, and 9, LLC, SJL/J, and A/J) develop spontaneous amyloidosis with aging; the precursor protein of amyloid fibrils is the intact apolipoprotein AII type C polymorphism (Gln^{5}, Glu^{20}, Val^{26}, Ala^{38} - Higuchi *et al.*, 1991).

2.2.5. Aβ_2M

β_2Microglobulin (β_2M) amyloidosis is a frequent complication in long term dialysis patients; its predominant clinical manifestations are carpal tunnel syndrome, and amyloid arthropathy with

chronic synovitis and progressive bone destruction. Amyloid fibrils are composed mainly of intact β_2M, but fragmentation has also been described (Gorevic *et al.*, 1985; Gejyo *et al.*, 1985; Linke *et al.*, 1991). β_2M, a member of the immunoglobulin superfamily, is a 11.8 kDa protein, with a high β-sheet content that is present in the surface of mammalian cells as a component of class I HLA antigens (Revillard *et al.*, 1989). Since β_2M is not effectively removed from plasma by conventional cuprophane dialysis membranes and β_2M spontaneously forms amyloid fibrils *in vitro* at high protein concentrations and low ionic strength (Connors *et al.*, 1985), it has been suggested that local concentration is a key factor in the pathogenesis of this disease.

2.2.6. AFib A

In a form of hereditary renal amyloidosis the amyloid precursor protein is a fibrinogen Aα chain variant; either FibAα Leu 554 or FibAα Val 526 (Uemichi *et al.*, 1994a); recently a nucleotide deletion at codon 524 of Fib Aα gene, resulting in frame shift and premature termination, has also been found associated with renal amyloidosis (Uemichi *et al.*, 1996a).

2.2.7. AL, AH

Primary amyloidosis is an uncommon disease that occurs more frequently in men and shows an increasing incidence rate with advancing age. It is generally associated with overproduction of monoclonal light chains as a result of plasma cell dyscrasias, with systemic deposition of amyloid; the organ distribution is highly variable, and, in some cases, localized deposits in the lung and skin were also observed (Kyle, 1991). The amyloid fibrils consist of either the whole molecule or fragments of monoclonal immunoglobulin light chains (5-23 kDa); the fragments are derived from the amino terminal region and consist of the variable region with or without a portion of the constant region. Among the amyloidogenic light chains, there is a prevalence of the λ over κ class and some λ isotypes also predominate (Solomon and Weiss, 1994; Ozaki *et al.*, 1994).

In the so called heavy-chain-associated amyloidosis the amyloid precursor was identified as immunoglobulin heavy chain fragments, corresponding mainly to the variable region (Eulitz *et al.*, 1990; Solomon *et al.*, 1994).

2.2.8. AGel

Familial amyloidosis, Finnish type is characterized by amyloid deposition in the cornea, cranial nerves and various internal organs (Meretoja, 1969). The amyloid deposits are composed of a protein fragment related to gelsolin, a calcium-dependent actin-modulating protein, that severs actin filaments, nucleates actin filament growth and cap barbed filament ends (Stossel *et al.*, 1985). It occurs in both intracellular and secretory forms. The amyloidogenic fragment is homologous to residues 173-243 of gelsolin, with a substitution of asparagine or tyrosine for aspartic acid at position 187 (Maury, 1991;De la Chapelle *et al.*, 1992). Recent studies, with COS-1 cells

transfected with both normal and mutant gelsolin cDNAs, indicate that the mutant secretory forms (but not the intracellular ones) are abnormally processed (Paunio *et al.*, 1996; Kangas *et al.*, 1996).

2.2.9. ALys

Hereditary non-neuropathic systemic amyloidosis was associated with lysozyme variants in two British families described by Pepys *et al.*, (1993); the mutations occur in highly conserved residues, namely threonine for isoleucine at position 56 and histidine for aspartic acid at position 67, and it appears that it is the intact protein that deposits. Lysozyme is a ubiquitous bacteriolytic enzyme present in external secretions, polymorphs, and macrophages.

2.3. ATTR amyloidosis

The most frequent ATTR amyloidoses are the autosomal dominantly inherited forms, that include familial amyloidotic polyneuropathy (FAP) and familial amyloidotic cardiomyopathy (FAC).

2.3.1. Familial amyloidotic polyneuropathy

FAP was first described by Andrade in 1952, in Portuguese patients, as a "peculiar" form of polyneuropathy characterized by systemic amyloid deposition with special involvement of the peripheral nerves. The disease has an age of onset usually in the third or fourth decade, with progression to death within 10 to 15 years; the major symptoms are peripheral sensory motor neuropathy, autonomic neuropathy and varying degrees of systemic organ involvement (Andrade, 1952). Extensive amyloid deposition is observed in the meninges, spinal and autonomic ganglia. In the peripheral nerves amyloid infiltrates the epineurium, perineurium and particularly the endoneurium. Amyloid deposits are also found throughout the connective tissues and in and around blood vessels, specially in capillaries and small arteries; no amyloid deposition is detected in the brain or spinal cord except in some cases for scarce subependymal deposits; likewise, in the liver very little amounts of amyloid, if any at all, are observed (Guedes, 1976).

In 1978, Costa and colleagues (Costa *et al.*, 1978) isolated the main protein constituent of the amyloid fibrils in Portuguese patients, and verified that it was immunologically related to transthyretin (TTR - formerly known as prealbumin); the isolated protein was characterized subsequently and a substitution of methionine for valine at position 30 - TTR Met 30 was found (Saraiva *et al.*, 1983). This substitution, that results from a point mutation in the TTR gene (Sasaki *et al.*, 1984), was shown to be the biochemical marker for FAP (Saraiva *et al.*, 1985).

TTR Met 30 associated FAP affects a large number of kindreds in Portugal (about 600), which is the largest focus in the world; other large foci include Japan, Sweden, Brazil and the Island of Majorca; in addition, FAP patients have been found in Italy, Greece, Cyprus and in the United States (Saraiva *et al.*, 1993). There is some heterogeneity in FAP, not only in the clinical presentation (Saraiva *et al.*, 1993) but also in age of onset as presented in table 3; gene dosage

appears not to influence the age of onset or the severity of the disease as observed in homozygotic individuals (Holmgren *et al.*, 1992).

Table 3 - FAP Met 30 age of onset

Focus	Mean age (range), years	References
Portugal	33.5 (17-78)	Sousa, 1995
Sweden	56.7 (26-84)	Sousa, 1995
Japan	34.2 (18-69)	Ikegawa *et al.*, 1991
Majorca	49 (20-84)	Munar-Quès *et al.*, 1996

The penetrance of FAP is incomplete, as carriers of the mutation may not develop the disease (Coelho *et al.*, 1994). These facts suggest that other factors, besides the mutation in the TTR molecule, must intervene in the pathogenesis of FAP.

Since the identification of the Met 30 variant, several other amino acid substitutions were identified in the TTR molecule, the majority of them associated with hereditary amyloidosis. This genetic heterogeneity is associated with different clinical phenotypes; it is possible, however, to group the different variants according to the dominant clinical manifestation, which can be a neuropathy, cardiomyopathy, vitreous opacities, a carpal tunnel syndrome or other.

The majority of the TTR variants are associated with a predominant clinical picture of neuropathy, as reflected in table 4, and various degrees of the other manifestations. Among these variants we can distinguish TTR Met 30, as the most frequent variant, and TTR Pro 55, as the variant associated with the most aggressive form of amyloidosis; it is characterized by an early age of onset, between 15 and 20 years, with quick progression to death in less 5 to 10 years. In addition to neuropathy, the carriers had cardiomyopathy and vitreous opacities (Jacobson *et al.*, 1992; Yamamoto *et al.*, 1994). Recently, and for the first time, a deletion of a codon in the TTR gene, resulting in the loss of a valine (TTR ΔVal 122), was reported to be associated with polyneuropathy (Uemichi *et al.*, 1996b). TTR Ile 50 is also an interesting case since it was described in association with neuropathy in one kindred, but with cardiomyopathy in another.

2.3.2. Familial amyloidotic cardiomyopathy

Cardiac amyloidosis is a frequent secondary manifestation in FAP, however a predominant clinical picture of cardiomyopathy is associated with some TTR variants, in the absence of neuropathy; the syndromes associated with these variants are referred to as FAC and usually have a age of onset in the fifth or sixth decade. Among FAC related variants, TTR Ile 122 and TTR Met 111 are particularly relevant. The former is found only in the black population occurring

Table 4 - Neuropathic TTR variants

Position	Substitution	Normal	Phenotype	References
10	Arg	Cys	N, C, V	Uemichi *et al.*, 1992; Benson and Uemichi, 1996
18	Glu	Asp	N	Booth *et al.*, 1996
24	Ser	Pro	N, CT, C	Uemichi *et al.*, 1995
30	Met	Val	N, C, V	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,V	Nakazato *et al.*, 1984
33	Leu	Phe	N	Ii *et al.*, 1991; Harding *et al.*, 1991
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,V	Jones *et al.*, 1991b
42	Gly	Glu	N	Ueno *et al.*, 1990a
47	Arg	Gly	N	Murakami *et al.*, 1992b
47	Val	Gly	N, CT, C	Booth *et al.*, 1994
47	Ala	Gly	N, C	Ferlini *et al.*, 1994
49	Ala	Thr	N, C, V	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, V	Jacobson *et al.*, 1992
58	His	Leu	N, CT , C	Nichols *et al.*, 1989; Benson and Uemichi, 1996
58	Arg	Leu	N, CT, V	Saeki *et al.*, 1991
60	Ala	Thr	N, CT, 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, CT, C	Izumoto *et al.*, 1992
71	Ala	Val	N, CT, C, V	Almeida *et al.*, 1993; Benson II *et al.*, 1993
77	Tyr	Ser	N	Wallace *et al.*, 1988
84	Ser	Ile	CT, C, V	Dwulet and Benson, 1986
89	Gln	Glu	CT, N, C	Almeida *et al.*, 1992
97	Gly	Ala	N, C	Yasuda *et al.*, 1994, Nakazato *et al.*, 1994
107	Val	Ile	N, CT	Jacobson *et al.*, 1994a; Uemichi *et al.*, 1994b
112	Ile	Ser	N, C	DeLucia *et al.*, 1993
114	His	Tyr	CT	Murakami *et al.*, 1994
114	Cys	Tyr	N,V	Ueno *et al.*, 1990b
Δ122	–	Val	N, C	Uemichi *et al.*, 1996b

N - Neuropathy; CT - Carpal Tunnel Syndrome, C - Cardiomyopathy; V - Vitreous Opacities; Nph - Nephropathy

with a high frequency (2.2% of heterozygotes - Jacobson, 1992), and the latter has the earliest age of onset (40 years) with death occurring within 1-3 years (Ranlov *et al.*, 1992)

Table 5 - Cardiopathic TTR variants

Position	Substitution	Normal	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; Saraiva *et al.*, 1990a
125	Ser	Pro	C	Ferlini *et al.*, 1996

N - Neuropathy; C - Cardiomyopathy

2.3.3. Others

Some TTR variants are associated predominantly with amyloidotic vitreous opacities, and others with "peculiar" clinical pictures, completely diverse from those reported above, such as central nervous system dysfunction, and liver amyloidosis (table 6).

Table 6 - "Peculiar" TTR variants

Position	Substitution	Normal	Phenotype	References
12	Pro	Leu	L, IH	Booth *et al.*, 1996
18	Asp	Gly	CND	Vidal *et al.*, 1996
30	Gly	Val	V, CND	Herbert *et al.*, 1994; Peterson *et al.*, 1995
69	His	Tyr	V	Zeldenrust *et al.*, 1994
84	Asn	Ile	V, C, CT	Skinner *et al.*, 1992

V - Vitreous Opacities; CT - Carpal Tunnel Syndrome, C - Cardiomyopathy; L - liver amyloid; CND - Central nervous system dysfunction, IH - intracerebral hemorrhage

2.3.4. Non amyloidogenic variants and compound heterozygotes

Amino acid substitutions in the TTR molecule not associated with amyloidosis have been described and are discriminated in table 7. Although non amyloidogenic, some of these substitutions are associated with abnormal thyroxine affinity, namely the substitution at position 109. Since some of them occur with high frequency, that might explain the existence of compound heterozygotes (table 8). TTR Ser 6 is considered to be a common polymorphism in Caucasians, due to the 12% frequency of this allele (Jacobson *et al.*, 1995).

Table 7 - Non amyloidogenic TTR variants

Position	Substitution	Normal	Phenotype	References
6	Ser	Gly	N	Fitch *et al.*, 1991; Jacobson *et al.*, 1995
74	His	Asp	N	Uemichi *et al.*, 1994c
90	Ans	His	N	Saraiva *et al.*, 1991
102	Arg	Pro	N	Almeida *et al.*, 1991b
104	Cys	Arg	N	Torres *et al.*, 1996
109	Thr	Ala	EHT	Moses *et al.*, 1990
109	Val	Ala	EHT	Izumoto *et al.*, 1993
119	Met	Thr	N	Harrison *et al.*, 1991; Alves *et al.*, 1993

N - normal; EHT - Euthyroid hyperthyroxinemia

Table 8 - Compound TTR variant heterozygotes

Allele 1	Allele 2	References
Ser 6	Met 30	Alves *et al.*, 1996a
Ser 6 - Ile 33	N	Jacobson and Buxbaum, 1994
Ser 6	Asp 45	Jacobson *et al.*, 1994b
Ser 6	Gly 54	Booth *et al.*, 1994
Ser 6	Met 111	Nordvåg *et al.*, 1995
Ser 6 - Cyst 114 *		Waits *et al.*, 1995
Ser 6	Met 119	Jacobson *et al.*, 1995
Asn 90	Met 30	Saraiva *et al.*, 1991
Asn 90 - Gly 42	N	Skare *et al.*, 1994
Asn 90	Met 119	Alves *et al.*, 1993
Thr 109	Met 119	Izumoto *et al.*, 1993
Met 119	Met 30	Alves *et al.*, 1996b
Ser 125	?	Ferlini *et al.*, 1996

N - normal; * it is not reported whether the mutations are in the same alle

2.3.5. Senile systemic amyloidosis

SSA is the most prevalent of the ATTR amyloidosis, affecting about 25% of individuals above the age of 80; it is usually recognized post mortem, but in some individuals cardiac insufficiency and conduction disturbances leading to death can occur (Cornwell *et al.*, 1983). Amyloid deposits are found in the atria and ventricles of the heart, and to a lesser extent in the lungs and other organs; the amyloid fibrils were shown to be composed of fragments of normal TTR (Westermark *et al.*, 1990a).

2.3.6. Biochemical features of transthyretin

TTR is a homotetrameric plasma protein, strongly conserved in evolution, being present in mammals, birds and reptiles (Richardson *et al.*, 1994). Its physiological functions are not quite understood; TTR binds the complex retinol-retinol binding protein (holo-RBP), thereby preventing glomerular filtration of RBP and playing an important role in the distribution of retinol to the tissues (Kanai *et al.*, 1968; Raz *et al.*, 1970), possibly by modulating the levels of holoRBP and free retinol (Sivaprasadarao and Findlay, 1988). The other major ligand of TTR is thyroxine (T_4), and, although the major T_4 plasma carrier in humans is thyroxine binding globulin, TTR has been suggested to be responsible for T_4 transport in the central nervous system, thus being essential for brain development (Schreiber *et al.*, 1990). However, the normal phenotype of TTR knock-out mice argues against this hypothesis, indicating that alternative mechanisms must exist (Episkopou *et al.*, 1993; Palha *et al.* 1994a). Other minor ligands of TTR include: noradrenaline oxidation products (Boomsma *et al.*, 1991), pterins (Ernström *et al.*, 1995), chicken lutein (Pettersson *et al.*, 1995), hemin and hemoglobin (Martone and Herbert, 1993a), polyhalogenated biphenyl compounds (Lans *et al.*, 1993) and possibly retinoic acid (Smith *et al.*, 1994).

Biosynthesis and catabolism

As most plasma proteins, TTR is synthesized predominantly by hepatocytes (Felding and Fex, 1982) and secreted into the serum where its normal concentration in humans varies between 20 and 35 mg/dl. The major site of extrahepatic TTR synthesis is the choroid plexus epithelium, accounting for the high concentration of TTR in the cerebrospinal fluid, 2 to 4 mg/dl (Soprano *et al.*, 1985; Herbert *et al.*, 1986), followed by the retinal pigment epithelium (Martone *et al.*, 1988; Cavallaro *et al.*, 1990). Pancreatic islets, particularly human A-cells (Jacobsson, 1989a), and, to a minor extent, pineal gland (Martone *et al.*, 1993), stomach, heart and skeletal muscle (Soprano *et al.*, 1985) are all places shown to express TTR mRNA.

TTR is expressed very early in fetal development; in humans TTR mRNA was detected in hepatocytes and choroid plexus epithelial cells from the eighth week, and in pancreatic islets from mid-term pregnancy (Jacobsson *et al.*, 1989b); in mice, the TTR mRNA was identified from the tenth day of gestation in hepatocytes, tela choroidea, and visceral yolk sac (Murakami *et al.*, 1987). The appearance of TTR mRNA in the developing eye was studied in the rat, and it was shown that TTR is expressed at day 16 (Mizuno *et al.*, 1992), in contrast to the choroid plexus where it was detected by day 11 (Soprano *et al.*, 1986).

The half-life of TTR is approximately 2-3 days in humans (Vahlquist *et al.*, 1973) and 10-13 h in rats (Peterson *et al.*, 1974). The major site of TTR degradation, as determined by studies in the rat (both liver and brain TTR), is the liver (35 - 40%) followed by the muscle (10-15%)

and skin (11%); kidneys appear to be responsible for only 6% of total TTR degradation (Makeover *et al.*, 1988)

Gene structure and regulation

Human TTR is encoded by a single-copy gene that was mapped to chromosome 18 (18 q11.2-q12.1 - Sparkes et al., 1987). The gene, represented in figure 2, spans approximately 7 kb and consists of four exons, of about 200 bp, and three introns (A, B and C); exon 1 codes for the signal peptide (20 amino acid residues) and the first three amino acid residues of the mature protein; exon 2 codes for residues 4 to 47, exon 3, 48 to 92, and exon 4, 93 to 127. Introns A and C contain two open reading frames of unknown significance. Several consensus sequences were found upstream the cap site namely, a TATA-box at position -30, a CAAT-box at position -101, and two overlapping sequences homologous to glucocorticoid responsive elements at positions -224 and -212. A polyadenylation signal was identified 123 bp downstream from the coding sequence (Tsuzuki *et al.*, 1985; Sasaki *et al.*, 1985).

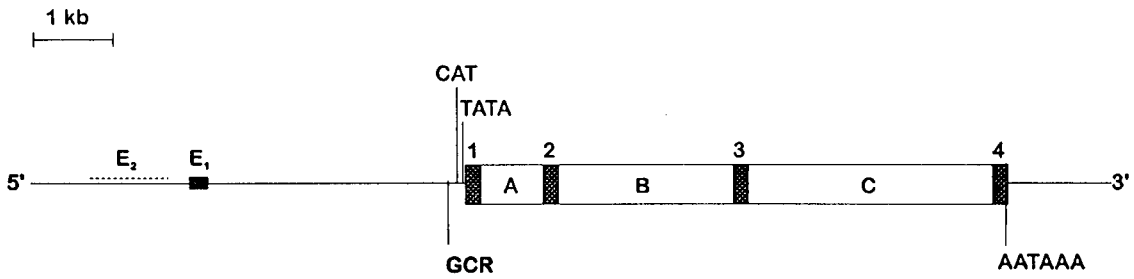


Figure 2 - Human TTR gene structure; exons are referred by numbers and introns by letters; E_1 - sequence homologous to mouse enhancer; E_2 - human specific enhancer ? ;GCR - glucocorticoid responsive element; CAT - CAAT box; TATA - TATA box; AATAAA - polyadenylation signal.

Experiments with hepatoma cells have identified two major cis-regulatory regions in the mouse TTR gene; a promoter proximal region at -70 to -202 relatively to the cap site, and an enhancer sequence at -1.86 to -1.96 kb; these DNA segments contain the binding sites for several liver specific nuclear proteins (Costa *et al.*, 1986, 1989). Furthermore, it was verified that the presence of these segments is sufficient for normal hepatic expression, but not for choroid plexus expression in transgenic animals; the latter required 3 kb of upstream sequences (Yan *et al.*, 1990), thus indicating that choroid plexus and liver use different *cis*- and/or *trans*-acting factors (Costa *et al.*, 1990); differential TTR gene regulation by liver and choroid plexus had already been suggested, since liver TTR mRNA levels decrease during the acute phase response, but not in the choroid plexus

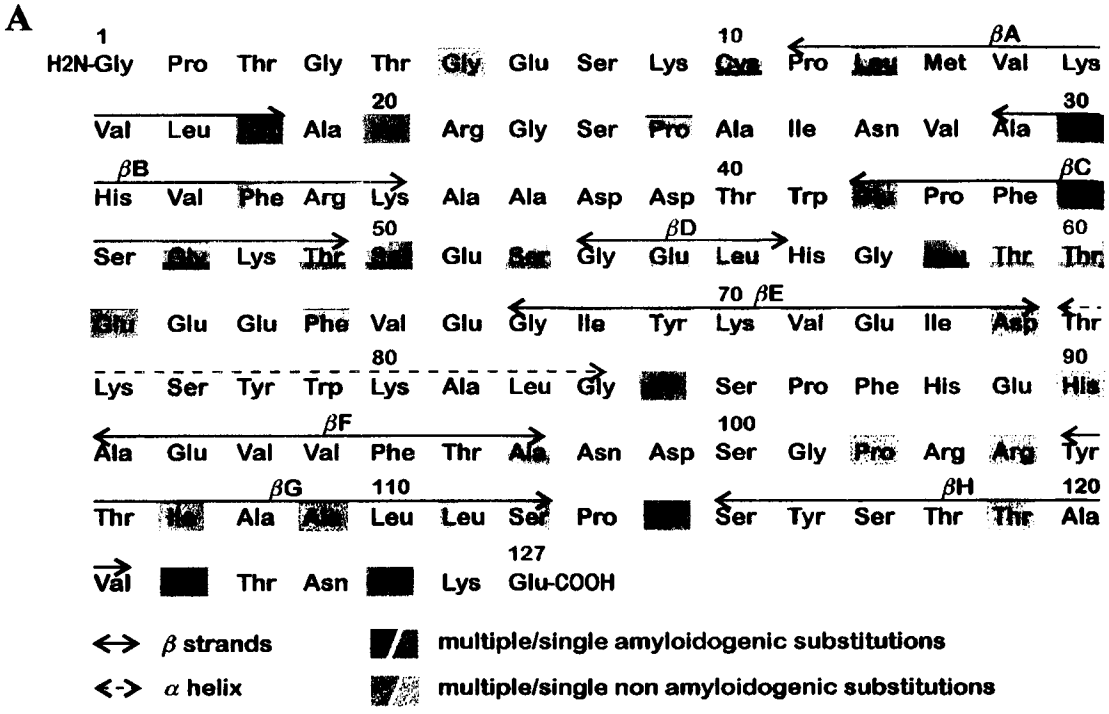
(Dickson *et al*., 1986) In regard to the regulation of the human gene much less is known; although a sequence homologous to the mouse enhancer was found at -3.4 kb, in Hep G2 cells it was a region between -3.9 and -4.9 kb that stimulated transcription by 2 to 3 fold (Sakaki *et al.*, 1989; Sasaki *et al.*, 1990). Furthermore transgenic mouse for the human TTR have shown that the *cis*-elements sufficient for liver and yolk sac expression were present in 600 bp upstream sequences, however for developmental, tissue-specific and quantitatively normal expression, 6 kb of upstream sequences are required (Nagata *et al.*, 1995), therefore it seems that the human TTR gene regulation is similar to that of the mouse.

Protein structure

The TTR monomer is constituted by 127 amino acids and has a molecular weight of 13,475 Da (Kanda *et al.*, 1974); its primary and secondary structures are represented in figure 3A, as well as the residues involved in substitutions in the natural TTR variants.

Forty five percent of the monomer amino acid residues are arranged in eight anti-parallel β sheet strands, designated A through H, with the exception of strands A and G that are parallel. These strands are assembled into a sandwich composed of two four strand sheets, DAGH and CBEF, stabilized by interactions between both sheets. Two monomers, associated through hydrogen bonding between strands F:F' and H:H', and between residues 116:92', form a pair of twisted eight strand sheets, one outer sheet (CBEFF'E'B'C') and one inner sheet (DAGHH'G'A'D'). Two dimers assemble with their inner sheets face-to-face, forming a cylinder with a central hydrophobic channel (figure 3B), where two binding sites for thyroxine exist, stabilized by interactions between loops AB of one dimer and the H strands of the other dimer (Blake *et al.*, 1974; 1978). The RBP molecules interact with one of the TTR dimers, hindering occupancy of the other dimer binding sites (Monaco *et al.*, 1995).

The three dimensional structures of the variants TTR Met 30, TTR Ile 122, TTR Thr 109 and TTR Ser 84 have been determined, revealing an overall structural homology with the normal tetramer. Major differences include: in the Met 30 variant, a higher spacing between DAGH and CBEF sheets to accommodate the bulkier Met 30, with a movement of the outer sheet into the solvent; the movement of DAGH originates a more elliptical central channel with a narrower width in the binding site of thyroxine at the A strand, thus explaining the low affinity of Met 30 tetramers for T_4 (Hamilton *et al.*, 1992; 1993); the opposite effect is observed in the Thr 109 variant (Steinrauf *et al.*, 1993). A higher distance between dimers is also reported for the Met 30 variant (Hamilton, 1992), along with a movement of the A strand exposing residue 10 to solvent (Terry *et al.*, 1993). As for the Ile 122 variant, small changes are observed in the dimers contact, resulting from an increase in the length of the hydrogen bonds between dimers, and within dimers (Damas *et al.*, 1996). Concerning the Ser 84 protein, the alterations reported refer mainly to the



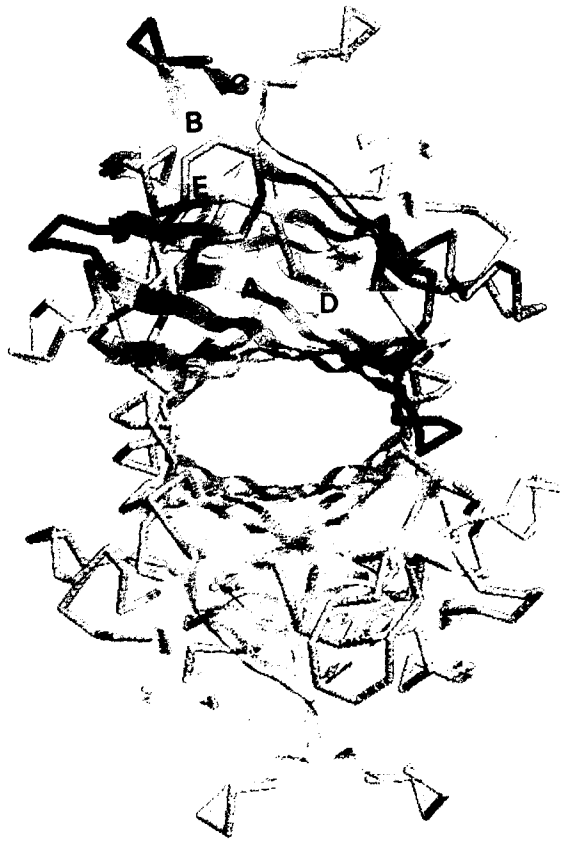


Figure 3 - TTR structure. **Panel A:** Monomer primary and secondary structures. **Panel B:** Ribbon diagram of the tetramer viewed along the axis of the thyroxine-binding channel. The diagram was generated with the program RasMol (R. Sayle, Glaxo Res. and Develop., UK), using the crystallographic coordinates deposited at Brookhaven's Protein Databank. The A monomer of one of the dimers is shaded in gray with indication of the β-strands designations. Positions of aminoacid substitutions in the natural TTR variants are indicated in panels A and B in orange (amyloidogenic) and blue (nonamyloidogenic).

narrowing of a cleft in the surface of the molecule, which is in the binding site region of RBP (Hamilton *et al.*, 1996). Recently the crystallization of the Pro 55 variant was reported (Sebastião *et al.*, 1996) and the preliminary data indicate major changes in the structure, namely the D strand is no longer hydrogen bonded to the A strand (Damas, personal communication).

The changes observed in the dimer interactions in the two amyloidogenic Met 30 and Ile 122 variants could lead to the destabilization of the tetrameric structure, thus facilitating fibril formation.

3. Models of amyloidogenesis

The mechanisms for amyloid fibril formation are unknown; however, several factors appear to contribute to fibrillogenesis, namely the amyloidogenic potential of the precursor protein, tissue factors and chaperone proteins. In figure 4 are represented possible pathways for the transformation of a normal soluble protein into an insoluble polymer, together with potential intervening factors. In this illustration the soluble amyloid protein precursor is converted into an amyloid subunit, by either a conformational modification, proteolysis or both. The amyloid subunit further polymerizes into amyloid fibrils that deposit in tissues. The conversion of the precursor into an amyloid subunit, or the polymerization step, can be induced and/or facilitated by chaperones such as SAP, GAGs, apo E or other still unknown factors. It has also to be considered the possible existence of factors that might prevent either the polymerization step, or the formation of an amyloid subunit.

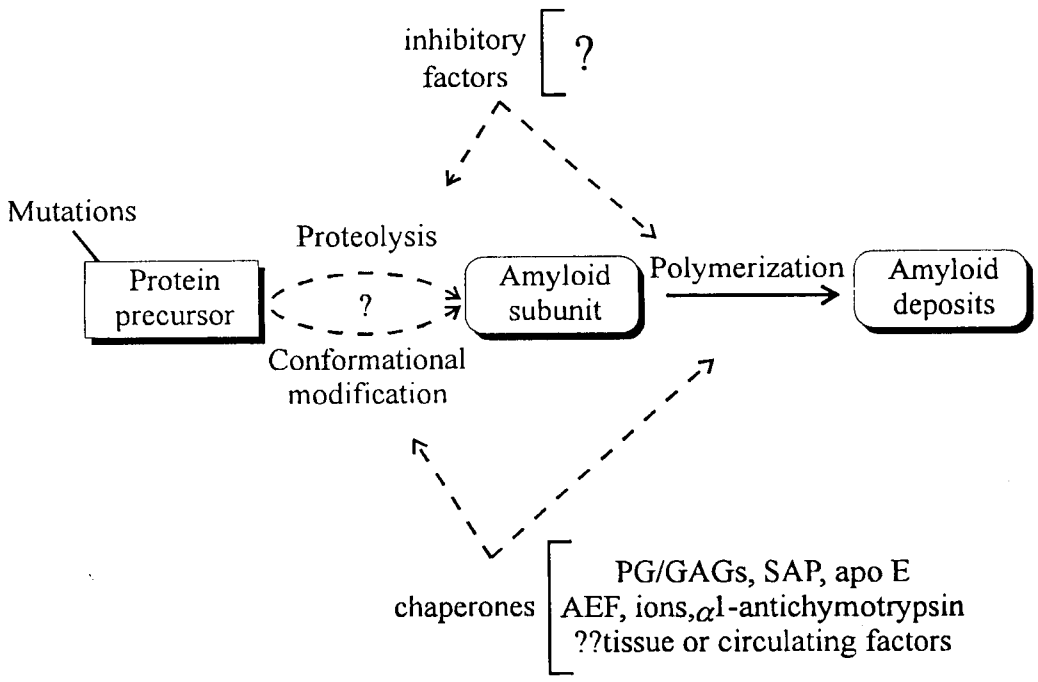


Figure 4 - Possible mechanisms of amyloidogenesis; the process is represented as a sequential one, but it might well be simultaneous.

3.1. Amyloidogenic potential

3.1.1 ATTR

In the case of ATTR two hypotheses have been raised to explain the amyloidogenic potential of the protein: the conformational hypothesis and the proteolytic hypothesis.

Conformational hypothesis

This hypothesis is based on the formation of new structural domains in the TTR molecule due to the amino acid substitutions, originating a molecule with an increased tendency to self-aggregation (Saraiva and Costa, 1991). In this line of thought, some mutations should be non-amyloidogenic, and that is the case of amino acid substitutions at positions 6, 90, 102, 109 and 119 (Saraiva, 1995). Three dimensional studies of the TTR Met 30 variant have indicated that residue 10 (cysteine) is slightly more exposed than in the native TTR molecule, thereby allowing the occurrence of intermolecular disulfide bridges. Based on this finding and on the existence of disulfide bridges in some amyloid fibrils (Felding *et al.*, 1985; Thylèn *et al.*, 1993), Terry *et al.*, (1993) have suggested that the TTR tetramers assemble into amyloid fibrils by intermolecular disulfide bridges, however the existence of an amyloidogenic variant lacking this cysteine residue, TTR Arg 10 (Uemichi *et al.*, 1992), and the *in vitro* production of amyloid-like fibrils from a recombinant TTR, where cysteine 10 was replaced by an alanine (McCutchen and Kelly, 1993), argue against the role of disulfide bridges in TTR amyloid formation.

Colón and Kelly (1992), based on the *in vitro* production of amyloid fibrils from native wild type TTR by acid mediated denaturation and during the refolding of partially denaturated TTR, have suggested that amyloid fibrils could be formed during the normal denaturation or folding pathways of the TTR molecule, by self-assembly of a denaturing/refolding intermediate (the amyloidogenic intermediate); thereby fibril formation and TTR denaturation/folding would be competitive processes. The study of the TTR acid denaturation pathway in the presence or absence of Z3-14 (N-tetradecyl-N,N-dimethyl-3-ammonio-1-propanesulfonate), a detergent that inhibits amyloid formation, by spectroscopic and SDS-PAGE analysis lead to the following model (figure 5): the partial acid denaturation of the native TTR tetramer, achieved in the lysosomal environment, produces a structurally rearranged amyloidogenic monomer, retaining most of its native secondary and some native tertiary structure that polymerizes into amyloid fibrils; further denaturation (by lowering pH) yields a monomer in the so called "A-state", with no native tertiary structure or ability for self-association.

Amyloidogenic mutations would affect the denaturation pathway, facilitating fibril formation by either destabilizing the tetramer, and/or making the intermediate form more accessible under milder acidic conditions. Study of the acid denaturation pathway of some TTR variants, namely variant Met 119, Met 30, Pro 55 and an engineered double mutant Met 119 - Met 30 have indicated that the amyloidogenicity of the variants was inversely correlated with the stability of the tetramer towards acid denaturation (McCutchen *et al.*, 1993, 1995), corroborating the above hypothesis.

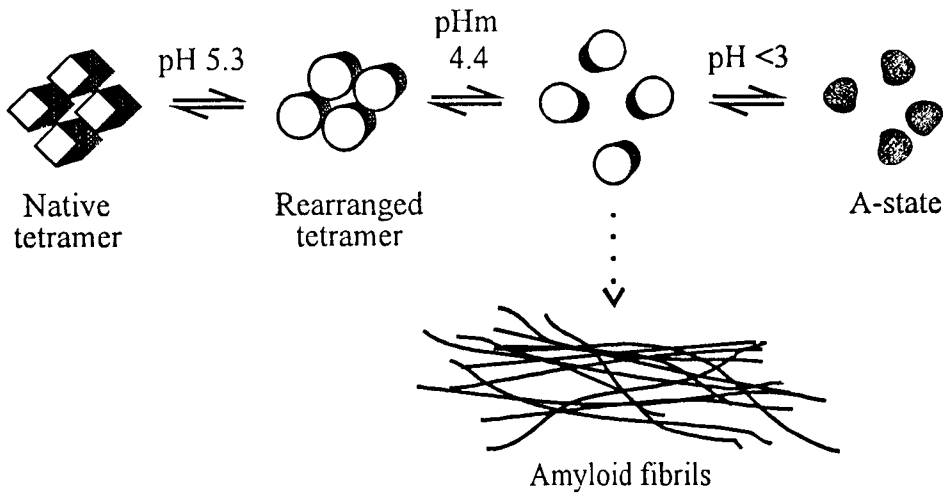


Figure 5 - Acid mediated denaturation pathway of native TTR with possible fibril formation. pHm - midpoint of tetramer to monomer equilibrium.

Kelly and Lansbury (1994) further suggested that in the amyloidogenic monomer strands C and D were displaced from their native position, being more exposed to solvent, and that the rearranged monomers would assemble head to tail into fibrils by interactions between strands A and H.

The involvement of strands C and D in amyloid formation was the subject of an independent study by Serpell and colleagues (1996) that pointed out the existence of a mutational hot spot on the CD region, by the statistical analysis of TTR variants. They further verified that recombinant TTR molecules with a triple substitution/deletion on strand D, formed amyloid fibrils *in vitro* at neutral conditions. Based on these findings, it was proposed that amyloidogenic mutations would alter the molecular structure at the edge of the two β-sheets that form the framework of the TTR molecule (C and D) allowing fibril formation by hydrogen bonding between altered TTR molecules (amyloidogenic intermediate). It was not excluded, however, the assistance of other factors such as fragmentation, molecular chaperones or pH changes, in fibril formation.

Proteolytic hypothesis

The analysis of amyloid fibrils from SSA and some FAP patients revealed the presence of TTR polypeptides, together with intact monomer in variable amounts; in SSA fibrils the fragments

resulted from the cleavage at positions 46, 49 and 52 of the TTR molecule, and represented the bulk of fibril protein (Cornwell *et al.*, 1988); as for FAP fibrils, fragments resulting from cleavage at position 49 were reported for TTR Met 30 carriers (Wahlquist *et al.*, 1991) TTR Ile 122 (Gorevic *et al.*, 1989) and TTR Ile 33 (Pras *et al.*, 1983; Nakazato *et al.*, 1984); amyloid fibrils from TTR Met 111 carriers were shown to contain TTR molecules cleaved at positions 46, 49 and 59 (Nordlie *et al.*, 1990; Hermansen *et al.*, 1995). These findings led to the hypothesis that proteolysis, as a result of physiological mechanisms such as aging, could trigger fibril formation by releasing amyloidogenic fragments that would serve as nidus for the polymerization of fibrils (Saraiva and Costa, 1991); mutations in the TTR molecule could make the tetramer more susceptible to disruption by proteolytic cleavage. In addition, two neutrophil proteases, human neutrophil elastase and cathepsin G, were identified in TTR amyloid deposits (Skinner *et al.*, 1986; Stone *et al.*, 1993). However, the production of *in vitro* amyloid fibrils by intact TTR (Gustavsson *et al.*, 1991, Colón and Kelly, 1992) suggests that proteolysis is not a necessary step for amyloid formation but a secondary event. Furthermore, in view of Kelly and Lansbury mechanism (referred to above) the fragmentation of the TTR molecule in the conserved positions 46, 49 and 52 (C strand and loop CD) further corroborates that this region is more exposed before, during or after fibril formation.

3.1.2. AA

The fact that AA proteins of various lengths, corresponding to half or two thirds of the amino acid terminal portion of SAA have been found in human amyloid deposits (Liepnieks *et al.*, 1995), and that amyloid fibrils are found in lysosomes, in experimental murine amyloidosis (Shirama and Cohen, 1975; Takahashi *et al.*, 1989), led to the idea that proteolysis was an integral part of the *in vivo* amyloid formation mechanism; according to this hypothesis, fibrils would be the result of impaired SAA degradation (Husby *et al.*, 1994b). However no differences in the degradation rates were observed between patients with or without amyloidosis; besides not only was the carboxy-terminal of SAA identified in murine deposits (Miura *et al.*, 1990; Kisilevsky *et al.*, 1994), but also intact SAA molecules have been found in extracted amyloid fibrils (Westermark and Sletten, 1982). In addition, intact SAA deposits as AA fibrils in the duck (Ericsson *et al.*, 1987), and intact recombinant SAA_1 forms amyloid like fibrils *in vitro* (Yamada *et al.*, 1994). Therefore, proteolytic cleavage appears to occur as a post-fibrillogenic event, not being crucial for fibril formation. In most species AA proteins derive from only one SAA isoform, that varies among species, suggesting that the primary structure of SAA molecules determines its amyloidogenicity; in humans, however, several isoforms were identified in amyloid deposits (Husby *et al.*, 1994b). *In vitro* studies with synthetic peptides revealed that the amino terminal residues (first 12 amino acids) were essential for amyloid formation (Westermark *et al.*, 1992,Yamada *et al.*, 1995) and determined the

amyloidogenicity of the molecule. Since human SAA isoforms are homogenous in this region, that could explain why no preferential isoform is deposited. However, a recent report indicates a higher predominance of SAA_1 isoforms over SAA_{2b} in six cases of reactive amyloidosis (Liepnieks *et al.*, 1995). It was also reported a higher allelic frequency of SAA_{1g} in AA amyloidosis patients than in the control population (Baba *et al.*, 1995). Therefore, other factors (pathological chaperones) must be involved in the pathogenesis of AA amyloidosis.

3.1.3. AL

Due to the inherent variability in light chain (L-chain) primary structure, it is very difficult to associate a single site specific amino acid residue with the amyloidogenicity of the protein. Although not explaining the amyloidogenicity of some L-chains, the initially proposed mechanism for amyloid formation in AL amyloidosis was by lysosomal proteolytic processing; this proposal was based on the presence of L-chain fragments in amyloid deposits of patients, (Glenner, 1980) and on the observation that proteolysis could induce *in vitro* amyloid formation from intact light chains (Epstein *et al.*, 1974). However, not only are intact L-chains also present in the amyloid deposits of some patients (Terry *et al.*, 1973), but also they can produce *in vitro* amyloid fibrils (Klafki *et al.*, 1993). Recently two theories emerged attempting to explain the amyloidogenic potential of L-chains (Solomon and Weiss,1995): Hurle *et al.*, (1994) suggested that specific amino acid replacements destabilize the tertiary structure of the V_L domain, leading to the formation of an intermediate with a different folding, more prone to aggregation than the native state; this theory was based on the fibrillogenic capacity of recombinant V_L constructs, that contained amino acid replacements in conserved positions, thought to destabilized the folded structure of the molecule. Stevens *et al.*, (1995) on the other hand proposed that native V_L domains non covalently associate into dimers, which stack into linear polymers that ultimately associate laterally forming stable fibrils; the basis for this theory came from the statistical analysis of the frequency of particular residues within V_L domains of 180 L-chains showing that specific positions appeared to be associated with amyloidosis; these positions were mainly distributed along the surface of the L-chain not being involved in the internal packing of the V_L domain; moreover the side chains of the residues in those positions would be available for intermolecular interactions stabilizing macroaggregates.

3.1.4. Polypeptide hormone derived amyloid

Among the polypeptide hormone derived (PHD) amyloids, the IAPP derived amyloid is the most studied; in humans, the deposition of amyloid in Langerhans islets of non-insulin-dependent diabetes (diabetes type II) patients is dependent not only on IAPP primary structure, since IAPP from some species, lacking a particular sequence, do not form amyloid deposits either *in vivo* or *in vitro* (Westermark *et al.*, 1990b), but also on the overexpression of IAPP, as high levels of IAPP were shown to be sufficient for amyloid formation in both transgenic cultured islets and

COS-1 cells producing human IAPP (Koning *et al.*, 1994; O'Brien *et al.*, 1995). However, transgenic animals overexpressing human IAPP have no amyloid deposits (Westermark *et al.*, 1995). One possible explanation would be that in diabetes type II the IAPP clearance and/or cellular trafficking mechanisms are defective, which would account for high concentrations of IAPP (extracellularly and intracellularly), thus facilitating fibril formation (Koning *et al.*, 1994; O'Brien *et al.*, 1994).

In relation to the other PHD-amyloids little is known, the occurrence of amyloidogenic sequences in the polypeptide hormones and the local concentration of the fibril precursor appear to be important, but again other factors must be involved (Westermark, 1994).

3.1.5. Aβ

Amyloid formation from APP was initially thought to result from a defective proteolytic pathway and/or membrane damage (Halverson *et al.*, 1990); however the existence of soluble Aβ in cerebral spinal fluid and in plasma (Seubert *et al.*, 1992; Shoji *et al.*, 1992) favoured an alternative mechanism, whereby the conformation and aggregation of Aβ or APP fragments is critical for amyloidogenesis (Frangione *et al.*, 1994). A general model for Aβ fibrillogenesis is represented in figure 6; soluble monomeric Aβ is converted into (or exists in equilibrium with) a β-sheet intermediate, that self-assembles initiating fibril formation (nucleation phase); further intermediate addition results in fibril growth, and the association of these fibrils originates fibers that precipitate into the amyloid deposits (growth phase).

This process can be envisioned as a one-dimensional crystallization whereby the nucleation phase is rate limiting, and thus the presence of a nucleus accelerates the process (Jarret and Lansbury,1993). The β-sheet intermediate has been suggested to be a dimer (Shen and Murphy,1995), or a pentamer/hexamer (LeVine 1995a), or, as recently reported by Soto *et al.*, (1995a), a monomer representing almost completely a β-sheet conformation. What promotes the conversion of soluble Aβ into the β-sheet intermediate is still unclear, and there are several candidates that can be divided in two categories: environmental and genetic factors. Most environmental factors were elucidated by *in vitro* studies of fibril formation from synthetic peptides, although it should be taken into account that sample preparation method and sample history affect the resulting aggregate concentration, size and morphology (Shen *et al.*, 1993, 1994).

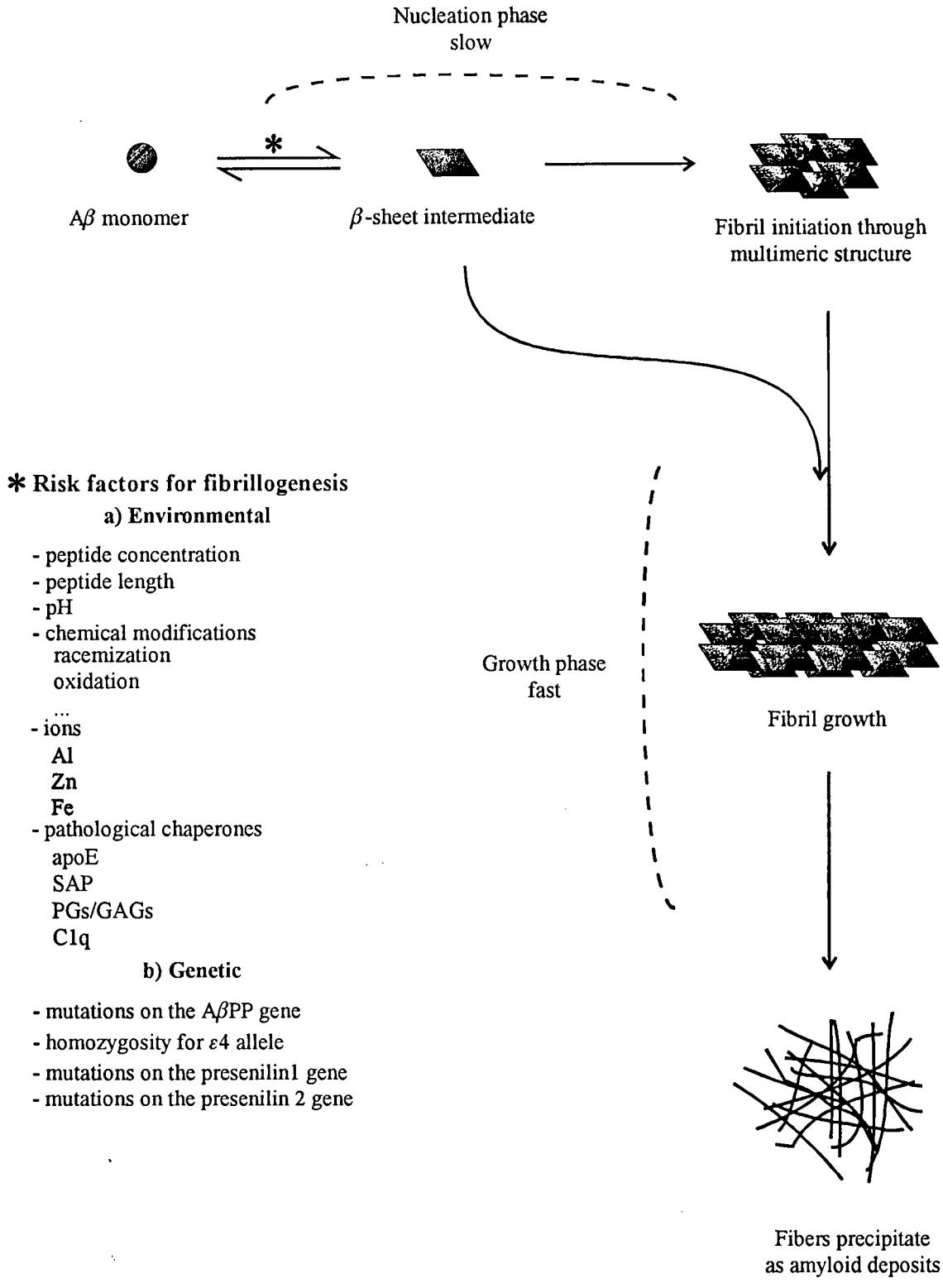


Figure 6 - Hypothetical model of amyloid formation by AβPP, with discrimination of possible intervening factors.

It has been shown that: i) aggregation is strongly dependent on peptide concentration, pH, time, temperature, ionic strength and seeding (Pike *et al.*, 1991; Shen *et al.*, 1993; Jarret *et al.*, 1993; Snyder *et al.*, 1994); ii) peptide length is also important, since longer β(1-42/43) nucleates and aggregates more rapidly than shorter β(1-39/40) (Jarret *et al.*, 1993; Snyder *et al.*, 1994); in effect, it was suggested that β(1-42/43) would initiate fibril formation and β(1-39/40) would be involved in the elongation of fibrils (Jarret *et al.*, 1993; Tamaoka *et al.*, 1994a). iii) Chemical modification of β peptides, such as racemization of aspartic and serine residues and amino acid oxidation by free radical attack oxidation promote aggregation (Tomiyama *et al.*, 1994a; Vyas and Duffy, 1995; Dyrks *et al.*, 1992, 1993; Hensley *et al.*, 1994). iv) Metal ions including aluminum, iron, and zinc were also suggested to play a role in Aβ fibrillogenesis, as well as chaperone proteins, namely apolipoprotein E, α_1-antichymotrypsin, serum amyloid P component, heparan sulphate proteoglycan, and complement protein C1q, as will be described below.

Genetic factors associated with Aβ amyloidosis include: i) the existence of mutations in the APP molecule, which appears to be associated with an earlier age of onset (Schellenberg, 1995); the substitution of glutamic acid by a glutamine at position 22 of Aβ, occurring in hereditary cerebral hemorrhage with amyloidosis of Dutch type (Levy *et al.*, 1990), was shown to accelerate fibril formation *in vitro* (Wisniewski *et al.*, 1991); furthermore transfection of cells with the double mutant APP Asn_{670}/Leu_{671} induces a several fold increase in Aβ production (Citron et al 1992; Cai *et al.*, 1993); similar transfection studies with APP Ile_{717} or Phe_{717} or Gly_{717} have shown that the ratio of β(1-42) production to β(1-40) was increased (Suzuki *et al.*, 1994; Tamaoka *et al.*, 1994b); ii) inheritance of the naturally occurring ε4 allele of the apoliprotein E gene, that increases the likelihood of development of late-onset Alzheimer's disease (AD) and lowers its age of onset (Corder *et al.*, 1993). Conversely the inheritance of apoE ε2 allele appears to confer a decreased risk of developing AD (Corder *et al.*, 1994). iii) Mutations in the presenilin 1 gene in chromosome 14, and in the presenilin 2 gene in chromosome 1 have recently been linked to early-onset familial AD (Sherrington *et al.*, 1995; Rogaev *et al.*, 1995; Van Broeckhoven, 1995) and it is suggested that mutations in these genes affect AβPP sorting, leading to a higher production of β(1-42) form (Kovacs *et al.*, 1995).

As for factors that might prevent the conversion of soluble Aβ into the β-sheet intermediate, it was reported by Wisniewski *et al.*, (1993) that cerebrospinal fluid inhibited amyloid formation from Aβ *in vitro*, leading to the hypothesis that sequestration of soluble Aβ by some proteins prevents amyloid formation. Several candidate proteins were considered, namely apoJ, TTR and apoE. TTR was shown to inhibit Aβ aggregation *in vitro* (Schwarzman *et al.*, 1994), and to prevent accumulation of Aβ deposits in cultured canine smooth muscle cells (Mazur-Kolecka *et al.*, 1995); under physiological conditions apoJ, a multifunctional glycoprotein present in nearly all body fluids, binds with a higher affinity soluble but not aggregated Aβ (Matsubara *et al.*, 1995; Golabek *et al.*, 1995). Further studies are needed to elucidate the role of these proteins in fibrillogenesis.

3.2. Pathological chaperones

The presence of the protein precursors is necessary for fibril formation; what determines the deposition of these precursors as amyloid fibrils in some individuals and the variation in time and anatomical distribution of the deposits is probably dependent on other factors, that may act as pathological chaperones. Pathological chaperones were defined as unrelated proteins that play a role in the aggregation/folding of amyloid precursors and/or bind to amyloid preventing its degradation (Wisniewski and Frangione, 1992); they can be either tissue or circulating factors; among these are:

3.2.1. Serum amyloid P component

Serum amyloid P component (SAP), is a homodecameric plasma glycoprotein with a known three dimensional structure (Emsley *et al.*, 1994) but whose physiological function is at present unknown; SAP binds in a calcium dependent manner several ligands, such as DNA, chromatin, C4-binding protein, glycosaminoglycans among others; it is also a calcium-dependent lectin and a constituent of glomerular basement membranes and elastic fibers (Pepys and Baltz, 1983; Butler *et al.*, 1990). SAP is associated with all amyloid deposits *in vivo* in a calcium dependent fashion (Pepys, 1988); it is therefore hypothesized to protect amyloid fibrils from *in vivo* degradation by masking its abnormal structure, and/or because it is highly resistant to proteolytic digestion in the presence of calcium (Kinoshita *et al.*, 1992). In effect, SAP was shown to protect amyloid fibrils from the digestion with several proteinases *in vitro*, but only when bound to the fibrils (Tennent *et al.*, 1995). In addition to this protective role, it was recently suggested that SAP affects *in vitro* aggregation of Aβ peptides; Janciauskiene *et al.*, (1995) reported that SAP inhibited Aβ(1-42) aggregation, however Hamazaki (1995) stated that SAP promotes Aβ(1-40) aggregation. This opposite effect is probably a result of the different experimental conditions used in both reports.

3.2.2. Proteoglycans and glycosaminoglycans

Proteoglycans (PGs) are complex macromolecules composed of glycosaminoglycan (GAG) chains, which are highly sulphated carbohydrates, covalently bound to a protein core. PGs are ubiquitous in the organisms, being present intracellularly, associated with the cell surface, or in the extracellular matrix. PGs play a role in various physiological processes such as: maintaining the structural integrity of tissues, mediating cell adhesion, motility, differentiation and even morphogenesis (Kjellén and Lindahl, 1991). PGs and/or GAGs are also present in all amyloids regardless of the precursor protein involved (Snow *et al.*, 1987), and belong mainly to the heparan sulphate proteoglycan (HSPG) class (Snow *et al.*, 1988; Husby *et al.*, 1994c). In an experimental model of AA amyloidosis it was observed that GAGs concurrently deposited with the amyloid protein (Snow and Kisilevsky, 1985), thus PGs/GAGs were hypothesized to play a role in amyloidogenesis.

According to Snow and Wight (1989) PGs/GAGs could:

i) initiate fibrillogenesis by promoting amyloidogenic protein conformations; this idea is supported by the observation that heparan sulphate increases the amount of β-pleated sheet structure of murine SAA_2 but not SAA_1 *in vitro* (McCubbin *et al.*, 1988); however, it can also be refuted by the *in vitro* formation of amyloid like fibrils *per se*, although in nonphysiological conditions (Gustavsson *et al.*, 1991; Yamada *et al.*, 1994; Maury and Nurmiaho-Lassila, 1992);

ii) stabilize amyloid fibrils by maintaining the amyloid precursor/fibril in an aggregated state; in effect, Fraser *et al.*, (1992) showed that heparan sulphate could induce extensive lateral aggregation of β-peptide with macrofiber formation; moreover *in vivo* experiments have indicated that HSPG promotes the deposition of Aβ peptide and contributes to the persistence of those deposits (Snow *et al.*, 1994). Glycosaminoglycans were also suggested to play a role in the maintenance of AA fibril structure through the sulphate moiety (Wong and Kisilevsky, 1990)

iii) prevent amyloid degradation either by enhancing the stability of pre-formed fibrils, or by interacting with proteases and/or protease inhibitors. That HSPG can protect some proteins against enzymatic degradation has already been observed by Saksela *et al.*, (1988); likewise, Gupta-Bansal *et al.*, (1995) reported that both heparan and chondroitin sulphate proteoglycans inhibit proteolytic degradation of fibrillary, but not nonfibrillary, Aβ by papain and cathepsin B.

iv) determine the anatomical distribution of the amyloid deposits, which could occur through the preferential binding to amyloidogenic precursors.

This importance of PGs/GAGs in amyloidogenesis lead to the hypothesis that possible therapeutic agents could be molecules that interfered with the PG/amyloid interactions. In effect, growing evidence supports this idea; sulphated polyanions were shown to inhibit the accumulation of the protease resistant isoform of prion protein in cell culture and to delay or abolish scrapie pathogenesis *in vivo* (Priola and Caughey, 1994); in addition it was shown that sulphated polyanions inhibited the binding of heparan sulphate to β-amyloid peptide *in vitro* (Leveugle *et al.*, 1994), and attenuated the neurotoxic effects of β-amyloid in cell culture (Pollack *et al.*, 1995; Sadler *et al.*, 1995); finally, anionic polysulphonates and disulphates were reported to inhibit AA amyloidogenesis *in vivo* (Kisilevsky *et al.*, 1995a), being the mechanism proposed for such inhibitions a competition between the exogenous GAGs/sulphonates with the endogenous proteoglycans.

3.2.3. Apolipoprotein E

Apolipoprotein E (ApoE) is a 34 kDa apolipoprotein present in all types of lipoprotein particles, involved in cholesterol transport (Mahley, 1988). Among other characteristics, it is the main lipoprotein synthesized in brain and it is involved in peripheral nerve regeneration (Poirier,1994). Apo E was shown to be present in both systemic and cerebral amyloid deposits

(Wisniewski and Frangione, 1992; Gallo *et al.*, 1994); and, in addition, apo E genotype has been linked to sporadic and late onset Alzheimer's disease (Roses, 1996), although it appears not to affect either the susceptibility to AA amyloidosis, or the age of onset in TTR Met 30 FAP individuals (Lovat *et al.*, 1995), or plaque formation in transmissible spongiform encephalopathies (Dranovsky *et al.*, 1995). Apo E was, therefore, suggested to play a role in mediating amyloidogenesis; in both canine and human smooth muscular cells apo E induced intracellular accumulation of β-peptide (Mazur-Kolecka *et al.*, 1995); *in vitro* experiments have shown that apo E not only interacts with Aβ peptides to produce morphologically distinct fibrils (Sanan *et al.*, 1994), but it also promotes amyloid fibril formation from Aβ peptides (Ma *et al.*, 1994; Wisniewski *et al.*, 1994; Castaño *et al.*, 1995a) and from synthetic peptides corresponding to amyloidogenic sequences of gelsolin and AA protein. Moreover this promoting effect is more pronounced with lesser amyloidogenic peptides, suggesting that apo E changes the conformation of the peptides, turning them more competent to form amyloid fibrils (Soto *et al.*, 1995b). It was also reported that apo E could inhibit Aβ aggregation *in vitro* (Evans *et al.*, 1995), this discrepancy from the above results was thought to be due to different experimental conditions, but it also raised the idea of a dual role for apo E, depending on local concentrations of apo E and amyloid precursors *in vivo* (Castaño *et al.*, 1995b).

3.2.4. Amyloid enhancing factor

Amyloid enhancing factor (AEF) is defined as an entity, that when injected in mice or hamsters, together with a inflammatory stimulus, reduces the induction time of AA amyloidosis from 2-3 weeks to 2-3 days. Its precise nature is not known, it appears to be proteinaceous since its activity is abolished by protease treatment, or denaturing conditions. AEF activity was found in several preparations, namely in amyloid fibrils, tissue extracts and peritoneal macrophages from amyloidotic animals and splenic extracts from normal and aged mice (Kisilevski *et al.*, 1995b). Recently, amyloid like fibrils were also shown to possess AEF like activity (Ganowiak *et al.*, 1994), suggesting that this activity may reside in a unique conformation of different protein molecules, and further supporting the idea of AEF as a nucleus that once formed, speeds up amyloid fibril formation. This effect would be similar to the increase observed in the rate of β-peptide fibrillogenesis *in vitro* by pre-formed fibrils (Jarret *et al.*, 1993).

3.2.5. Additional factors

Other factors have been suggested to play a role in particular syndromes; these include α_1-antichymotrypsin, complement proteins and metal ions. The former is a protease inhibitor found in close association with amyloid deposits in Alzeimer's disease (Abraham *et al.*, 1988), that was shown to decrease the rate of β(1-40) aggregation and to disaggregate preformed fibrils *in vitro* (Fraser *et al.*, 1993; Eriksson *et al.*, 1995); however, a promoting effect on β(1-42) aggregation *in vitro* was also mentioned (Ma *et al.*, 1994). Complement protein C1q is also found in Aβ deposits

(Rogers *et al.*, 1992) and it is reported to enhance Aβ aggregation by a nucleating phenomenon (Webster *et al.*, 1995). Zinc, aluminum and iron were all shown to enhance β-peptide aggregation *in vitro* (Mantyh *et al.*, 1993; Bush *et al.*, 1994; Kawahara *et al.*, 1994); in addition, β peptide has binding sites for zinc (Bush *et al.*, 1993), and aluminum not only appears to enhance β-peptide neurotoxicity (Kuroda *et al.*, 1995), but it is also considered an environmental risk factor for Alzheimer's disease (McLachlan *et al.*, 1991). Recently, zinc and calcium were also mentioned to enhance *in vitro* fibril formation from IAPP (Westermark *et al.*, 1996).

It is noteworthy to emphasize the variability of effects (sometimes opposite) observed in β-peptide aggregation *in vitro* by some chaperones; that is most certainly due to the peptide-handling procedures used and the different techniques employed to monitor and characterize β-peptide aggregation.

In summary, the process of amyloid formation is definitely a multifactorial one, where an amyloidogenic precursor is necessary, and, by either a proteolytic event, and/or a conformational modification, in conjunction with the above described chaperones and/or with other as yet undefined factors, leads to the appearance of insoluble protein fibrils as amyloid. Further elucidation of amyloid pathogenesis, and ultimately an effective treatment, depends in part on the availability of *in vivo* experimental models, i.e. animal models.

4. Experimental models for the study of amyloidogenesis

4.1. Animal models

Animal models are very useful for the study of human diseases, both genetic and acquired, not only to elucidate the mechanisms of the pathological processes, but also to test therapeutic strategies. In addition, they can be used to analyze the physiological role of a particular protein. Since the majority of the human pathologies has no natural animal models, it was necessary to design ways of reproducing in animals particular phenotypes, and that was achieved either by inducing a particular physiological condition, or by altering the animals germ line through transgenic technology.

In relation to amyloidosis, the most frequent form occurring in a number of species is senescence associated amyloidosis, in which the amyloid precursors are SAA, ApoAI and ApoAII (Higuchi *et al.*, 1991; Johnson *et al.*, 1992). Deposition of Aβ in the dog, polar bear, and monkey, but not in rodents, has been described (Selkoe *et al.*, 1987) with some neuropathological characteristics of aging and Alzheimer's disease. Sporadic cases of other amyloidosis were also reported such as AL amyloidosis in cats, horses and a dog (Liepnieks *et al.*, 1991; 1994). Induced animal models were obtained for AA, AL and Aβ, and transgenic mice were produced for ATTR, Aβ, and AIAPP.

4.1.1. Induced animal models

It is known for long that AA amyloidosis can be experimentally induced in mice, by using a variety of stimulus, namely alveolar hydatid cyst infection (Du and Ali-Khan, 1990), casein, azocasein, silver nitrate (Skinner *et al.*, 1977), or endotoxins to produce sustained inflammation. This model has given a great contribution for the understanding of AA pathogenesis (Husby *et al.*, 1994b); it was used to study, among other things: the amyloidogenicity of the SAA isoforms deposited (Meek *et al.*, 1986; Hérbert and Gervais, 1990; Sipe *et al.*, 1993), whether proteolysis is a pre- or post-fibrillogenesis event (Shirama and Cohen, 1975; Kisilevski *et al.*, 1994), the role of pathological chaperones (Lyon *et al.*, 1991; Ailles *et al.*, 1994), and the paradigm of AEF (Kisilevski *et al.*, 1995b).

Concerning AL amyloidosis, Solomon and coworkers (Solomon *et al.*, 1992), described the experimental induction of human AL amyloid deposits in mice, whereby the repeated injection of human light chains (either λVI or κI), isolated from myeloma and amyloidosis patients urine, lead to the deposition of this material (together with mouse SAP) as amyloid in renal blood vessels, parenchymal tissue as well as in other organs, with a pattern similar to the one found in patients. No amyloid deposition was observed in control mice injected with non amyloid associated light chains. It was further observed that dehydration of the animals prior to the infusion of light chains, accelerated amyloid formation and diminished the amounts of light chains necessary for amyloid formation. This model was suggested to be a mean to study not only the amyloidogenicity of light chains but also the pathogenesis of the disease.

The unique tentative induced animal model for Alzheimer's disease was reported by Snow *et al.*, (1994) whereby the infusion of synthetic Aβ(1-40) and a specific heparan sulphate proteoglycan in rat brain led to the formation of amyloid deposits adjacent to the infusion; these deposits were shown to contain Aβ and the proteoglycan, but no other neuropathological changes were observed. Although successful in obtaining amyloid deposition this model is very limited as compared to the transgenic animal models developed, as described below.

4.1.2. Transgenic animals

The most widely used strategy to produce transgenic animals, has been the microinjection of foreign DNA into the pronuclei of embryos, that are subsequently implanted into pseudopregnant females. The efficiency of pronuclear microinjection is relatively high, about 30% of the progeny carry the transgene. Another strategy also applied, although presenting some limitations on the transgene size, has been the use of retroviral vectors, containing the gene of interest, to infect embryos; and recently, the genetic manipulation of pluripotent embryonic stem cells and successful incorporation into blastocytes has also allowed the generation of inactivating mutations in individual genes (gene knock out).

ATTR

There are no natural models for FAP and therefore several groups used transgenic mouse technology as an attempt to obtain an animal model for this pathology.

In 1986 Sasaki *et al.* described the generation of the first transgenic mice producing human TTR Met 30; these animals carried the structural gene for the human TTR Met 30 variant under the control of the mouse metalothionein gene promoter (MT1-TTR Met 30) and, when induced with zinc, had the human protein in the circulation. Expression of the transgene was found in several tissues, and varied among the transgenic strains. Liver and kidney had very low levels of transgene expression which was explained to be probably due to the integration of additional plasmid sequences in the fusion construct (Sasaki *et al.*, 1989).

In 1987 Wakasugi *et al.* described the production of transgenic mice carrying the same type of construct, the structural gene for the human TTR Met 30 variant under the control of the mouse MT promoter (MT2-TTR Met 30); in these animals amyloid deposition was observed from 6 months of age in the mucosa of the small intestine. The amyloid deposits were shown to contain the human protein and mouse amyloid P component, as assessed by immunological methods, and as animals aged systemic deposition was observed; however brain, peripheral nerves and hematopoietic tissues were spared. This pattern of amyloid deposition was similar to the one found in human FAP autopsy cases with the exception of the peripheral nervous system, which in the latter, is particularly affected (Yi *et al.* 1991, Araki *et al.*, 1994).

Transgenic mice carrying the structural gene for the human TTR Met 30 variant under the control of its own regulatory regions were also produced by Yamamura's group (Yamamura *et al.* 1987, Nagata *et al.* 1995). In one of these strains (0.6-hTTR Met30), the human gene was driven by 600 bp regulatory regions upstream the cap site restricting transgene expression to the liver and yolk sac. Systemic amyloid deposition was observed from 15 months of age (Shimada *et al.*, 1989).

To obtain proper transgene expression levels and specificity, the 6 kb of upstream human TTR regulatory regions were used to induce the human gene (6-hTTR Met30). The expression of the transgene was shown to be developmental, tissue-specific and quantitatively normal. The human protein levels in the circulation were higher than in the other strains and amyloid was observed in 9 month old animals (Nagata *et al.*, 1995). Again, the peripheral nervous system was spared although the protein was highly expressed in the choroid plexus (Maeda *et al.*, 1996).

As the amyloid deposits in these transgenic animals contained, besides human TTR, mouse SAP, which is an acute-phase reactant in mice, and, in order to study the relationship between SAP and amyloidogenesis, MT2-TTR Met 30 mice were subjected to repeated injections of *Escherichia coli* lipopolysaccharide over time. Induction of SAP by acute inflammation appeared

not to change either the onset or the extent of amyloid deposition (Murakami *et al.*, 1992c). Double transgenic animals were also produced by mating MT2-TTR Met 30 mice with transgenic mice carrying the human SAP structural gene with its own regulatory regions to investigate the effect of human SAP in the process of amyloid deposition in the MT2-TTR Met 30 mice. The onset, progression and tissue distribution of amyloid deposition in these double transgenic animals did not differ from the MT2-TTR Met 30 mice, thus indicating that human SAP does not play a role in the deposition of amyloid in these animals (Tashiro *et al.*, 1991).

Recently Yokoi *et al.* (1996) described a transgenic mouse strain devoid of endogenous mouse TTR and carrying the 6-hTTR Met 30 construct (6-hMet 30); amyloid deposition was similar to the 6-hTTR Met 30 transgenic strain, indicating therefore that endogenous mouse TTR does not intervene in the deposition of the human protein as amyloid.

Finally, transgenic mice as models for other TTR related amyloidosis were also described, although none of them possessed TTR reactive amyloid deposits (Teng *et al.*, 1996; Waits *et al.*, 1996). Table 9 summarizes the current transgenic mice strains produced as models for FAP and its main characteristics. Several factors remain unexplained, namely the non deposition of amyloid in some transgenic strains: MT1-TTR Met 30 and TTR Pro 55 (the TTR Ser 84 are not old enough); and the absence of amyloid deposition in the peripheral nervous system in the strains that develop systemic amyloidoses.

Table 9 - TTR transgenic strains

Transgenic Strain	Genetic Background	Promoter	[human TTR] mg/dl	Tissue Expression	TTR amyloid deposition start age (months)	TTR amyloid deposition organs affected	References
MT1-TTR Met30	(C57BL/6xC3H)F1 x Balb C	mouse metalothionein	0.25 - 1.4	intestine, testis, brain, heart	-	negative	Sasaki *et al.*, 1986 1989
MT2-TTR Met30	C57BL/6	mouse metalothionein and hTTR promoter	1.0 - 4.8	liver, brain, kidney, heart, lung, skeletal muscle	4/6	heart, GI tract, kidney, skin, thyroid vesicular glands	Wakasugi *et al.*, 1987 Shimada *et al.*, 1989 Yi *et al.*, 1991
0.6-h TTR Met30	C57BL/6	0.6kb hTTR regulatory regions	0.2 - 3.0	liver, yolk sac	15	np	Yamamura *et al.*, 1987 Shimada *et al.*,. 1989
6-h TTR Met30	C57BL/6	6 kb hTTR regulatory regions	0 - 17.1	liver, yolk sac, choroid plexus, kidney	9	kidney, esophagus,heart, stomach, intestine, lung, spleen	Nagata *et al.*, 1995 Maeda (pc)
6-h Met30 (knock-out for mTTR)	MF1x129/Sv//Ev	6 kb hTTR regulatory regions	20 - 60	liver, yolk sac, choroid plexus, kidney	11	Esophagus, stomach, heart, lung, liver, bladder, intestine, kidney, thyroid , spleen	Yokoi *et al.*, 1996 Episkopou *et al.*, 1993 Maeda (pc)
TTR Ser84	C57BL/6xC3H	7 kb hTTR regulatory regions	25	liver, yolk sac, choroid plexus	-	negative	Waits *et al.*, 1996
TTR Pro55	C57BL/6xDBA/2	all known hTTR regulatory regions	2 - 4	liver, eye, brain	-	negative	Teng *et al.*, 1996

np - not published; pc - personal communication

Aβ

Transgenic mice as models for Alzheimer's disease have been produced by several groups that have made use of a variety of experimental strategies and mice strains. The DNA constructs tested included the entire AβPP human gene (Pearson and Choi, 1993; Lamb *et al.*, 1993), the human AβPP cDNA of the different isoforms with (Games *et al.*, 1995) or without known mutations (Quon *et al.*, 1991; Yamaguchi *et al.*, 1991; Hsiao *et al.*, 1995), the sequence encoding the carboxy terminal 100 amino acids of human AβPP (Kammesheidt *et al.*, 1992; Sandhu *et al.*, 1991; Kawabata *et al.*, 1991; Fukuchi *et al.*, 1993; Araki *et al.*, 1995) and the coding sequence for human or mouse Aβ (Wirak *et al.*, 1991; LaFerla *et al.*, 1995); and the expression of the transgene in abnormal locations was also experimented (Quon *et al.*, 1991; LaFerla *et al.*, 1995). The observed phenotypes were diverse and some of them were not reproducible (Wirak *et al.*, 1992); the most relevant strains and their main characteristics are summarized in table 10.

Table 10 - Aβ/AβPP transgenic strains

Transgene	Promoter	Characteristics	References
Mur Aβ	Mouse NF-L	Over production of β-peptide intracellularly; marked neurodegeneration, apoptosis and gliosis; premature death	LaFerla *et al.*, 1995
104-CT AβPP	brain dystrophin	Accumulation of Aβ immunoreactivity in stroma and neuropil and thioflavine S staining in some vessels	Kammesheidt *et al.*, 1992
100-CT AβPP	JC virus	Aβ immunoreactivity in brain	Sandhu *et al.*, 1991
$A\beta PP_{695}$	Metallothionein IIA	Slight learning impairment	Yamaguchi, *et al.*, 1991
Hum$A\beta PP_{695}$, mur $A\beta PP_{695}$	Prion protein	Premature death (earlier with human APP); acceleration of a senescent CNS disorder of FVB/N mice	Hsiao *et al.*, 1995
$A\beta PP_{751}$ cDNA	Rat neuronal-specific enolase	Overexpression of transgene in neurons; diffuse β amyloid deposits; presence of abnormal tau protein in neurons; gliosis; structures similar to dystrophic neurites; impaired memory and learning acquisition; phenotype more significant with age	Quon *et al.*, 1991; Higgins *et al.*, 1995; Moran *et al.*, 1995
AβPP Phe 717	PDGF β-chain	Expression of the 3 APP isoforms; amyloid deposition by 6 months; gliosis; dystrophic neurites	Games,*et al.*, 1995
400kb AβPP	AβPP	No neuropathology or behavioural anomalies	Pearson and Choi, 1993; Lamb *et al.*, 1993

CT-carboxy terminal; NF-L - neurofilament light chain; CNS - central nervous system; PDGF - platelet-derived growth factor; Hum - human; mur - murine.

Despite the fact that some transgenic strains have neuropathological and/or behavioural similarities with AD, none of them completely models the AD phenotype, as in the case of ATTR transgenic mice; these animals are nonetheless useful to elucidate the role of AβPP expression. Mice with a null mutation in the AβPP gene, although viable and fertile, showed reactive gliosis and behavioural abnormalities (Zheng *et al.*, 1995); it should be very interesting to study the effect of the human gene in these knock-out mice.

AIAPP

Transgenic mice expressing human islet amyloid polypeptide in β cells, have been produced by using constructs of genomic human IAPP under the control of rat insulin 1 or 2 promotor (Fox *et al.*, 1993; Höppener *et al.*, 1993; Verchere *et al.*, 1994). Although overproduction of the human peptide was achieved in β cells, none of these strains developed islet amyloidosis so far. In an effort to mimic human diabetes, several experimental approaches were followed namely; promoting β cell stress by pancreatectomy and treatment with glucocorticosteroids, inducing hyperphagia or obesity, and by crossing the transgenic animals with the *ob* and *db* mouse models of diabetes; no amyloid deposition was however observed (Westermark *et al.*, 1995).

In summary, the transgenic mice produced as animal models for the human amyloidoses do not completely represent the phenotype of these diseases. Several reasons could account for that failure; i) multigenecity as in the case of Alzheimer's disease; although abnormalities in AβPP lead to amyloid deposition, other genes are involved in the etiology of the disease (so far apoE alleles, and presenilins genes), this could probably be solved by producing animals transgenic for the combination of intervening genes, ii) interference with the endogenous genes, that can be avoided by using knock-out animals, or iii) specie-specific factors, which is more difficult to overcome, since the production of transgenic primates has obvious limitations.

4.2. Cell culture models

One other valuable system to study and model human pathologies is the use of genetically modified cells, whereby an expression vector containing the gene of interest, is introduced into the cells by transfection, lipofection, retroviral infection or electroporation; the principal advantages of this system are the technical ease and the possibility of dissecting the influence of different factors in particular cellular pathways, which is much more difficult to perform in an animal not only because of the complexity of the organisms, but also because there are several factors that can not be controlled. Cell culture systems as models for some amyloidosis (Aβ, Agel, AIAPP, AL) have been produced and in some cases provided important information regarding the function of the amyloid precursor protein, and possible mechanims of the disease.

In relation to Aβ amyloidosis several cell systems were developed; COS cells overexpressing the carboxy-terminal 100 amino acids of AβPP were shown to produce intracellular amyloid-like deposits, and were considered a candidate model system to evaluate inhibitory factors (Maruyama *et al.*, 1990); COS cells (Tamaoka *et al.*, 1994b), human neuroblastoma cells (Suzuki *et al.*, 1994), MDCK cells (De Strooper *et al.*, 1995), rat hippocampal neurons (De Strooper *et al.*, 1995), and human kidney 293 cells (Yang *et al.*, 1995) were also used to express wild type and/or mutant AβPPs allowing the study of protein synthesis, intracellular trafficking and processing, thus giving insights into the amyloidogenesis mechanisms. COS-1 cells have also been used to dissect the possible mechanisms of gelsolin amyloidogenesis (Paunio *et al.*, 1996; Kangas *et al.*, 1996). As for the PDH amyloids, some cellular systems expressing human IAPP have been reported; COS-1 cells overexpressing human, but not rat, IAPP developed intracellular amyloid deposits that lead to cell death (O'Brien *et al.*, 1995) thereby indicating amyloid toxicity; furthermore, although the IAPP transgenic mice do not develop amyloid deposition (as described above), primary cultures of the transgenic islets gave rise to intra and extracellular amyloid deposits (Koning *et al.*, 1994). Recently rat and human mesangial cells were shown to accumulate intra- and extracellular amyloidogenic light chains added to the cultures in the presence of AEF (Tagouri *et al.*, 1996) providing a model to study renal AL amyloidosis.

In summary, several tools are available not only to study the mechanisms of amyloidogenesis, but also for the development of therapeutic strategies and/or drugs, each presenting advantages and disadvantages; *in vitro* studies are very important to elucidate structure and to give indications on what affects fibril formation but they never mimic the *in vivo* situation; animal models should be the ideal situation but as previously described they fail to completely reproduce the human phenotypes; as for the cell culture systems, although easier to manipulate and control, they are limited *per se*.

5. Therapeutic agents

A considerable effort has been made in the search of effective therapeutic drugs for the amyloidoses; promising agents have been proposed, although little is known about their mechanisms of action. Rifampicin and rifamycins were shown to inhibit *in vitro* Aβ(1-40) aggregation and to prevent its toxic effects in cultured rat PC12 cells (Tomiyama *et al.*, 1994b); hexadecyl-N-piperidinium bromide was reported to specifically inhibit Aβ(1-40) aggregation *in vitro*, but not TTR or IAPP aggregation, possibly by binding to monomeric Aβ and preventing self-assembly processes (Wood *et al.*, 1996); sulphated polyanions and polysulphonates as potential therapeutic agents have already been discussed in this chapter, section 3.2.1; finally 4'-iodo-4'-deoxydoxorubicin (IDOX), a cytotoxic drug that binds specifically and with high affinity to several types of amyloid, was shown to reduce the amounts of splenic amyloid deposits

in the murine AA model (Merlini *et al.*, 1995); this drug was further used in AL patients, with encouraging results of amyloid resorption in some cases; it appears that IDOX, by binding to the amyloid fibrils, prevents subsequent deposition of precursor and facilitates or induces proteolytic resorption of the deposits (Gianni *et al.*, 1995).

Part II

Experimental Research

Experimental Research

Introduction

In the past few years the study of the amyloid diseases has suffered a great evolution, not only at the structural and biochemical levels of the proteins involved as fibril precursors, and possible chaperones, but also in the experimental models developed to elucidate the pathogenesis of amyloidosis; in spite of the efforts, the mechanisms of amyloid formation remain elusive.

Taking the above exposed into consideration, we have developed and used *in vitro* assays and a cell culture system to study the process of amyloid formation from TTR at different conditions and the influence of possible chaperone factors. We have also characterized a transgenic mouse strain that does not develop amyloidosis to assess possible causative factors.

Aims of the project

1 - Study the effect of factors influencing the process of amyloid formation from TTR in vitro (chapters 1, 2)

- Development of an *in vitro* assay for amyloid evaluation and quantification
- Production of amyloid-like fibrils from soluble TTR
- Analysis of the TTR amyloid formation process
- Examination of the effect of influence of exogenous factors in the process of amyloid formation
- Investigation of the role of TTR mutations in amyloidogenesis

2 - Investigate the transgenic strain MT1-TTR Met 30 lacking amyloid deposition and determine possible intervening factors (chapter 3)

- Characterization of transgene expression, and of the human TTR Met 30 produced by the transgenic line MT1-TTR Met 30
- Search for amyloid deposition in long-term induced animals

3 - Production of genetically modified cells producing TTR Met 30 and development of models for the study of amyloidogenesis (chapter 4)

- Production of genetically modified cells secreting human TTR and TTR Met 30 by retroviral infection
- Use the genetically modified cells to: develop cell culture models and *in vivo* models for the study of amyloidogenesis.

Chapter 1: *In vitro* amyloid fibril formation

Aims

- Production of amyloid-like fibrils from soluble TTR by acidification and optimization of the process.
- Development of an *in vitro* assay to assess intervening factors in the process of amyloid formation from TTR.
- Search for methods, other than acidification, for amyloid-like fibril formation.

Introduction

Amyloid deposition is a characteristic feature of a complex group of diseases known as amyloidoses, which include, among others, familial amyloidotic polyneuropathy (FAP) and Alzheimer's disease (Ghiso *et al.*, 1994). These deposits are composed of 7-10 nm wide, rigid, nonbranching protein fibrils with variable lengths and a typical twisted β-pleated sheet structure, presenting unique tinctorial properties such as apple-green birefringence under polarized light and yellow green fluorescence, upon staining with Congo red and thioflavine S respectively, (Glenner and Page, 1976).

Several apparently nonrelated proteins can be found as main constituents of amyloid fibrils, and this chemical heterogeneity is associated with specific clinical forms of amyloidosis. Other components present in the amyloid deposits include serum amyloid P component (Pepys, 1988), proteoglycans (Snow *et al.*, 1987) and apolipoprotein E (Wisniewski and Frangione, 1992).

In two forms of hereditary amyloidoses, FAP and familial amyloidotic cardiomyopathy (FAC), and in senile systemic amyloidosis (SSA), the amyloid precursor protein is transthyretin (TTR) (Costa *et al.*, 1978; Nordlie *et al.*, 1988; Saraiva *et al.*, 1990a; Saraiva *et al.*, 1992: Westermark *et al.*, 1990a), a homotetrameric plasma protein of 55,000 Da that binds thyroxine and retinol binding protein.

The molecular mechanisms leading to the polymerization of TTR and its deposition as amyloid fibrils are still unknown. Several TTR mutations have been found associated with FAP and FAC (Saraiva, 1995), but this genetic heterogeneity does not correlate with the clinical expression (Saraiva *et al.*, 1993); furthermore, in SSA it is the normal TTR that deposits as amyloid (Westermark *et al.*, 1990a).

In order to develop an *in vitro* assay to study intervening factors in amyloid formation from TTR, we started by testing a fluorometric method with thioflavine T (Naiki *et al.*, 1989) as a quantitative assay for TTR amyloid evaluation, and, for that purpose, used amyloid isolated from post-mortem tissues of FAP patients. We then produced amyloid-like fibrils from soluble TTR by a modification of described acidification methods (Gustavsson *et al.*, 1991) and optimized the process in terms of protein concentration, pH and incubation media. In addition, we studied the effect of seeding and the stability of the amyloid-like fibrils. Finally, we tested other methods for the production of amyloid-like fibrils from TTR, namely metal catalyzed oxidation, as described for β-peptide (Dyrks *et al.*, 1992).

Materials and Methods

Isolation of amyloid fibrils

Amyloid fibrils were isolated post-mortem from the thyroid of a FAP individual (FAP XX) and from the heart of an individual with heterozygous Ile 122 related FAC (Saraiva *et al.*, 1990a) following standard procedures (Costa *et al.*, 1978). Briefly, the tissues were minced into small pieces and homogenized in 0.1 M phosphate-buffered saline (PBS) in a VirTis-45 homogenizer, and the suspension was centrifuged at 12,000 *g* for 30 min. The sediments were resuspended in PBS, homogenized in a Potter-Elvehjem apparatus and centrifuged again in the same conditions; the last steps were repeated twice and the final sediments were resuspended in double-destilled water (ddH_2O). Nine homogenizations in water followed, with collection of the supernantants, after which a centrifugation was performed with those supernatants at 27,000 *g* for 4 to 5 hours. The pellets containing the higher percentage of amyloid, as assessed by Congo red staining, were pooled and stored at 4°C; part of the thyroid preparation was stored as lyophilized material.

Lyophilized FAP kidney amyloid fibril preparations from other FAP patients, already available in the laboratory, were also used.

Isolation and purification of TTR

TTR used in these studies was produced in an *E.coli* expression system (McCutchen *et al.*, 1993; a kind gift from Dr. J.F.Kelly, Texas A&M University). The protein was isolated and purified by preparative gel electrophoresis after ion exchange chromatography, as described by Almeida *et al.*, (1996), followed by a gel filtration step carried out in a 1.5x50 cm BioRad column packed with Biogel P-100 (BioRad) equilibrated with 0.1 M KH_2PO_4, 0.1 M Na_2SO_4. Isolated proteins were stored at -20°C in ddH_2O.

Analysis of native amyloid fibrils

Amyloid fibril preparations were subjected to sodium dodecyl sulphate gel polyacrylamide electrophoresis (SDS-PAGE) in 15% gels with a discontinuous buffer system (Laemmli, 1970). Electrophoretically separated proteins were transferred to nitrocelullose membranes (Towbin *et al.*, 1979) and blots probed with a 1:200 dilution of either peroxidase labeled anti-human TTR (Binding Site) or anti-human SAP (DAKO). When using anti-human SAP, the blots were further incubated with a 1:1000 dilution of peroxidase labeled anti-rabbit immunoglobulins (Binding Site).

Fractionated amyloid fibrils were analyzed by SDS-PAGE, in linear 15-20% acrylamide gradient gels for Ile 122 fibrils and 15% acrylamide gels for kidney fibrils.

Amyloid-like fibril production

The *in vitro* production of amyloid-like fibrils was achieved with different methods and preparations, namely:

1) From TTR: i) As described by Colon and Kelly (1991): 200 μg/ml TTR in 50 mM Tris pH 7.5, 100 mM KCl were acidified to pH 4.5 in steps of less than 0.4 units, by the addition of hydrochloric acid solutions (HCl - 5%, 2% and 0.5%). After 30 min incubation at room temperature, solutions were analyzed by Congo red staining. ii) Essentially as described by Gustavsson *et al.*, (1991): 3 mg/ml TTR were incubated in 10% acetic acid at room temperature but for a period of 2 weeks.

2) From synthetic TTR peptides as described by Gustavsson *et al.*, (1991): 10 mg/ml synthetic peptides were incubated in either 1 or 10% acetic acid at room temperature for 24 or 48 hours. TTR synthetic peptides used were: Y649 (residues 28 to 33 of human TTR Met 30; asn-val-met-his-val-phe-spacer arm-cys) and PEG1 (residues 46 to 55 of human TTR; ser-gly-lys-thr-ser-glu-ser-gly-glu-leu), synthesized by automatic solid-phase synthesis (Macromolecular Analysis Service, Univ. Birmingham, UK).

3) From insulin by the method of Bourke and Rougvie (1972); briefly, 10 mg/ml bovine insulin (Sigma) in 0.5% acetic acid pH 2.0 (with HCl), were incubated for 2 hours at 85°C, frozen at -80°C and heated at 50°C. After 5 freeze/thaw cycles amyloid-like fibrils were sedimented by centrifugation at 14,000 rpm in a microfuge, and analyzed by Congo red binding assays.

4) From polylysine by the method of Sarkar and Doty (1966); 10 mg poly-L-lysine hydrobromide (70,000-150,000 Da from Sigma) were dissolved in 1 ml ddH_2O, and 5 M sodium hydroxide added until pH 11.0; after an incubation of 20 min at 50°C, the suspension obtained was cooled to room temperature and analyzed by Congo red binding assays.

Amyloid formation studies

All the assays were carried out in triplicate and at room temperature, in the following conditions:

1) concentration dependence curve - TTR at various concentrations (0.5, 1.25, 2, 3, 4 and 5 mg/ml) was incubated in 40 µl of 0.1 M sodium acetate pH 4.0 for 96 hours.

2) pH dependence curve - TTR at a concentration of 2 mg/ml was incubated in 40 µl of 0.1 M citric acid/0.2 M sodium hydrogenophosphate buffer at the following pHs: 2.6, 3.0, 3.6, 4.0, 4.6, 5.0, 5.6 for 96 hours.

3) buffer testing - 2 mg/ml TTR was incubated for 72 hours in 20 µl of each of the following buffers at pH 4.0: 0.1 M sodium acetate, 0.1 M potassium hydrogenoftalate or 0.1 M citric acid/ 0.2 M sodium hydrogenophosphate.

4) kinetic studies - incubation times of the kinetic experiments ranged from zero to 100 hours and were performed as follows: TTR stocks in sterile ddH_2O were distributed into Eppendorf tubes and stored at -20°C. At each time point (starting at the corresponding longer incubation time), tubes were brought to room temperature, and 1 M acetate buffer pH 4.0 was added (final concentrations, 2 mg/ml TTR in 50 µl 0.1 M acetate buffer). At the end of the incubation times samples were analyzed by fluorometry.

5) seeding experiment - this experiment was carried out as described for the kinetic experiments, with the exception that tubes were added a master mix containing the appropriate seed and acetate buffer pH 4.0. Two types of seed were used in these experiments: i) isolated amyloid-like fibrils prepared by incubating 200 µg TTR in 100 µl of 0.1 M acetate buffer pH 4.0, for 120 hours and centrifuging the suspension obtained, after neutralization with a 25% ammonia solution, at 65,000 *g* for 4 hours. The sediment was resuspended in 40 µl ddH_2O and half of it was used as seed. ii) the whole volume of an amyloid-like fibril suspension, prepared by incubating 80 µg TTR in 40 µl of 0.1 M acetate buffer pH 4.0, for 48 hours.

6) stability testing - To 12 Eppendorf tubes containing amyloid-like fibrils, produced from 2 mg/ml TTR in 25 µl 0.1 M sodium acetate pH 4.0 for 9 days, 175 µl Tris-buffered saline were added. Triplicates were then frozen at each desired incubation time (0, 5, 11 and 15 days), and samples were thawed just before fluorometry.

TTR monomer isolation by isoelectric focusing

Isoelectric focusing (IEF) was performed in 4 M urea-polyacrylamide gels with 5% ampholytes pH 4-6.5, as described (Altland *et al.*, 1981). 1 mg TTR was applied on a native preparative gel electrophoresis and the gel slice containing TTR was applied onto the IEF gel.

IEF was carried out at 1200 V for 6 hours. A piece of the gel was then stained and destained to identify the monomer bands. Both oxidized and normal monomer bands were excised, electroeluted in an elutrap (Schleicher & Schuell) and desalted by quick spin dialysis (G25 coarse Sephadex, Pharmacia).

Protein quantification

Protein concentration was determined by using bicinchoninic acid (BCA assay reagent, Pierce) or the BioRad Protein Assay (based on the Bradford dye binding procedure). Standard curves of bovine serum albumin (range 50-250 μg/ml) were used in each assay.

Peptide concentration of Y649 amyloid-like fibrils was determined, after a 6.5 hours incubation of the fibrils in 7 M guanidine hydrochloride/ 0.1 M phosphate pH 8.0, by quantifying free sulfhydryl groups using Ellman's reagent (Pierce) and a standard curve of L-cysteine (range 50-500 μM) in the same guanidine solution.

Total protein content of thyroid amyloid fibrils was determined by quantitative amino acid assay (Protein Chemistry Core Facility at Columbia University, New York).

Congo red binding assays

Congo red stock solutions (approximately 700-900 μM) were prepared by dissolving the dye (Aldrich) in 100 mM KCl, 50 mM Tris pH 7.5, filtering the resulting solutions through 0.45 μm filters (Schleicher & Schuell) and determining the exact concentration by measuring absorbance at 488 nm (ε_{488}= 595 $g^{-1}dm^3cm^{-1}$, as referred to in Merck Index).

Native (100-500 μg/ml) and amyloid-like fibrils (10-50 μg/ml) were incubated with 7 μM Congo red and absorbance spectra (400-600nm) were taken in a Shimadzu UV-160A dual beam spectrophotometer. Stained samples were also examined under polarized light in a Olympus polarization light microscope.

Thioflavine T based fluorometric assays

The dye stock solutions were prepared by dissolving thioflavine T (Aldrich) in 50 mM glycine-NaOH pH 9.0, at an approximate concentration of 400-500 μM; the solutions were filtered through 0.22 μm filters (Schleicher & Schuell) and concentration determined by absorbance measurement (ε_{411}= 2.2x10^4 $M^{-1}cm^{-1}$as experimentally determined).

Fluorescence spectroscopy was performed on a Shimadzu RF-5001PC spectrofluorophotometer in the scan mode, with an assay volume of 1 ml. In all studies excitation spectra (400-500 nm) were taken with high sensitivity, and emission fixed at 482 nm. Excitation and emission slits were set at 5 and 10 nm, respectively, and scan speed was set to medium. The reaction mixture contained 3 μM thioflavine T in 50 mM glycine-NaOH pH 9.0 (Naiki *et al.*, 1989). Spectra were obtained at room temperature (range 16 - 22°C) within 2-3 minutes after the addition

of sample to the reaction mixture. Measurements were performed until concordant duplicates were obtained for each sample. Between samples the cell was washed with ddH_2O.

Electron microscopy

Samples from isolated amyloid-like fibrils (prepared as described in the seeding experiments) were dried on carbonized formvar coated grids (TAAB Laboratories Equipment Limited), negatively stained with 1% uranyl acetate and examined in a JEOL 100CX electron microscope with an acceleration voltage of 70 kV.

Other methods

Linear regression, correlation coefficient, averages and standard deviations were used for statistical analysis.

Results

To optimize the experimental conditions for amyloid-like fibril formation from TTR it was necessary to use specific methods for amyloid evaluation and quantification; such methods had to be tested with good preparations of native amyloid fibrils, which were first characterized.

1. Characterization of amyloid fibrils: constituents and molecular weight of the fibril protein

Lyophilized preparations of amyloid fibrils extracted post-mortem from FAP kidneys were available in the laboratory; in addition, amyloid fibrils from a FAP thyroid were isolated. We have also isolated amyloid fibrils from a FAC heart in which the associated TTR variant was Ile 122 (Saraiva *et al.*, 1990a). These amyloid fibril preparations were then partially characterized by SDS-PAGE immunoblots probed with anti-TTR and anti-SAP antibodies. Serum amyloid P component is a common constituent of amyloid fibrils, and was detected in the thyroid and Ile 122 amyloid fibril preparations as shown by the presence of a band at about 25,000 Da reactive with anti-SAP antibody (figure 1); in the kidney preparations tested, however, we have not detected SAP, which is probably due to the low amounts of amyloid present in these preparations, since SAP has been previously reported in Met 30 kidney fibrils (Damas *et al.*, 1995). As far as TTR is concerned, two major bands were observed in all fibril preparations, one close to the 14,300 Da marker band, corresponding to the TTR monomer, and another close to the 29,000 Da marker band which corresponds to the TTR dimer. The TTR monomer band in the 122 amyloid fibrils appeared to be broader as compared to the thyroid amyloid fibrils (figure 1), suggesting the presence of lower molecular weight species.

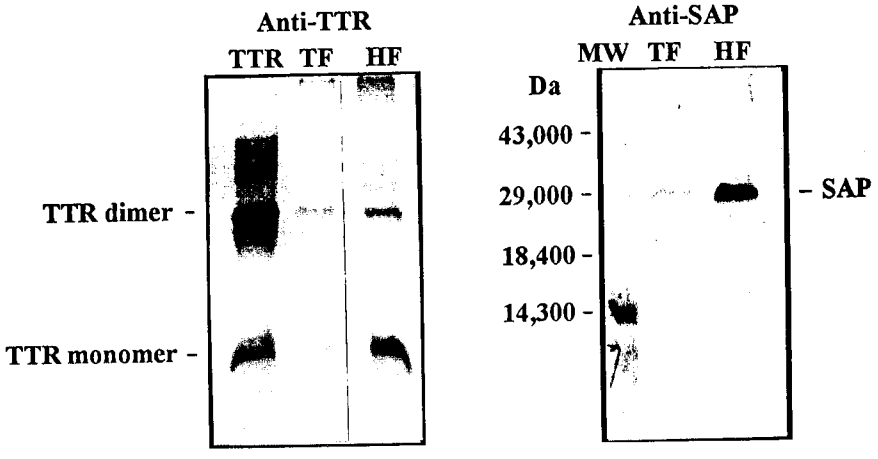
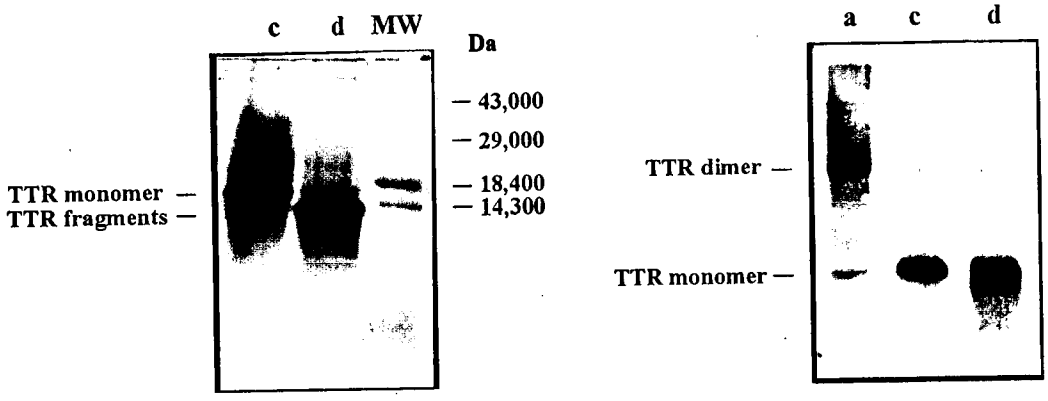


Figure 1 Immunoblotting analysis of Met 30 (thyroid) and Ile 122 (heart) amyloid fibrils. MW - low molecular weight standards; TF - thyroid amyloid fibrils; HF - heart amyloid fibrils; TTR - semi-purified TTR preparation as control.

Amyloid fibrils from FAC, SSA and some FAP individuals are known to contain, besides the TTR monomer, fragments resulting from cleavage in the region between residues 46 to 52 of the TTR monomer (Saraiva and Costa, 1991). The Ile 122 amyloid fibrils had already been shown to contain TTR fragments, by SDS-PAGE immunoblot analysis of fractionated amyloid material in Sephadex G-100 columns (Saraiva *et al.*, 1990b). We investigated the presence of TTR fragments in a kidney Met 30 amyloid fibril preparation by following similar methodology. The Ile 122 preparation was used as a control.

In figures 2 and 3 are represented the elution profiles obtained for both amyloid fibril preparations and the analyses performed on the respective fractions. Three different areas can be considered in the Ile 122 elution profile (figure 2), that, when analyzed by SDS-PAGE and immunoblotting, were shown to correspond to TTR dimers (fraction a), monomers (fraction c) and fragments smaller than 14 kDa (fraction d). The latter fraction was sequenced and shown to contain fragments corresponding to TTR starting at positions 46 and 52. The elution profile obtained for the kidney Met 30 fibrils (figure 3) is significantly different, with a single evident peak (fraction c) corresponding mainly to the TTR monomer; when analyzing the region immediately after this peak (fraction d), it is possible, however, to detect minor fragments similar in size to the ones obtained for Ile 122 fraction d (figure 3, immunoblotting analysis). Therefore, fragmentation also occurs in Met 30 kidney amyloid fibrils, albeit at a much lower level than those of Ile 122 amyloid fibrils.



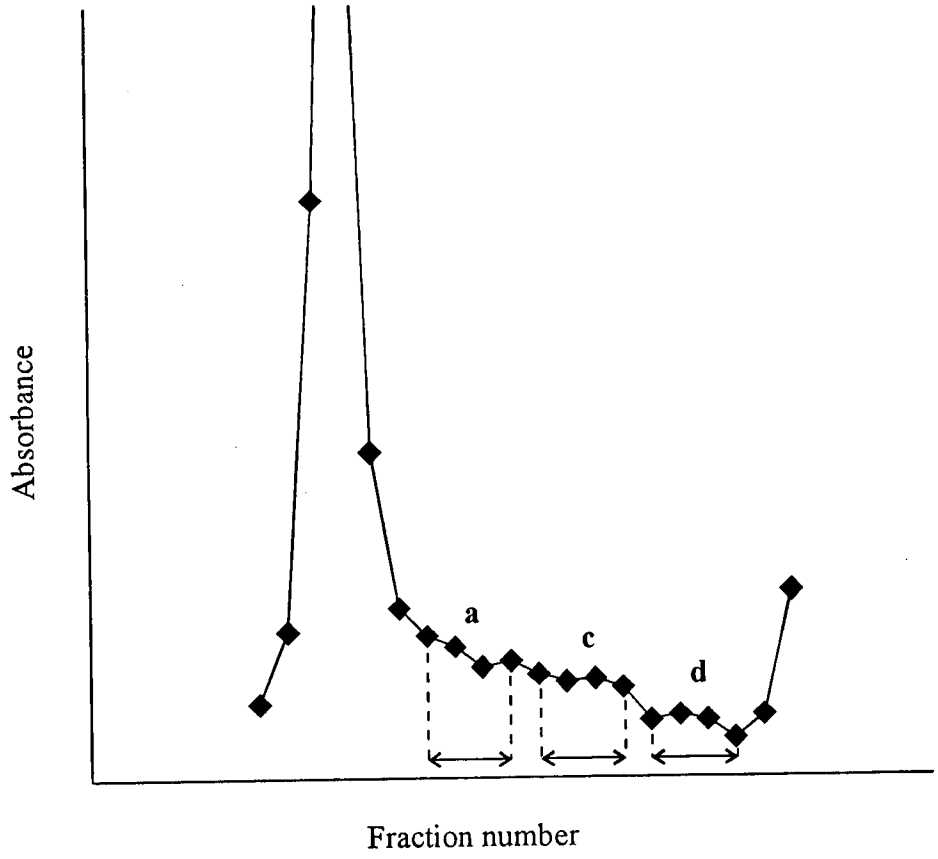


Figure 2 - Elution profile on Sephadex G-100 column of Ile 122 amyloid fibrils. On the upper part of the figure are represented the analysis performed on the fractions; left side - silver stained 15-20% acrylamide gradient gel of fractions c and d; right side - immunoblot analysis of fractions a, c and d.

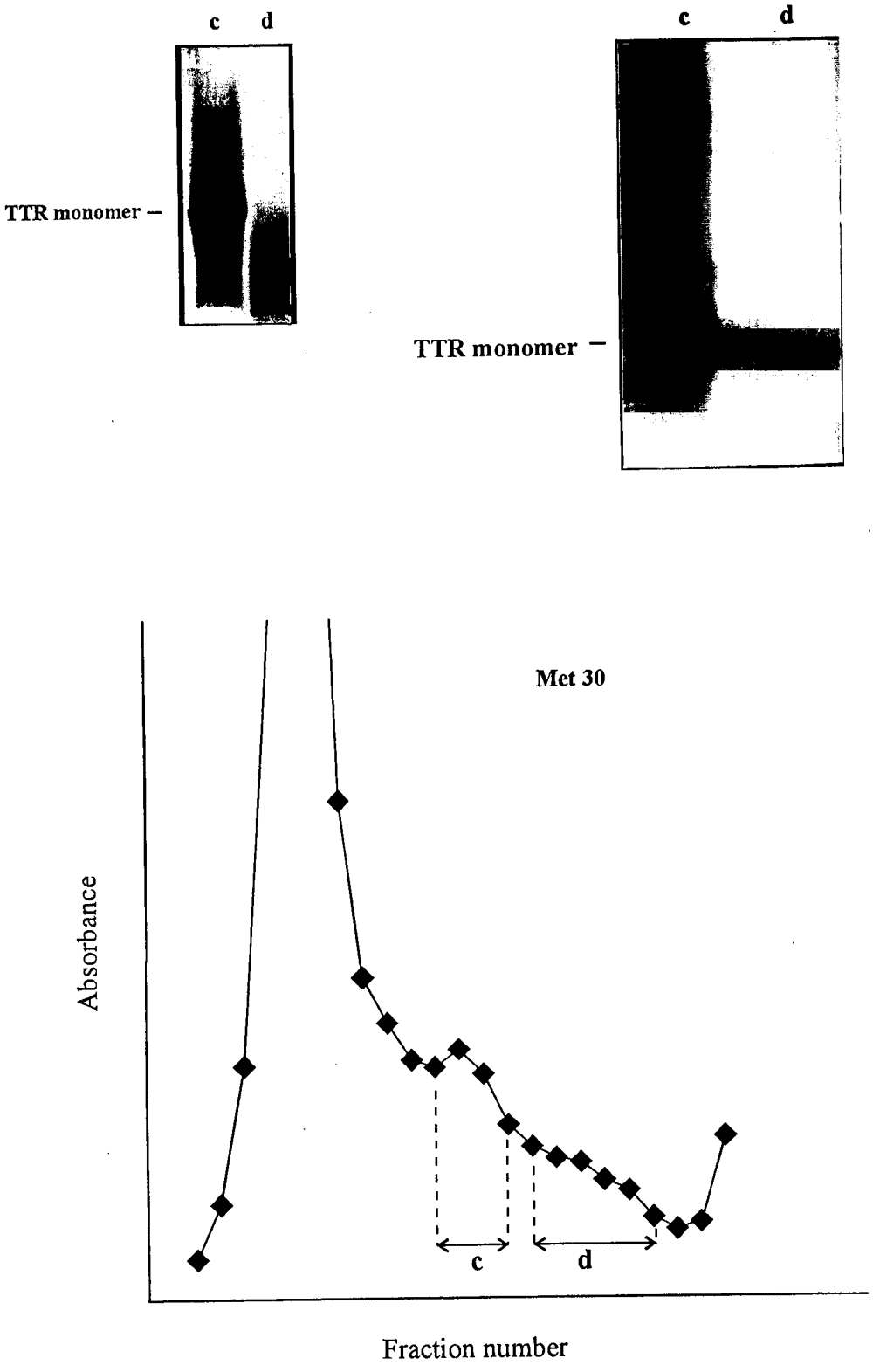


Figure 3 - Elution profile on Sephadex G-100 column of kidney Met 30 amyloid fibrils. On the upper part of the figure are represented the analyses performed on the fractions. left side - silver stained 15% acrylamide gel; right side - immunoblot analysis.

2. Amyloid evaluation by Congo red staining: effect of the physical-chemical properties of the fibril preparations

Amyloid deposits, both *in vivo* and *in vitro*, are usually identified by the characteristic apple green birefringence displayed under polarized light, upon binding to Congo red. Thus we analyzed, under polarized light, several Congo red stained fresh and lyophilized fibril preparations, namely fresh thyroid and heart fibril preparations, and lyophilized thyroid and kidney fibril preparations. The fresh preparations presented massive apple green birefringence, as observed in figure 4 for the thyroid sample; on the contrary, the lyophilized fibril preparations presented much less birefringence. This could be justified by lower amounts of amyloid present in the latter preparations; however the fresh and lyophilized thyroid preparations had the same origin, and therefore, the reason for the poorer results obtained with the lyophilized versus fresh preparations is probably related to an altered conformation of the fibrils due to the dehydration process; in effect these lyophilized preparations consist mainly of large clumps, hard to disaggregrate. To obtain a more homogeneous suspension, a lyophilized kidney preparation was homogenized in a glass Potter Elvejhem homogenizer; after Congo red binding, a decrease in the green birefringence as compared to the non homogenized preparation was observed, hence indicating that this kind of treatment, in addition to disaggregate the clumps, further disrupts fibril stacking or induces fibril fragmentation.

Extensive treatment with EDTA has been reported to solubilize up to 20% dry weight vitreous TTR amyloid fibrils (Herbert and Martone, 1993), therefore lyophilized kidney and fresh thyroid amyloid fibril preparations were subjected to one hour incubation in 150 mM NaCl (saline wash) followed by centrifugation at 14,000 rpm in a microfuge, and an overnight incubation of the pellet in 100 mM EDTA pH 7.2 with agitation at room temperature. The resulting fibril pellet, the supernatant and the saline wash were analyzed by immunoblotting, and as can be observed in figure 5 for the kidney fibrils, the TTR monomer is detected in the saline wash supernatant and in higher amounts in the supernatants after EDTA treatment, thus indicating that some degree of solubilization had occurred. Quantification by thioflavine T fluorometry, using the standardized conditions described below, with thyroid fibrils quantified before and after EDTA treatment, revealed a solubilization of 17%.

The EDTA treated fibrils retained the tinctorial properties of amyloid as detected by polarization microscopy after Congo red staining; in the case of the kidney fibrils with a higher level of birefringence than before the EDTA treatment, thus suggesting a more loosen structure of the lyophilized fibril clumps (figure 6).

Figure 4 - Polarization microscopy of Congo red stained thyroid amyloid fibrils (90× magnification).

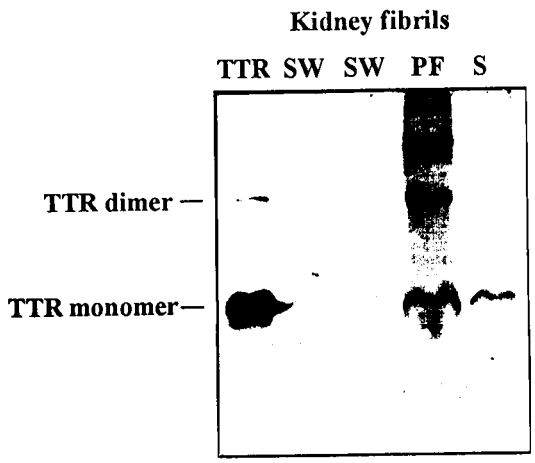


Figure 5 - Immunoblotting analysis of EDTA treated kidney amyloid fibrils. SW - saline wash supernatant, FP - fibril pellet after EDTA treatment, S - supernatant after EDTA treatment, TTR - isolated TTR.

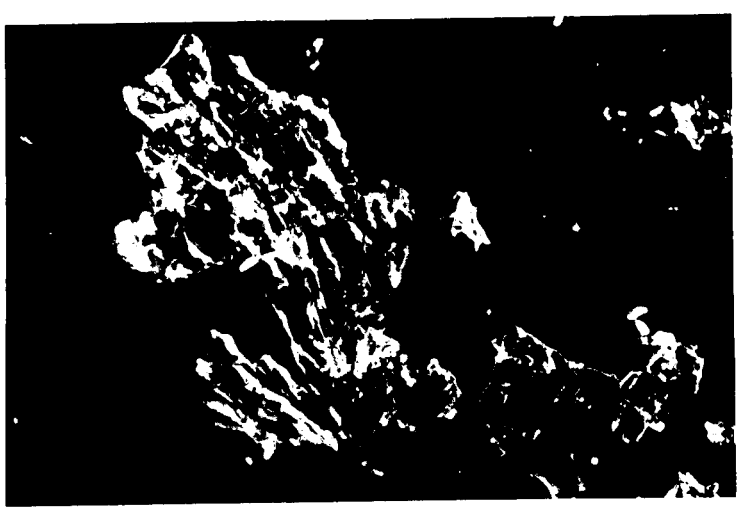


Figure 6 - Polarization microscopy of Congo stained kidney amyloid fibrils treated with EDTA (37× magnification)

In summary, the characteristic apple green birefringence under polarized light of Congo red bound to amyloid is influenced by physical and chemical treatments of the amyloid preparations, therefore other amyloid detection methods were next experimented.

3. Evaluation of Congo red binding to native and synthetic amyloid fibrils by spectrophotometry

Klunk *et al.*, (1989) described a quantitative method to evaluate the binding of Congo red to amyloid-like fibrils obtained from insulin, based on the spectral changes induced on Congo red absorbance spectra, namely a shift of 5-10 nm in the Congo red absorbance maxima and the appearance of a maximal spectral difference at 540 nm. In order to search for these hyperchromic shifts in the absorbance spectra we analyzed the following preparations:

- fresh thyroid and lyophilized kidney Met 30 amyloid fibrils
- acidified preparations of two TTR synthetic peptides, an heptapeptide (Y649) and a nonapeptide (PEG1)
- acidified preparations of soluble recombinant wild type TTR as described by Gustavsson *et al.*, (1991)
- acidified preparations of soluble recombinant wild type TTR as described by Colon and Kelly (1991) - "slow acidification"
- amyloid-like fibrils prepared from insulin and polylysine, as positive controls to the spectrophotometric method

Macroscopically it was observed that the acidified preparations (of TTR and TTR peptides) remained as clear solutions, with the exception of the Y649 preparation in which large precipitates were visualized. To these solutions (the total volume or 1/10 volume in the case of Y649) Congo red was added and the absorbance spectra taken. Samples were then centrifuged at 14,000 rpm in a microfuge and, when present, the pellets analyzed by polarization microscopy.

Both the thyroid and kidney amyloid fibrils produced the expected shifts in the absorbance spectra when bound to Congo red; the same occurred with the Y649 acidified preparation and with insulin and polylysine amyloid-like fibrils.

As seen in figure 7, no significant differences were observed among these samples. A more pronounced hyperchromic shift was detected with the thyroid amyloid fibrils as compared to the kidney amyloid fibrils and to the Y649 preparation, probably due to higher amounts of amyloid in the thyroid sample. Also higher backgrounds were obtained with the native amyloid preparations as compared to the amyloid-like fibrils which could be caused by non specific binding of Congo red to other components present in the native amyloid fibrils, such as tissue contaminants.

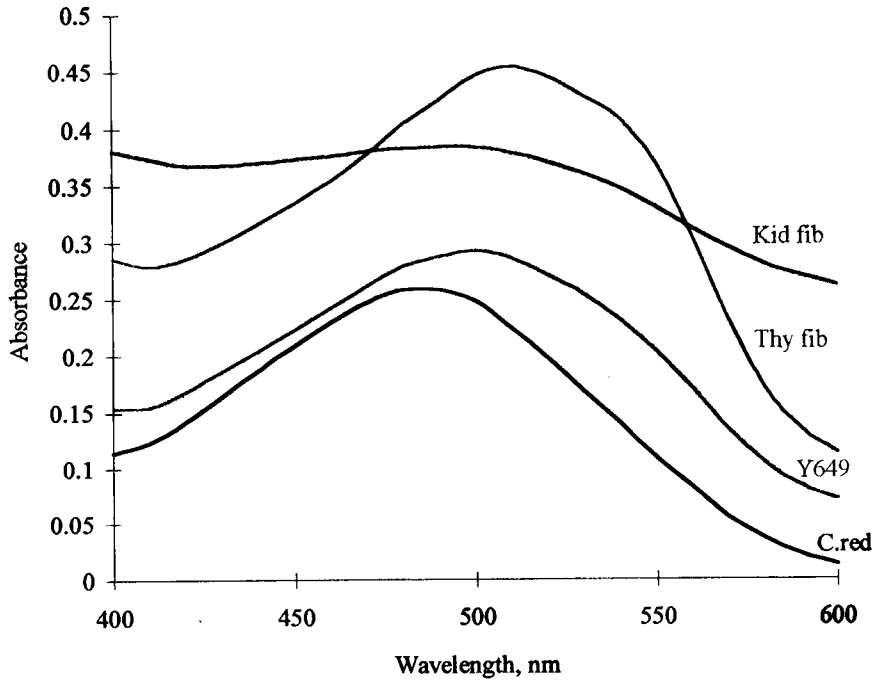


Figure 7- Absorbance spectra of a 6.7 μM Congo red solution alone (C.red), in the presence of a suspension of thyroid amyloid fibrils (Thy fib), or of kidney amyloid fibrils (Kid fib), or of the acidified Y649 preparation (Y649).

The PEG1 peptide and the other TTR acidified preparations originated overlapping spectra with the free dye. When these preparations were centrifuged it was possible to visualize a very small sediment in the "slow acidified" TTR preparation. Polarization microscopy of this sediment revealed non dichroic rod-like structures that we considered not to represent amyloid.

The Congo red stained Y649 acidified preparation was also centrifuged, and the sediment when analyzed in polarized light microscopy showed apple-green birefringence (figure 8); subsequent electron microscopy analysis confirmed the presence of fibrillary structures resembling amyloid fibrils (not shown).

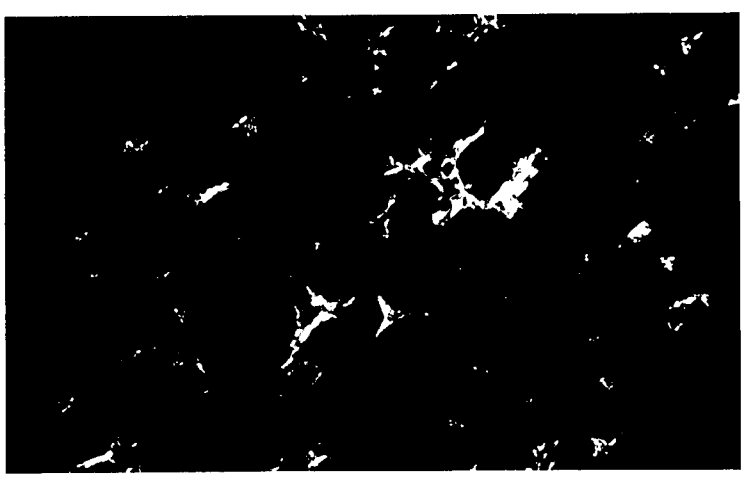


Figure 8 - Polarization microscopy of the Congo red stained Y649 acidified preparation. Y649 at a concentration of 10 mg/ml in 1% acetic acid (90× magnification)

In summary, only the Y649 peptide, which corresponds to TTR strand B, produced amyloid-like fibrils under the conditions tested; the fact that PEG1 peptide, corresponding to the bend between TTR strands C and D, appeared not to form amyloid is consistent with the hypothesis that a β-pleated sheet structure is necessary for amyloid formation (Gustavsson *et al.*, 1991). The other TTR preparations appeared also not to produce amyloid, thus indicating that the experimental conditions used may not be the ideal for amyloid formation, or alternatively Congo red staining as a detection method may not be sensitive enough. As a result we then pursued more sensitive methods for amyloid detection and other experimental conditions for amyloid formation.

4. Thioflavine T fluorometry as a quantitative method for TTR amyloid

In 1989, Naiki *et al.*, developed a quantitative method for murine senile amyloid protein (fAS_{SAM}) and murine amyloid protein A (fAA), using thioflavine T based fluorometry. This methodology is based on the appearance of novel excitation and emission maxima, at 450 nm and 482 nm respectively, upon binding of thioflavine T to amyloid fibrils, and has been applied for other amyloid proteins such as gelsolin (Maury *et al.*, 1994) and β-peptide (LeVine, 1993).

The suitability of this fluorometric assay for TTR amyloid was investigated with thyroid Met 30 amyloid fibrils (both the fresh and the lyophilized preparations) and amyloid-like fibrils obtained from TTR peptide Y649.

Upon addition of thioflavine T to these preparations, novel excitation and emission maxima were observed at 448 nm and 482 nm respectively (figure 9).

The pH dependence of the thioflavine T fluorescence signal upon binding to Y649 amyloid fibrils was studied by using different incubation buffers, namely 50 mM TrisCl pH 7.5 and pH 8 and 50 mM glycine pH 9; since maximal fluorescence was observed at pH 9, this was the pH chosen for the fluorometric assays.

We further tested the stability of the fluorescent signal produced by thioflavine T - thyroid amyloid fibril complexes and verified that within 1 hour there were no significant changes in the readings.

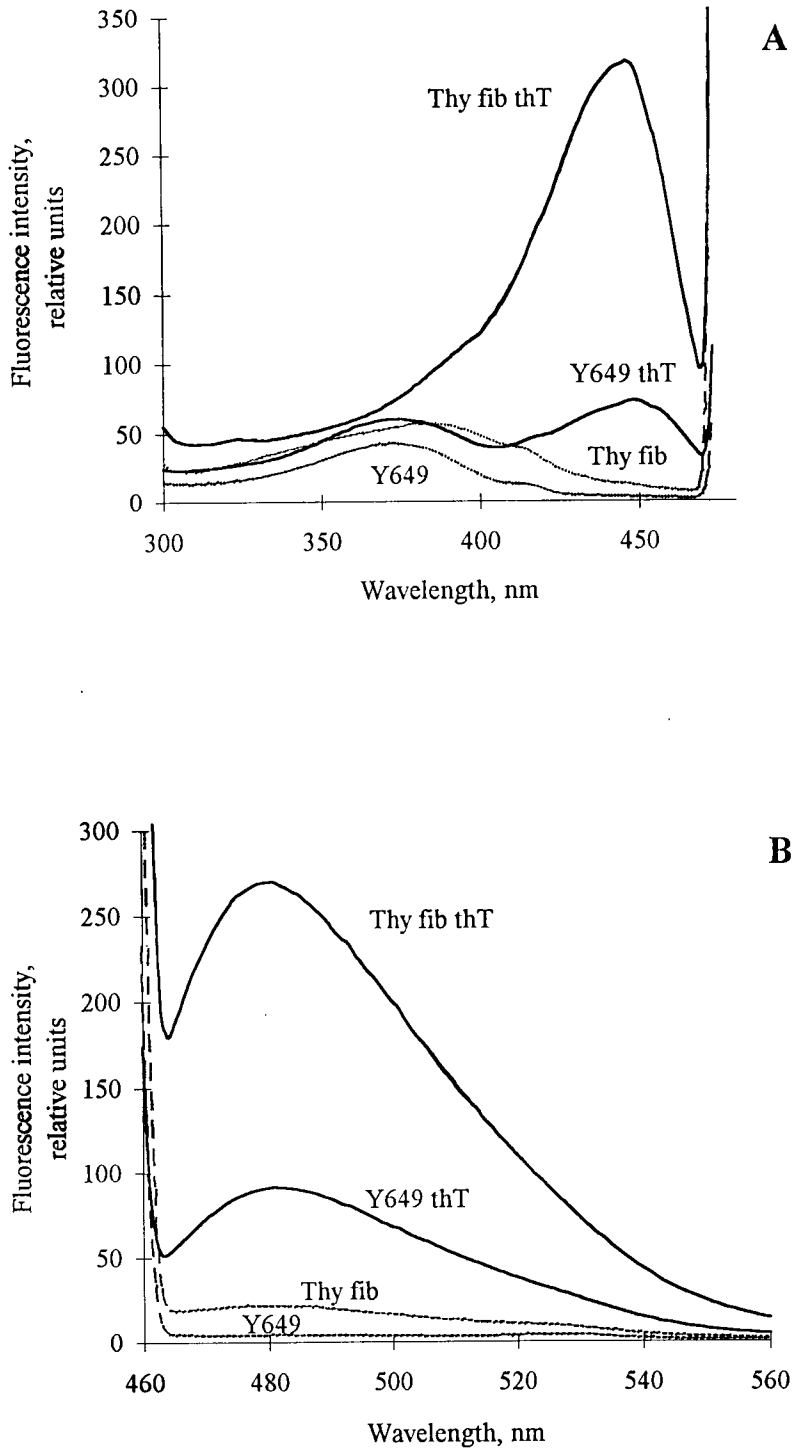


Figure 9 - Fluorescence spectra of thioflavine T - TTR amyloid fibril complexes. Thy fib thT - thyroid amyloid fibrils with thioflavine T; Y649 thT - amyloid-like fibrils with thioflavine T; Thy fib - thyroid amyloid fibrils alone; Y649 - amyloid-like fibrils alone. **Panel A:** Excitation spectra, λem fixed at 482 nm. **Panel B:** Emission spectra, λex fixed at 450 nm.

Fluorescence variation at different thioflavine T concentrations was studied using fresh and lyophilized thyroid amyloid fibrils and amyloid-like fibrils, in order to determine the apparent affinity constants of binding (K_a). In figure 10A are represented the saturation curves obtained and in figure 10B the corresponding double reciprocal plots used for K_a determination.

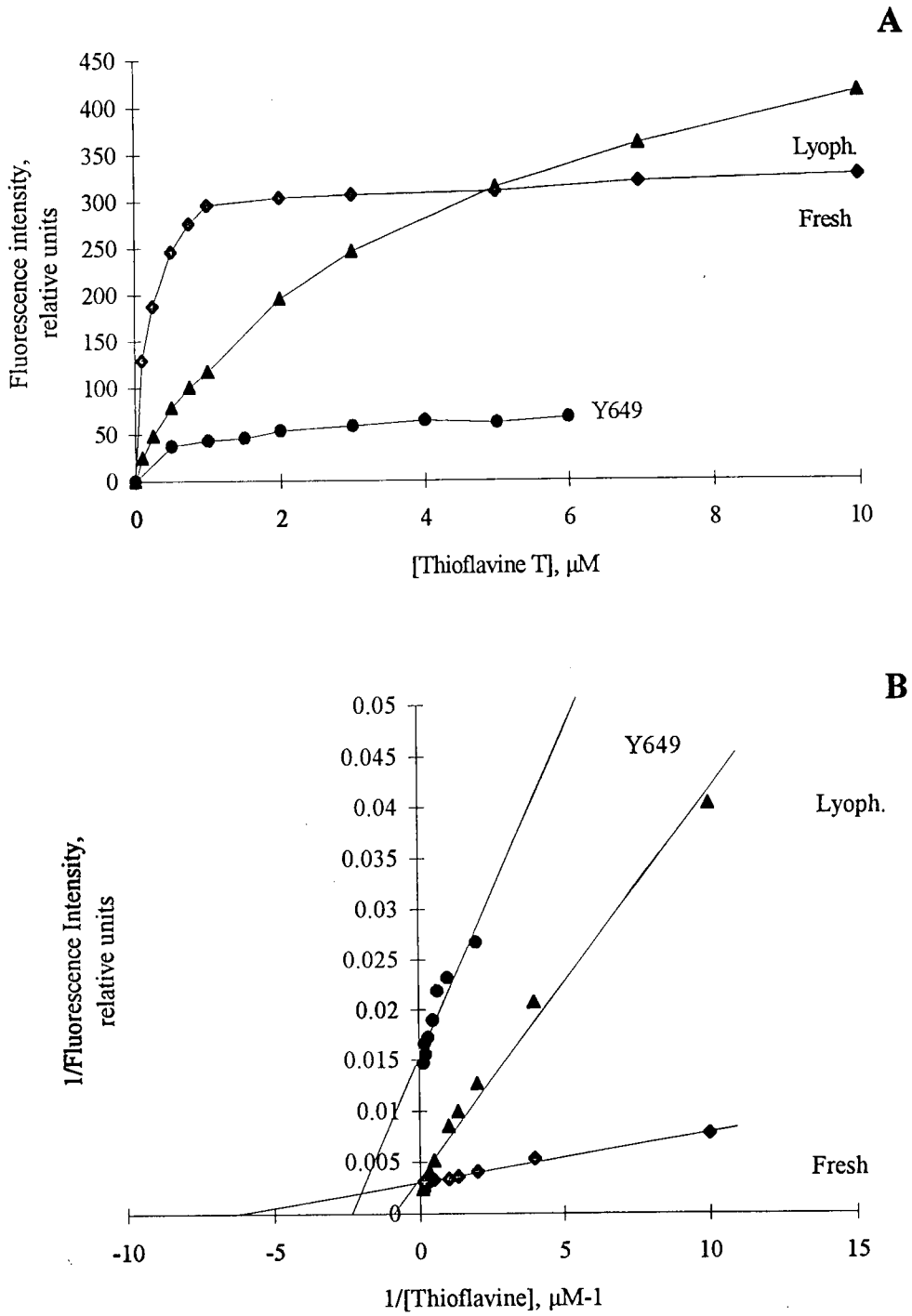


Figure 10 - Determination of the apparent affinity constants of thioflavine T binding to native and amyloid-like fibrils. Fresh - fresh thyroid amyloid fibrils; Lyoph - lyophilized thyroid amyloid fibrils; Y649 - amyloid-like fibrils. **Panel A:** Thioflavine T concentration dependence. **Panel B:** Double reciprocal representation of thioflavine T binding to amyloid fibrils.

Thioflavine T bound to fresh fibrils (Ka = 0.16 μM) with higher affinity than to lyophilized fibrils (Ka = 1.09 μM), and to amyloid-like fibrils with an intermediate affinity (Ka = 0.42 μM), thus implying that the affinity of the amyloid fibrils for thioflavine T is dependent upon the sample origin and processing.

Based on the above results, we selected 3 μM as the thioflavine T concentration to use in the subsequent studies.

Dependence on fibril concentration, for the binding of thioflavine T, was investigated with thyroid lyophilized fibrils and Y649 amyloid-like fibrils; it was verified that, for concentrations up to 5 μg/ml, of both lyophilized fibrils (referring to the total protein content of the fibrils, as determined by amino acid analysis) or amyloid-like fibrils, there was a corresponding linear increase in fluorescence (figure 11).

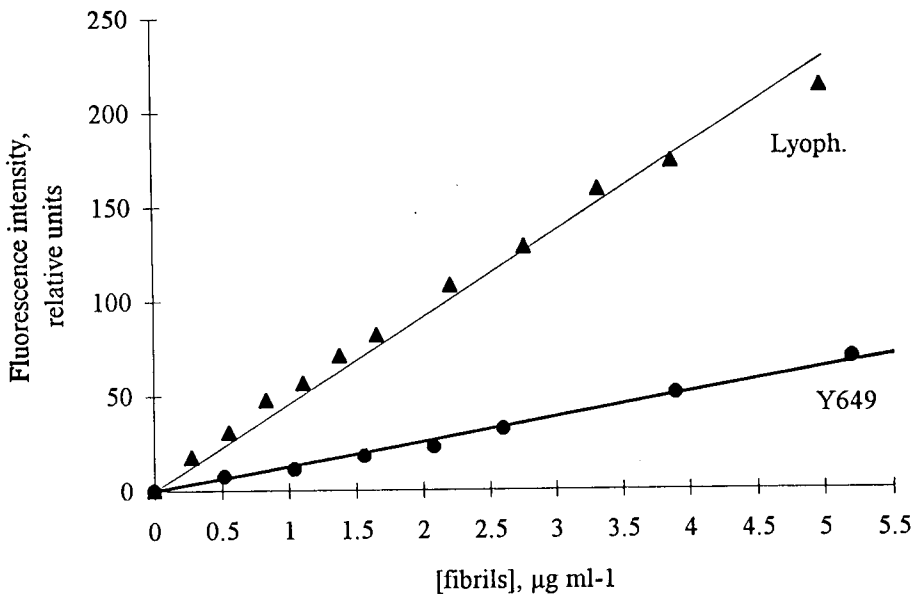


Figure 11 - Dependence of amyloid fibril concentration on the binding to thioflavine T. Used 3 μM thioflavine T in 50 mM glycine pH 9, λex at 448 nm and λem at 482 nm. Lyoph. - lyophilized thyroid fibrils; Y649 - amyloid-like fibrils.

Since amyloid fibrils are susceptible to solubilization by guanidine hydrochloride treatment, we incubated both thyroid and Ile 122 amyloid fibrils in 6 M guanidine hydrochloride, 20 mM DTT, 1 mM EDTA, 1 M Tris pH 8.8 for 3 hours at 37 °C and 12 hours at room temperature, and analyzed the resulting solutions by fluorometry. A decrease in fluorescence intensity of about 50% was observed, thus indicating that the disruption of amyloid fibril structure diminishes thioflavine T binding.

We therefore concluded that this thioflavine T fluorometric method is appropriate for TTR amyloid detection and quantification; however, standardized conditions (thioflavine T concentration and time interval of the assay) should be used as well as control samples.

5. Amyloid formation from soluble TTR

Having optimized the experimental conditions for amyloid quantification, our next aim was to produce amyloid from soluble wild type TTR.

In vitro amyloid formation from TTR synthetic peptides (Gustavsson *et al.*, 1991; Jarvis *et al.*, 1993) and from the whole TTR molecule (Baba *et al.*, 1991; Gustavsson *et al.*, 1991; Colon and Kelly 1991, 1992) had already been described, by acidification of the protein solutions. We started by following the experimental conditions described by Colon and Kelly (1991); however, no amyloid formation from wild type TTR was detected as assessed by Congo red binding assays (described above). The experimental conditions described by Gustavsson *et al.*, (1991) were then tested with both recombinant wild type TTR and the synthetic TTR peptides Y649 and PEG1. As previously referred to, Y649 but not PEG1 (10 mg/ml in 1 or 10% acetic acid), produced amyloid-like fibrils as assessed by Congo red binding, electron microscopy and thioflavine T fluorometry. Wild type TTR acidified preparations (3 mg/ml in 10% acetic acid), albeit not inducing any hyperchromicity on Congo red spectrophotometry, when analyzed by thioflavine T fluorometry produced a novel excitation maxima (ex max) at 436 nm. This maxima was considered to represent amyloid, since similar variations in the ex max of other amyloid-thioflavine T complexes have been observed (LeVine, 1993; Castaño *et al.*, 1995a; Naiki and Nakakuki,1996).

To exclude non specific thioflavine T binding, trichloroacetic acid was used to precipitate a solution of wild type TTR (1.4 mg/ml in 10% TCA; 20 min at 0°C); the suspension obtained was analyzed by fluorometry together with the TTR solution. No novel maximas were detected with any of these preparations, indicating that thioflavine T fluorometry is specific for amyloid, not detecting soluble protein or amorphous precipitates.

With the aim of producing amyloid in less extreme conditions, i.e. at a higher pH, we incubated wild type TTR at a concentration of 2 mg/ml, in 0.1M sodium acetate pH 4.0, for 2 days at room temperature. The suspensions so obtained were centrifuged at 65,000 *g* and the sediments investigated for the presence of amyloid by Congo red staining and thioflavine T fluorometry.

The absorbance spectra of the Congo red stained sediments presented the characteristic hyperchromic shifts (figure 12) and, under polarized microscopy, it was possible to observe apple green birefringence in some structures (not shown).

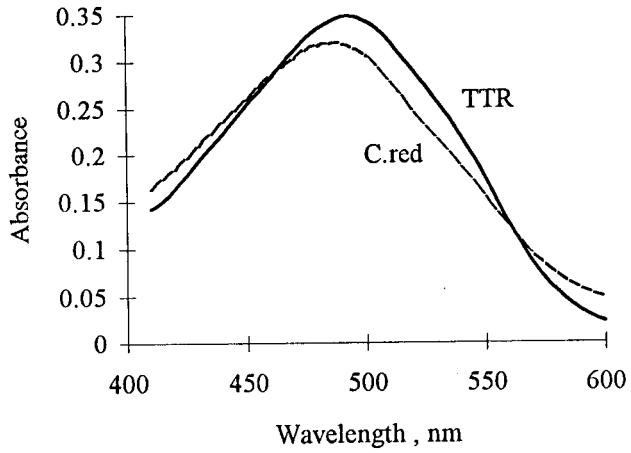


Figure 12 - Congo red binding to amyloid-like fibrils produced from wild type TTR. Absorbance spectra of a 7 μM Congo red solution alone - C.red; or in the presence of amyloid-like fibrils - TTR.

When bound to thioflavine T, these sediments also produced the new ex max at 436 nm (figure 13).

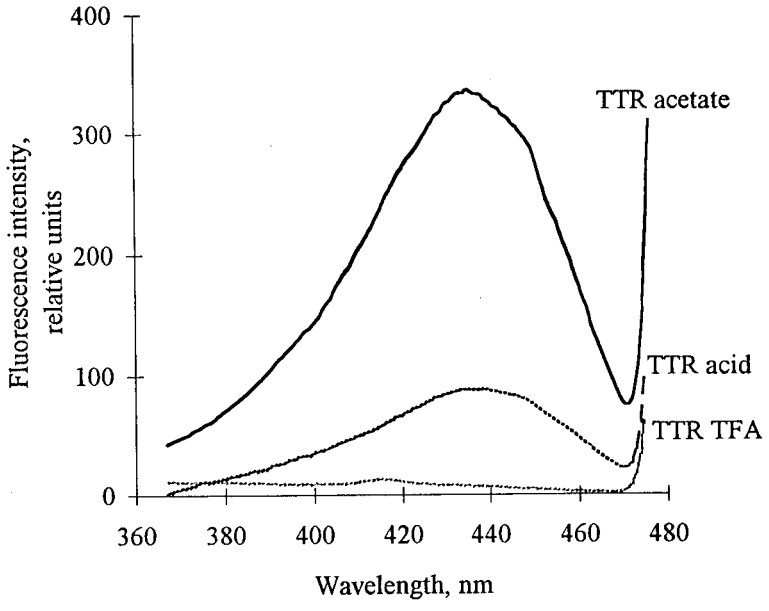


Figure 13 - Excitation spectra of amyloid-like fibrils bound to thioflavine T. Used 3 μM thioflavine T in 50 mM glycine pH 9, λem fixed at 482 nm. TTR - amyloid-like fibrils produced from TTR at pH 4, TTR acid - amyloid-like fibrils produced from TTR in 10 % acetic acid, TTR TFA - trichloroacetic acid precipitated TTR solution

Analysis of the sediments by electron microscopy revealed the presence of long, unbranched fibrils with an approximate diameter of 9.8 nm, very similar to native Met 30 amyloid fibrils as observed in figure 14.

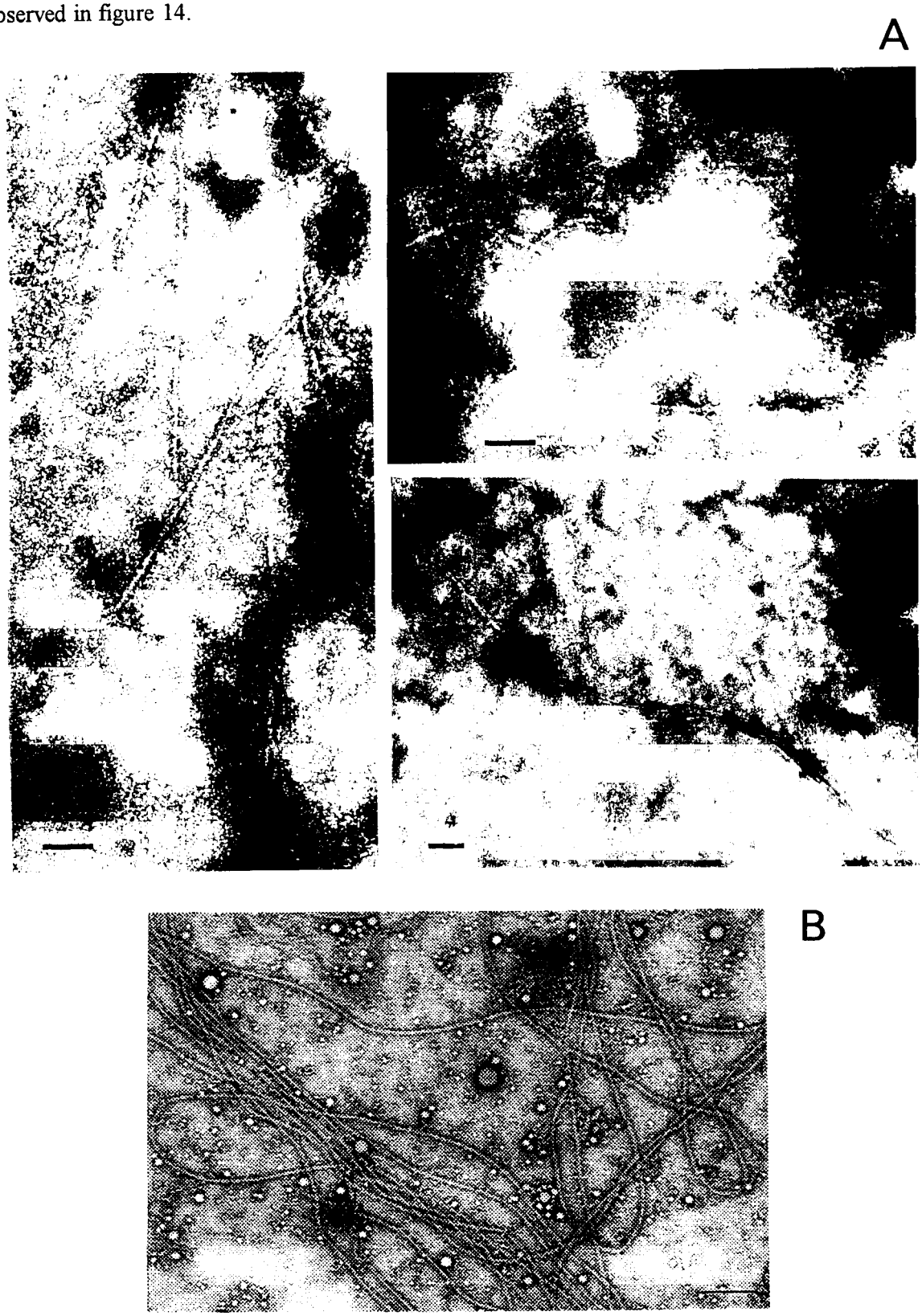


Figure 14 - Electron micrographs of negatively stained amyloid fibrils. **Panel A:** amyloid-like fibrils produced from TTR. The scale bar represents 100 nm; **Panel B:** Met 30 amyloid fibrils from a FAP vitreous (taken from Serpell *et al.*, 1995).

The process of amyloid formation was then quantitatively investigated. Protein concentration dependence studies, at pH 4.0 in acetate buffer, showed that the highest yield of amyloid formation was obtained with protein concentrations of 2-3 mg/ml. Concentration values lower than 1.25 mg/ml produced very low amounts of amyloid, and values higher than 3 mg/ml produced again lower amounts of amyloid, suggesting that at lower concentrations, amyloid fibril formation is a concentration dependent process and that higher concentrations are rate limiting (figure 15).

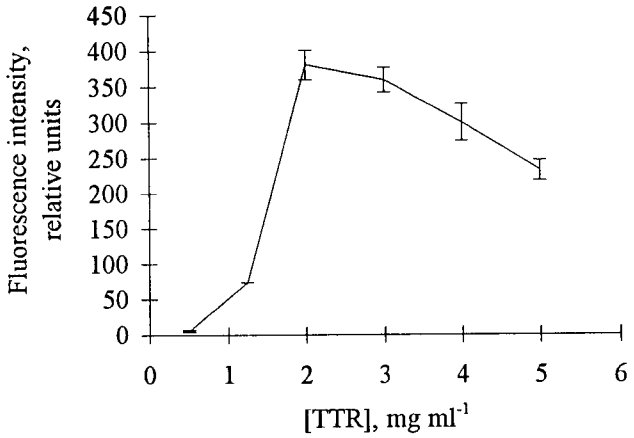


Figure 15 - Amyloid-like fibril formation from TTR by thioflavine T fluorometry. TTR concentration dependence curve. Fluorescence measured with λex at 436 nm and λem max at 482 nm. Values are reported as the mean of three assays ± standard deviation (in arbitrary units).

We next studied pH dependence by incubating 2 mg/ml TTR in citrate/phosphate buffer pH 2.6 to 5.6. It was observed that TTR formed amyloid between values of 2.6 to 4.6 with a peak at pH 3.6 (figure 16).

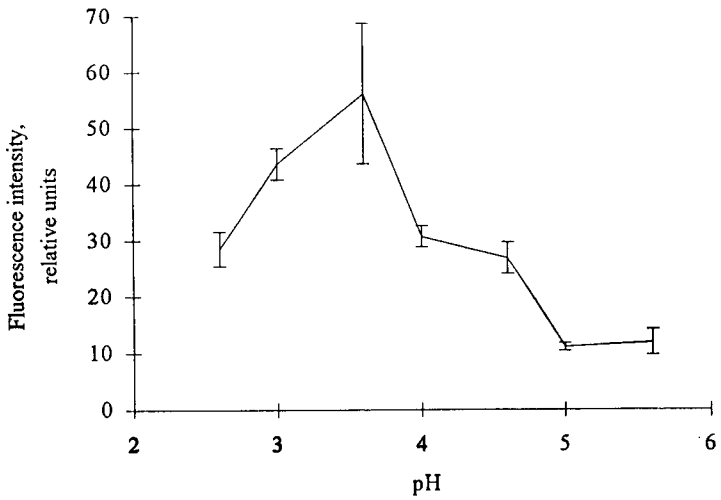


Figure 16 - Amyloid-like fibril formation from TTR by thioflavine T fluorometry. pH dependence curve. Fluorescence measured with λex at 436 nm and λem max at 482 nm. Values are reported as the mean of three assays ± standard deviation (in arbitrary units).

Although fluorescence intensities are expressed in relative units, in this experiment we found less amyloid formation at pH 4.0 than in the concentration dependence studies. Since this fact might be due to buffer composition we tested the production of amyloid at pH 4.0 in different buffers, namely potassium hydrogenoftalate, sodium acetate and sodium citrate/phosphate. Higher fluorescence intensities were observed with sodium acetate as compared to the other buffers. To verify whether the citrate/phosphate interfered with the fluorometric assay the following experiment was performed: amyloid-like fibrils formed from 2 mg/ml TTR in acetate buffer pH 4.0 for 72 hours were quantified by thioflavine T fluorometry, and immediately after citrate/phosphate buffer was added (at the corresponding concentration used in the amyloid formation assays) and the mixture quantified again. A diminution of about 37% in the fluorescence intensity was observed upon addition of citrate (figure 17), thus confirming an interference with the fluorometric assay. Sodium acetate appears, therefore, to be the ideal buffer to use in these amyloid formation experiments, however, the pH range of this buffer limits its application when studying pH dependence.

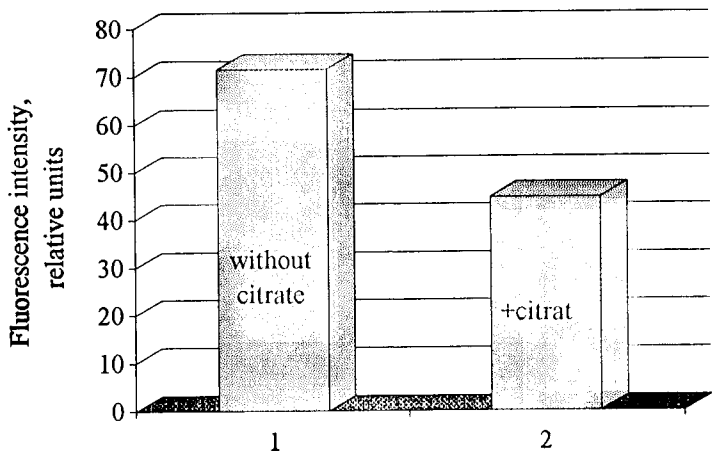


Figure 17 - Citrate/phosphate buffer effect on thioflavine T fluorometry. Amyloid-like fibrils produced in acetate buffer pH 4.0 before (1) and after the addition of 0.1 M citrate/phosphate buffer (2).

The kinetics of the amyloid formation process were then determined using 2 mg/ml TTR in sodium acetate pH 4.0 with incubation times ranging from zero to 100 hours. As can be seen in figure 18, amyloid formation is almost immediate. After a very short rate determining step (lag time) higher rates are observed in the first 20 to 30 hours after which there is a decrease in the amyloid formation rate. Therefore, incubation times of 48 hours yield reasonable amounts of amyloid.

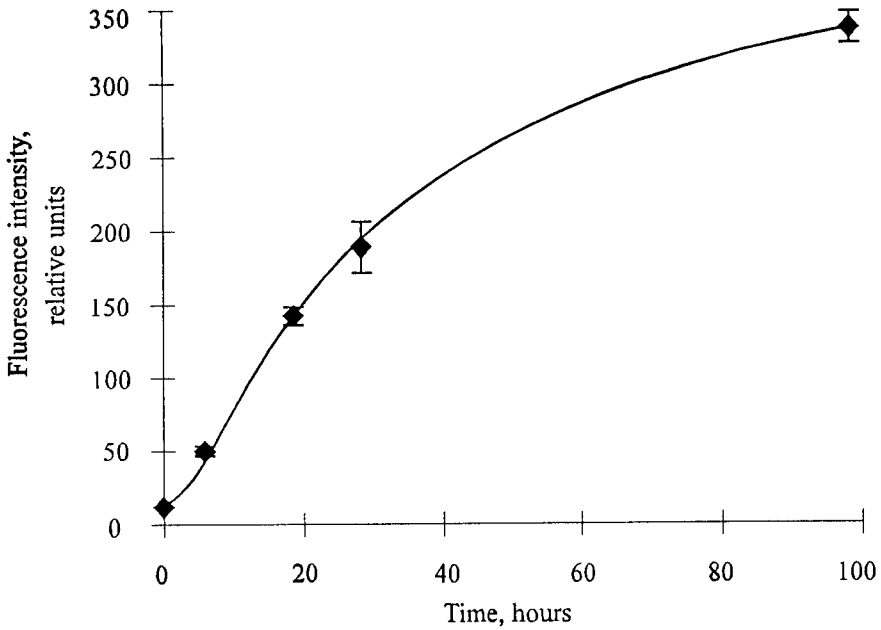


Figure 18 - Time course formation of amyloid-like fibrils from TTR in acetate buffer pH 4.0. Fluorescence measured with λex at 436 nm and λem max at 482 nm. Values are reported as the mean of three assays ± standard deviation (in arbitrary units).

The experimental conditions chosen to obtain amyloid from TTR, for the majority of the subsequent studies, were 96 hour incubations of 2 mg/ml TTR in acetate buffer pH 4.0, at room temperature.

Under these conditions the apparent affinity constant was determined, as described above for native amyloid fibrils, to be 1.33 μM (figure 19).

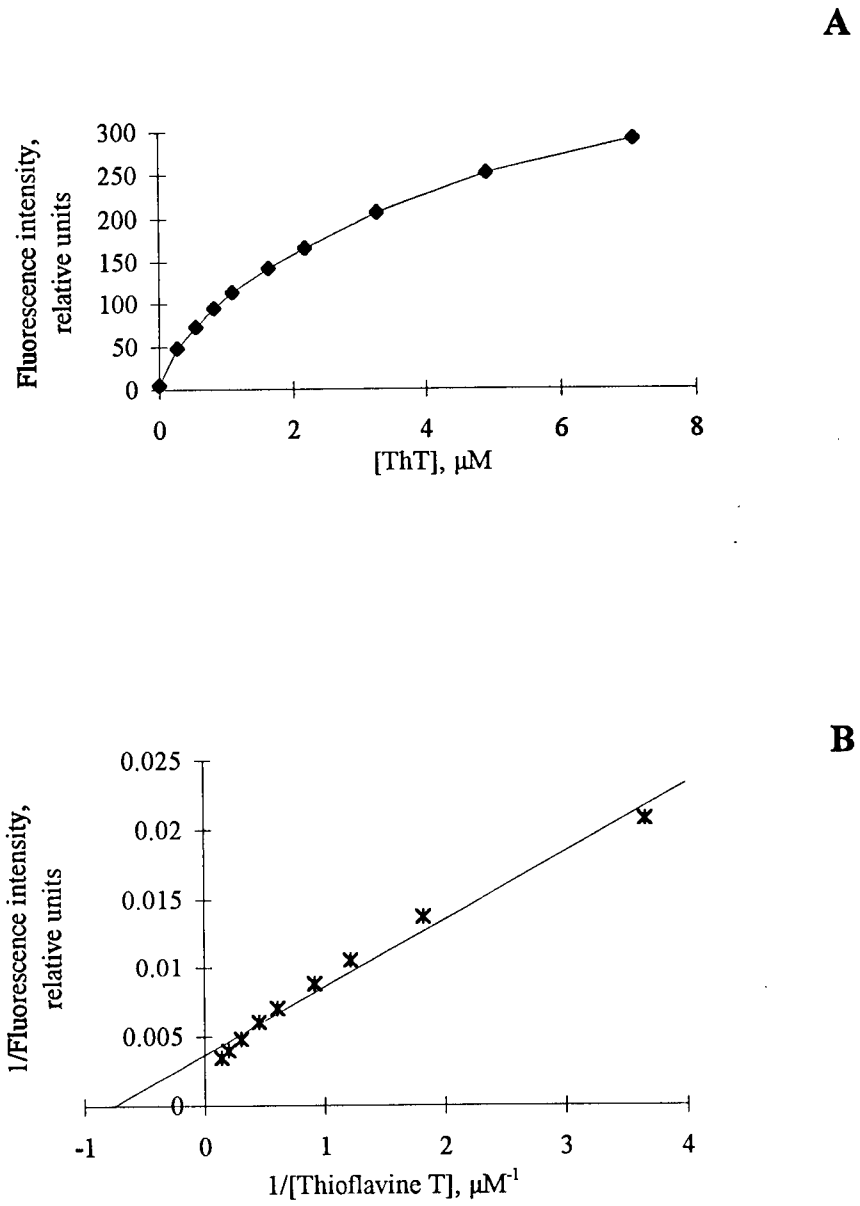


Figure 19 - Determination of the apparent affinity constant of thioflavine T binding to amyloid-like fibrils from TTR. **Panel A:** Thioflavine T concentration dependence. **Panel B:** Double reciprocal plot representation of thioflavine T binding to amyloid-like fibrils

The time dependent stability of the amyloid-like fibrils formed from TTR, under the conditions described above, was studied by raising pH to seven with either phosphate buffered saline or tris buffered saline, and evaluating the amount of amyloid present over a period of two weeks. Although there is a loss of fluorescence intensity with time, only 28.4 % is lost over this period of time (figure 20).

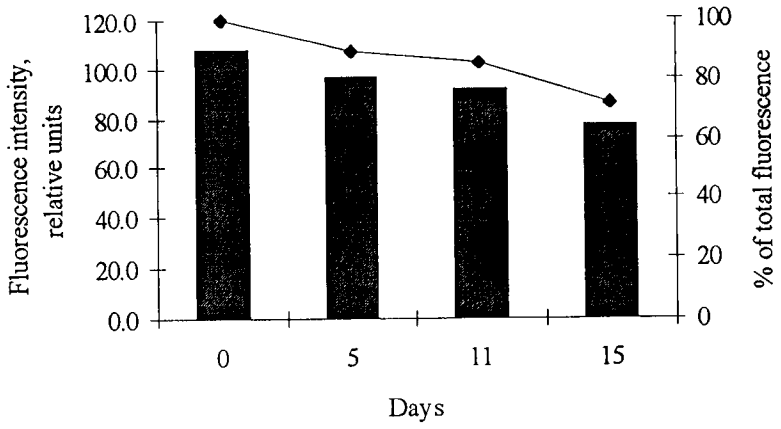


Figure 20 - Time dependent stability of amyloid-like fibrils formed from TTR in acetate buffer pH 4.0. Bars indicate the fluorescence intensity of the samples and the line represent the percentage of total fluorescence. Fluorescence measured with λex at 436 nm and λem at 482 nm. Values are reported as the mean of three assays.

During the optimization of the process of amyloid formation from TTR, several recombinant protein batches were used and some heterogeneity, as to the amounts of amyloid formed under the same conditions, was observed; i.e. the different protein batches, although having a similar behaviour in terms of concentration dependence and kinetics of amyloid formation, could produce different amounts of amyloid under the same conditions, thus absolute correlations should not be drawn among different experiments. In addition, with one or two particular batches no amyloid formation was detected under any of the experimental conditions tested. It is therefore necessary to use controls of the same protein batch when investigating other experimental conditions in the process of amyloid formation, such as studying the influence of exogenous factors as will be described below.

In summary, the experimental conditions for amyloid formation from soluble TTR were optimized in terms of protein concentration, pH and incubation buffer (2 mg/ml in acetate buffer pH 4.0). The kinetics of the process suggested that 48 hours of incubation is the minimum necessary to produce reasonable amounts of amyloid and that the amyloid formed under these conditions is relatively stable at room temperature for at least for 15 days.

To verify whether the process of amyloid formation could be accelerated by nucleation, we studied the kinetics of the process in the presence of two types of "seed"; S1- isolated *in vitro* amyloid-like fibrils (sediment obtained after ultracentrifugation); S2- an acidified preparation of TTR (after an incubation at pH 4.0 of 48 hours).

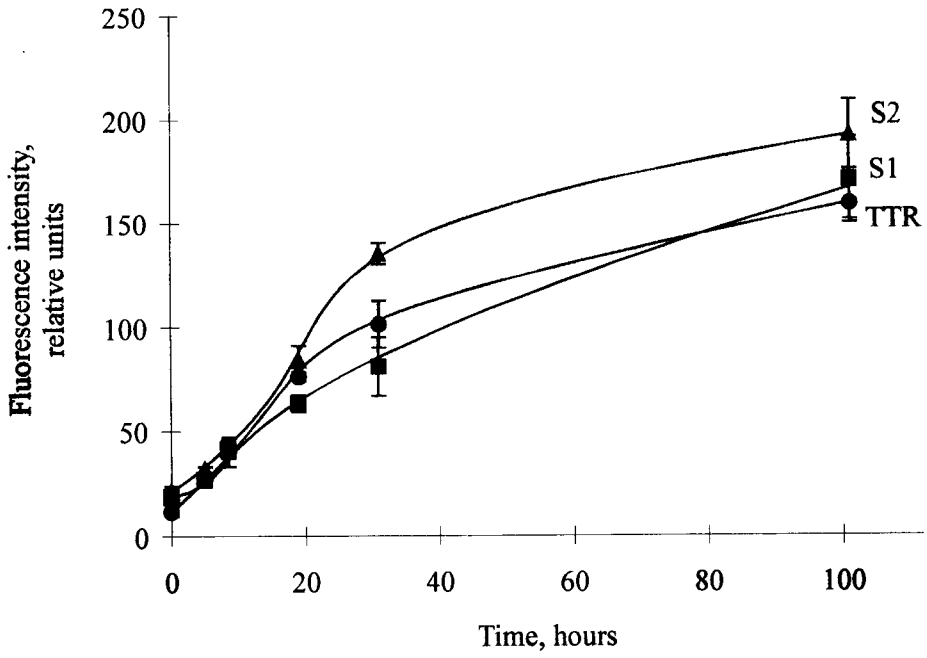


Figure 21 - Seeding effect in amyloid formation from TTR. Wild type TTR was incubated in acetate buffer pH 4.0 alone (TTR), in the presence of isolated amyloid-like fibrils (S1) or in the presence of an acidified TTR solution (S2). Fluorescence measured with λex at 436 nm and λem max at 482 nm. Values are reported as the mean of three assays ± standard deviation (in arbitrary units).

As can be seen in figure 21, no significant differences were observed in the lag times (first 10 hours) of amyloid formation in the presence of seeding or in its absence, hence indicating that under the conditions tested, the amyloid formation process appears to be nucleation independent. After the initial incubation period (20 hours) higher amounts of amyloid are detected when using the acidified preparation as seed (S2) as compared to the other samples; this is probably due to the higher TTR concentration in these samples.

6. Testing alternative methods to obtain amyloid from TTR, namely protein oxidation

We have optimized amyloid formation from TTR at a non physiological pH (with the possible exception of the lysosomal compartment); hence, other experimental conditions were searched to obtain amyloid from TTR at a more physiological pH.

Protein oxidation leading to aggregation has been reported for β-A4 (Dyrks *et al.*, 1992), and thus, in order to verify whether TTR oxidation would induce amyloid formation, several approaches were followed.

Initially we tested the conditions described for β-A4 metal-catalyzed oxidation (Dyrks *et al.*,1992): TTR at a concentration of 2 mg/ml, in either PBS or acetate buffer pH 5, was incubated with 0.5 mg/ml hemoglobin and 1 mM hydrogen peroxide, for either 1 hour or overnight at 37 °C. No amyloid formation was detected in these samples by thioflavine T fluorometry.

Since it is possible to separate normal from oxidized TTR monomers in isoelectric focusing (the oxidized monomer is more acidic than the normal one - Alves *et al.*, 1991) we have isolated both the normal and oxidized TTR monomers from preparative isoelectric focusing gels. Both proteins, at either 0.3 mg/ml or 0.7 mg/ml, were then incubated at room temperature for one week. Upon thioflavine T fluorometry no amyloid was detected in any of the samples. It is possible that either the concentrations used are too low to form amyloid or, since the protein was treated with 8 M urea, it might be present in an altered conformation, not conductive to amyloid formation.

In summary neither metal catalyzed oxidations of TTR nor the use of isolated oxidized species induced any amyloid formation, at least under the experimental conditions tested.

Discussion

In amyloid fibrils from FAP, FAC (Nordlie *et al.*, 1988; Gorevic *et al.*, 1989) and SSA (Westermark *et al.*, 1990a) patients, fragments of the TTR molecule together with the whole monomer (in variable proportions) have been found. The cleavage positions are mostly constant and encompass the region between residues 46 and 52 of the TTR (part of strand C and CD loop). In addition, neutrophil proteases such as human neutrophil elastase and cathepsin G have been identified in amyloid fibrils (Skinner *et al.*, 1986; Stone *et al.*, 1993). Altogether, these results lead to the concept that proteolysis (for instance as a result from aging processes) could trigger TTR fibril formation, by releasing amyloidogenic fragments that could serve as nucleus for further polymerization of fragments and monomers (Saraiva and Costa, 1991). That changes in the secondary structure of TTR occur after treatment with proteases, was shown by Walsh *et al.* (1994). However, there is no experimental data proving whether proteolysis is a pre-requesite for amyloid fibril formation or is a post-polymerization event. Our results concerning the evaluation of fragmentation in Met 30 amyloid fibrils as compared to Ile 122 amyloid fibrils, showed that very little fragmentation had occurred in the Met 30 amyloid fibrils, thus we would suggest that proteolysis, if a pre-event in certain amyloid diseases, it is probably not a general mechanism for fibrillogenesis in the ATTR amyloidosis.

The production of *in vitro* amyloid-like fibrils from different amyloid precursors has been an important tool for the study of amyloidogenesis *via* several approaches, namely: by the definition of amyloidogenic regions of protein precursor molecules using either synthetic peptides corresponding to fragments or the whole protein, as in the case of gelsolin (Maury *et al.*, 1994), islet amyloid polypeptide (Westermark *et al.*, 1990b), serum AA protein (Westermark *et al.*, 1992; Yamada *et al.*, 1994), L-chains (Hurle *et al.*, 1994), β-peptide (Wisniewski *et al.*, 1991; Castaño *et al.*, 1986) and TTR (Gustavsson *et al.*, 1991; McCutchen and Kelly, 1993); by the study of ions and other factors in the production of amyloid as in the case of β-amyloid (Bush *et al.*, 1994;

Kawahara *et al.*, 1994; Exley *et al.*, 1995; Fraser *et al.*, 1992; Dyrks *et al.*, 1992) and IAPP (Chargé *et al.*, 1995); by the study of aggregation kinetics as in the case of synthetic β-peptides (Jarret *et al.*, 1993; Shen *et al.*, 1993; Snyder *et al.*, 1994; Tomski and Murphy, 1992) and prion (Come *et al.*, 1993); and by structural analysis of the aggregates formed as in the case of β-peptide (Spencer *et al.*, 1991) and calcitonin (Bauer *et al.*, 1995).

The detection of amyloid-like fibrils is usually achieved by electron microscopy and Congo red staining; in some instances staining with thioflavine S (Kawahara *et al.*, 1994) and ultrastructural characterization by X-ray diffraction analysis were also used (Jarvis *et al.*, 1993; Inouye *et al.*, 1993). As for the quantification of amyloid-like fibrils, two methods have been used: a spectrophotometric method developed by Klunk *et al.*, (1989) to quantify Congo red binding to insulin amyloid-like fibrils was used to evaluate amyloid formation from β-A4 peptides (Wood *et al.*, 1995; Shen *et al.*, 1993); and a fluorometric assay with thioflavine T, initially developed by Naiki *et al.*, (1989), that has been used to quantify different types of native and synthetic amyloid fibrils.

We started by testing the available methodologies for *in vitro* amyloid detection and quantification. The characteristic green birefringence under polarized light of Congo red bound to amyloid, although amyloid specific, cannot be used to quantify amyloid, since it is dependent upon the sample physical-chemical properties, as judged from the differences observed with lyophilized and fresh fibrils; as for Congo red spectrophotometry, we successfully detected native FAP amyloid fibrils (both fresh and lyophilized) and amyloid-like fibrils from synthetic peptides, however, it was not possible to detect amyloid formation from TTR in conditions previously reported to originate amyloid-like fibrils.

The suitability of thioflavine T fluorometry as a quantitative method, already described for other amyloids, was by us tested and optimized, with both native TTR amyloid and amyloid-like fibrils derived from a TTR synthetic peptide, and later applied to amyloid-like fibrils from the whole TTR molecule. The affinity of thioflavine T to the amyloid fibrils was dependent not only on the identity of the fibrils (K_a 0.16 - 1.33 μM, fresh native < amyloid-like from peptide < amyloid-like from TTR), but also on the physical-chemical state of the fibrils, namely on the dehydration of the fibrils (K_a 0.16 - 1.09 μM, fresh native < lyophilized native). Affinity variation upon the identity of fibrils has been reported, being suggested that side chain substitutions can modulate the interaction with the dye (LeVine, 1995b); as for the dehydration of the fibrils, it probably affects either fibril conformation and/or originates a more aggregated state of the fibrils, altering the accessibility of the thioflavine T molecules to their binding sites. Regardless of the affinity variation, the spectral changes induced by the different amyloid fibrils were the same, thus corroborating the hypothesis that the thioflavine T interaction with amyloid fibrils is dependent on the quaternary

structure of the amyloid fibrils, but not on the nature of the precursor protein (LeVine, 1995b). The molecular basis for the large 120 nm red shift of the excitation maximum is not known, it probably reflects a stabilization of the electronic ground state of the dye that could be due to the extensive hydrogen bonding in the stacked β-sheets. LeVine (1995b) reported a higher K_a for the interaction of thioflavine T with amyloid-like fibrils produced from the whole TTR molecule (i.e. 5.2 μM) however not only the protein used was from a different source (commercially available TTR from serum) but also other experimental conditions were employed. Thioflavine T fluorometry is, therefore, a suitable method for amyloid quantification as long as it is validated for sample preparation.

The production of *in vitro* amyloid formation from synthetic peptides and the whole TTR molecule has already been described by acidification of the protein solutions (Gustavsson *et al.*, 1991; Colon and Kelly, 1991, 1992). We used a modification of these methods to produce amyloid-like fibrils from TTR, and characterized them by Congo red staining (both by polarization microscopy and spectrophotometry), and electron microscopy. The process of amyloid formation was then thoroughly studied at different experimental conditions, namely of pH, TTR concentration and incubation buffer, by quantitative thioflavine T based fluorometry. We found that TTR forms amyloid in a pH range of 2.6-4.6, with higher yields of amyloid being obtained at pH 3-3.6. Colon and Kelly (1992) reported amyloid formation from TTR at pH 3.3-4.5, but not at pH 2.5, having attributed this observation to the transition from tetramer to monomer with loss of the tertiary structure, occurring at pH 2.5. Under the conditions used we clearly have detected amyloid formation at pH 2.6, which indicates that the thioflavine T assay is more sensitive than the turbidimetry assays used by these authors; furthermore, the latter technique is not amyloid specific as it measures the amount of insoluble protein in suspension. On the other hand thioflavine T fluorometry does not detect amorphous aggregates as assessed with TCA precipitates. The concentration of the protein is also an important factor in these *in vitro* studies, as protein concentrations below 0.75 mg/ml originate very little amyloid; protein concentrations of 2-3 mg/ml appear to maximize the amounts of amyloid formed and above 5 mg/ml, again less amyloid is obtained, probably because highly populated solutions might favour some random aggregation, instead of oriented polymerization into amyloid fibrils. Gustavsson *et al.*, (1991) used 10 mg TTR/ml to produce amyloid-like fibrils but, as reported, very low yields were obtained; as for Colon and Kelly (1992) who used 0.6 mg TTR/ml, it is not possible to quantitatively compare their amounts of fibrils with ours, since the evaluation methods used were different.

Several reports suggest that amyloid precursors assemble into amyloid fibrils by a nucleation-dependent polymerization process, such as the assembly of microtubules or fibril formation by hemoglobin S (Jarret and Landsbury, 1992; Jarret and Landsbury, 1993;

Ashburn and Landsbury, 1993; Come *et al.*, 1993). Such mechanism presupposes the thermodinamically unfavourable formation of a nucleus, and thus rate limiting step, followed by rapid growth/polymerization. Increasing the critical concentration of monomeric species, or addition of a seed, accelerates the process by shortening or eliminating the rate determining step. When we studied the kinetics of amyloid formation from TTR we noticed the existence of a very short lag time, that appeared not to be affected by the presence of homologous pre-formed amyloid-like fibrils (seed). Therefore, under the conditions tested, the process of amyloid formation from TTR apparently is not dependent on nucleation. Colon and Kelly (1992), when studying the time course of TTR amyloid formation as a function of pH, observed lag phases of differing durations, which at pH 4 was of about 30 minutes, and concluded that the process should be described as nucleation dependent; however, no experiments of seeding were performed by these authors to confirm those results; in addition, the experimental conditions used (temperature, ionic strength, protein origin) were different from ours and it is reported, at least for β-peptide, that aggregation kinetics are dependent on concentration, sample origin, ionic strength and temperature (Snyder *et al.*, 1994).

We considered the optimal conditions for the production of amyloid-like fibrils from TTR to be 2 mg TTR/ml in 0.1 M acetate buffer pH 4 for 96 hours at room temperature. In these conditions, the fibrils were rather stable at neutral pH for at least two weeks; and the only care that should be taken is to include controls of the same protein batch, as marked variability in the protein behaviour is sometimes observed.

The acidic pH at which our assays are performed leads to some limitations; since it is not a physiological pH (except for the lysossomal compartment) it does not simulate the *in vivo* situation, and we can not test protein factors such as P component and apolipoprotein E, which were recently described respectively to inhibit and accelerate amyloid formation from β-peptide (Janciauskiene *et al.*, 1995; Castaño *et al.*, 1995a; Wisniewski *et al.*, 1994); or antibodies, and natural ligands of TTR, such as thyroxine and retinol binding protein, unless other experimental conditions are developed for the production of TTR amyloid-like fibrils.

We have used metal catalyzed oxidation, as reported for βA4, and isolated oxidized TTR, in attempts to induce amyloid-like fibril formation from TTR, but none of these approaches was successful; the two amyloid precursors are highly dissimilar; unlike TTR, β-peptide is an unstable peptide, forming spontaneously amyloid at neutral pH (Kirschner *et al.*, 1987) and it is also reported to be a self generator of free radicals (Hensley *et al.*, 1994). Therefore, conditions that lead to β peptide aggregation might have no effect on TTR; however, since we have not measured free radical generation when using metal catalyzed oxidation, we do not know the efficiency of that process; it is possible that the experimental conditions used were not the most appropriate for TTR, and further studies should elucidate that point.

In conclusion although the system developed by us has some limitations, it can be used to assess the influence of factors, other than proteins, on TTR amyloid formation. Potential therapeutic factors, such as rifampicin or 4'-iodo-4'-deoxydoxorubicin, already shown to inhibit fibrillogenesis from other amyloid precursors (Tomiyama *et al.*, 1994b; Merlini *et al.*, 1995), can easily be tested with our assay. Other factors such as metal ions, sulphate ions, and the differential amyloidogenic potential of TTR variants in amyloidogenesis, as assessed by fluorometry, will be described in the following chapter.

Chapter 2: Modulating factors in TTR amyloidogenesis

Aims

- Investigation of the effect of exogenous factors in the process of amyloid formation, such as metal and sulphate ions.
- Investigation of the role of TTR mutations in amyloidogenesis.

Introduction

Familial amyloidotic polyneuropathy (FAP) is an autosomal dominant disease characterized by the extracellular deposition of transthyretin (TTR) in several organs and tissues as amyloid. Several TTR variants have been identified in the plasma and amyloid deposits of FAP patients, and this genetic heterogeneity is associated with different clinical phenotypes; the major clinical manifestation is neuropathy with a differential involvement of other organs/tissues such as heart and vitreous (when the heart is the predominantly affected organ the syndrome is designated as familial amyloidotic cardiomyopathy - FAC); some variability in the age of onset and organ involvement can nevertheless be found with some TTR variants (Saraiva, 1991). Non amyloidogenic TTR variants have also been described (Saraiva 1995).

The mechanisms underlying the pathogenesis of FAP and FAC are unknown; however, it is clear that the existence of mutations in the TTR molecule favour the deposition of the protein as amyloid; the clinical heterogeneity associated with the Met 30 and Ile 50 mutations (Saraiva 1991; Nishi *et al.*, 1992; Saeki *et al.*, 1992), and the fact that normal TTR also deposits as amyloid in senile systemic amyloidosis (Westermark *et al.*, 1990a) suggest the involvement of other factors, genetic or not, in amyloidogenesis.

Using the *in vitro* assay of amyloid formation from soluble TTR described in the previous chapter, we investigated the influence of different concentrations of zinc, aluminum and magnesium in the process of amyloid formation from TTR, since it has been described that metal ions accelerate the aggregation of β-peptide (Bush *et al.*, 1994; Kawahara *et al.*, 1994). We have also tested the effect of sulphate ions in this process, as sulphated glycosaminoglycans are described as common constituents of amyloid deposits (Snow and Wight, 1989).

In order to study the influence of mutations on the TTR molecule we further evaluated the relative amyloidogenic behaviour of some TTR variants towards amyloid formation in a

pH dependence assay. The variants chosen were: TTR Met 30, the most prevalent TTR mutation associated with FAP; TTR Pro55, that results from a leucine for proline substitution at position 55 and gives rise to a particularly aggressive form of the disease (Jacobson *et al.*, 1992); TTR Met 111 and TTR Ile 122, two variants associated with FAC resulting, respectively, from a methionine for leucine substitution at position 111 (Nordlie *et al.*, 1988) and from a isoleucine for valine substitution at position 122 (Saraiva *et al.*, 1990a); and TTR Met 119, a non amyloidogenic variant, with a methionine for threonine substitution at position 119 (Harrison *et al.*, 1991).

Materials and Methods

In these studies we used recombinant TTR proteins produced in an *E. coli* expression system and TTR isolated from normal serum. Recombinant bacteria producing TTR Met 30, TTR Met 119, TTR Ile 122 and TTR Met 111 (Furuya *et al.*, 1991) were provided by Dr. Yoshiuki Sakaki, Tokyo University. Recombinant bacteria producing wild type TTR and TTR Pro 55 (McCutchen *et al.*, 1993) were a gift from Dr. JF Kelly, Texas A&M University. Both serum and recombinant proteins were isolated and purified as described in the previous chapter.

Testing exogenous factors in amyloid formation

i) Metal ions effect - 2 mg/ml wild type TTR in 40 µl 0.1 M sodium acetate pH 4.0 was incubated for 96 hours in the presence of the following concentrations of each ion: 0.5, 1, 10, and 50 mM of $AlCl_3$, $MgCl_2$ or $ZnCl_2$ respectively. Samples were then analyzed by fluorometry.

ii) Sulphate effect - 12 Eppendorf tubes containing 2 mg/ml wild type TTR in 20 µl 0.1 M sodium acetate were prepared and to a set of three tubes 50 mM Na_2SO_4 was added; twenty four hours later 50 mM Na_2SO_4 was added to another set of three tubes; and finaly, at the end of 100 hours incubation time, 50 mM Na_2SO_4 was added to another set of tubes and all samples were analyzed by fluorometry. To verify the aggregation effect of sulphate, wild type TTR at a concentration of 2 mg/ml was incubated for 96 hours in 0.1 M sodium acetate pH 4.0 in the presence of 50 mM Na_2SO_4, the resulting suspensions were centrifuged at 14,000 rpm in a microfuge for 20 min, and both sediments and supernatants were analyzed by fluorometry.

Assays of variant amyloidogenicity

pH dependency assays - Each of the recombinant proteins at a concentration of 2 mg/ml was incubated in 40 µl 0.1 M citric acid/ 0.2 M sodium hydrogenophosphate buffer at the following pHs: 2.6, 3.0, 3.6, 4.0, 4.6, 5.0 and 5.6 for 96 hours. When using serum TTR the incubation buffer was 0.1 M sodium acetate at the following pHs: 3.8, 4.2, 4.6 and 5.2 for 96 hours. Samples were analyzed by fluorometry without any further treatment.

Kinetic assays - Kinetic experiments were performed as described in the previous chapter, with each of the following recombinant proteins: TTR Met 30, TTR wild type and TTR Pro 55. Final concentrations were 2 mg/ml rec TTR in 0.1 M acetate buffer pH 4.6 with 50 mM Na_2SO_4, in a total volume of 75 µl. A kinetic experiment without Na_2SO_4 was carried out with TTR Pro 55. At the end of the incubation times samples were centrifuged at 14,000 rpm in a microfuge, supernatants used for protein quantification and pellets analyzed by fluorometry.

Other methods

Fluorometric assays with thioflavine T, electron microscopy and protein quantification were performed as described in the previous chapter.

Results

1. Study of possible intervening factors in the process of amyloid formation

1.1. Influence of metals ions

TTR was reported to have zinc binding affinity; in addition, zinc has also been reported to promote aggregation of TTR (Martone and Herbert, 1994), of β-peptide (Bush *et al.*, 1994) as well as of IAPP (Westermark *et al.*, 1996). The same effect on β-peptide was reported for aluminum (Kawahara *et al.*, 1994). Thus, we studied the influence of $AlCl_3$, $ZnCl_2$, $MgCl_2$ and NaCl in the process of amyloid formation from TTR.

In the first place we analyzed whether these ions interfered with the thioflavine T fluorometric assay in the same way as described in the previous chapter for the citrate/phosphate buffer; i.e. each ion, at different concentrations, was added to TTR amyloid-like fibrils immediately after fluorometry quantification, and the mixture was quantified again. It was observed that both aluminum, zinc and magnesium interfered with the thioflavine T assay, though at different concentrations; for concentrations higher than 1 mM (10, 50 mM) aluminum reduced the fluorescence of thioflavine T bound to amyloid fibrils, whereas only concentrations higher than 10 mM of zinc and magnesium interfered with the assay; as for sodium, no effect was observed for concentrations up to 150 mM. We have therefore tested the following concentrations of ions in the process of amyloid formation: 500 µM and 1 mM of aluminum, 500 µM, 1 mM and 10 mM of zinc and magnesium. No significant effect was observed with any of these concentrations in the process of amyloid formation as seen in figure 1.

Therefore, under the conditions tested none of these ions appears to affect the process of amyloid formation from TTR.

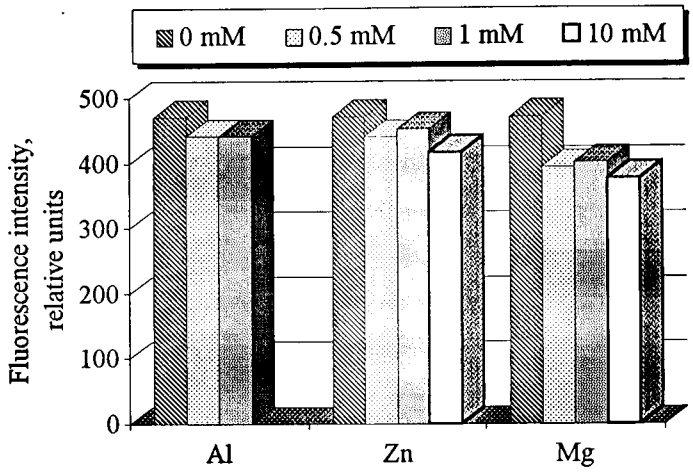


Figure 1 - Influence of metal ions in the process of amyloid formation from TTR. TTR at a concentration of 2 mg/ml was incubated in acetate buffer pH 4.0 for 96 hours alone (0 mM) or in the presence of 0.5 mM or 1 mM of each $AlCl_3$, $ZnCl_2$ or $MgCl_2$, or 10 mM of $ZnCl_2$ or $MgCl_2$.

1.2. Influence of sulphate ions

Native amyloid fibrils are known to have associated glycosaminoglycans, which are highly sulphated carbohydrates and that are hypothesized to play a role in amyloidogenesis (Snow and Wight, 1989). In order to study the influence of sulphate ions in the process of amyloid formation we started by testing whether 50 mM Na_2SO_4 interfered with the fluorometric assay, as described above for the metal ions study; no significant diminution of fluorescence intensity was detected upon the addition of sulphate to the reaction mixture containing amyloid-like fibrils and thioflavine T. TTR was then incubated at the standard conditions (2mg/ml, in sodium acetate pH 4.0) with 50 mM sodium sulphate. By fluorometry, it was observed that less amyloid was formed in the presence of sulphate as compared to the control without sulphate, indicating therefore an inhibition of amyloid formation. To confirm these results, a similar experiment was performed, which is represented in table 1; TTR was incubated at the standard conditions; i.e. 2 mg/ml in acetate buffer pH 4.0 and 50 mM sodium sulphate was added at different incubation times, namely at the beginning of the experiment (SO_4), after 24 hours (SO_4 24) and at the end of the experiment (SO_4 0); the control without sulphate is TTR. As can be observed, in the presence of sulphate less amyloid was detected, but the time of addition of sulphate was a determining factor, in that more fluorescence was detected with shorter sulphate incubation times.

Table 1 - Sulphate effect on fibril formation

Sample designation	Time of incubation before adding SO_4	Time of incubation with SO_4	Fluorescence intensity *
TTR	100 h	No SO_4	137.34 ± 0.58
SO_4	0 h	100 h	70.57 ± 6.4
SO_4 24	24 h	76 h	112.09 ± 3.3
SO_4 100	100 h	2-3 min	136.08 ± 1.3

* in relative units ± standard deviation

In a different experiment, we added 50 mM sulphate to amyloid-like fibrils produced by a 96 hour incubation (2 mg/ml in acetate buffer pH 4.0) and incubated another 24 hours before fluorometry. No differences were observed between these samples and the controls without sulphate. Altogether these results indicate that the effect of sulphate on fibril formation appears to occur in the exponential phase of polymerization, either by inhibiting the polymerization of the protein into amyloid, or by favouring the formation of other conformational species.

In addition, macroscopically the suspensions of amyloid-like fibrils formed in the presence of sulphate are much more turbid than the ones in the absence of sulphate, suggesting the presence of higher molecular weight aggregates. Sedimentation by centrifugation at low speed (14,000 rpm in a microfuge) originates pellets, that when analyzed by thioflavine T fluorometry

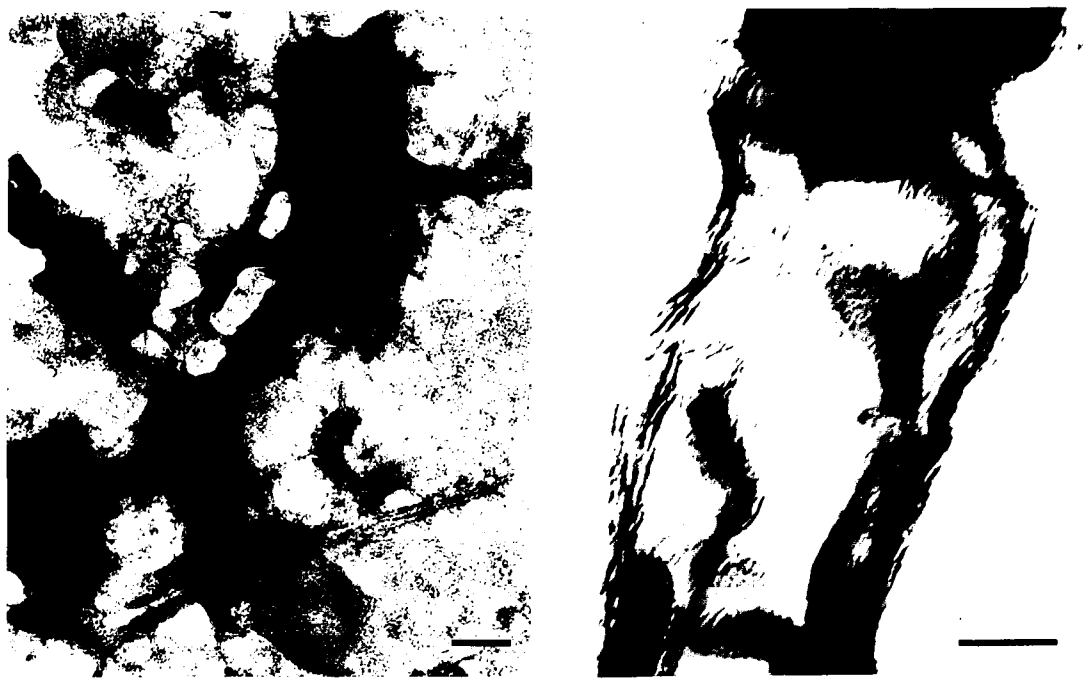


Figure 2 - Electron micrograph of sediments produced by TTR in 1% acetic acid with 10 mM sodium sulphate after 96 hours incubation and sedimentation of aggregates by centrifugation at 14,000 rpm. The scale bar represents 100 nm.

reveal the presence of amyloid (with no amyloid detected on the supernatants). Electron microscopy of these sediments shows the presence of macrostructures in which fibrils, dimensionally similar to amyloid fibrils (9.8 nm diameter), can be individualized (figure 2).

In conclusion, sulphate not only interferes with the amyloid formation process but also, apparently, promotes the formation of macroaggregates.

2. Amyloidogenicity of different TTR proteins

The behaviour of TTR of different origins, and of different TTR variants towards amyloid formation was next investigated.

2.1. Serum TTR *versus* recombinant TTR

Since the above conditions refer to a recombinant protein we studied the pH dependence of amyloid formation from human TTR isolated from sera (sTTR) by incubating sTTR at a concentration of 2 mg/ml in 0.1 M acetate buffer pH 3.8-5.2 (the widest range possible) for 96 hours at room temperature. As control, we used wild type TTR in the same conditions. At the end of the incubation time samples were analyzed by fluorometry. Both proteins were shown to form amyloid

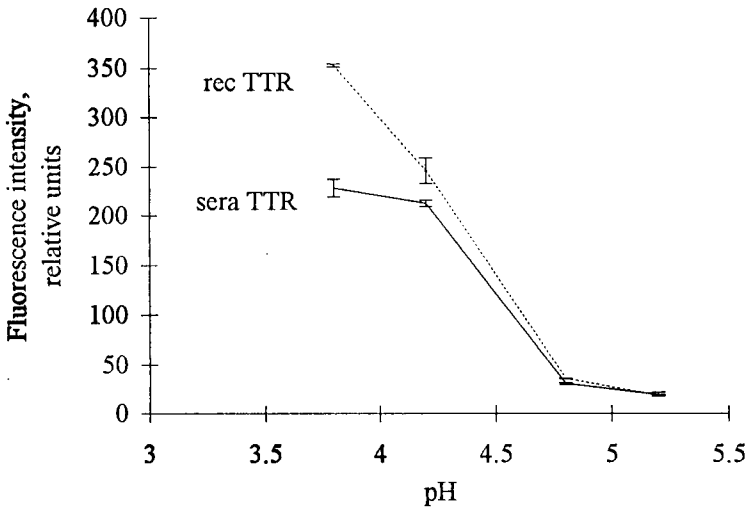


Figure 3 - Amyloid-like fibril formation from wild type TTR (rec TTR) and serum TTR (sera TTR) as a function of pH. Fluorescence measured with λex at 436 nm and λem at 482 nm. Values are reported as the mean of three assays ± standard deviation (in arbitrary units).

in the same pH range, with the recombinant TTR producing higher amounts of amyloid at pH 3.8 (figure 3).

Wild type and serum TTR presented, therefore, similar "amyloidogenic" behaviour.

2.2. Neuropathic and non amyloidogenic variants

We have selected some TTR variants that represent a wide range of phenotypes within the TTR hereditary amyloidosis, and studied the susceptibility of these variants towards amyloid formation as a function of pH. The buffer used in these experiments was citrate/phosphate due to its broader pH range. Initially, the variants studied were TTR Met 30, TTR Pro 55, and TTR Met 119. Eighty micrograms of normal and variant TTR at a concentration of 2 mg/ml were incubated in citrate/phosphate buffer, pH range 2.6-6.0, for 96 hours at room temperature. At the end of the experiment samples were used for fluorometric determination. As can be observed in figure 4, under the conditions tested, the Met 119 variant formed little amounts of amyloid and in more acidic conditions than the wild type TTR, i.e., between pH 2.6-3.5, with higher amounts of amyloid formed at pH 3.0, indicating a lower degree of amyloidogenicity when compared to the wild type TTR. The Met 30 variant formed higher amounts of amyloid than the wild type protein and in a wider range of pH, i.e. between 2.6-5.0; it showed, however, the same peak of maximum amyloid formation at pH 3.6. As for the TTR Pro 55 variant, amyloid was formed in the pH range of 3.0-6.0 (pH 2.6 was not tested), with high amounts produced at pH 3.6-5.0.

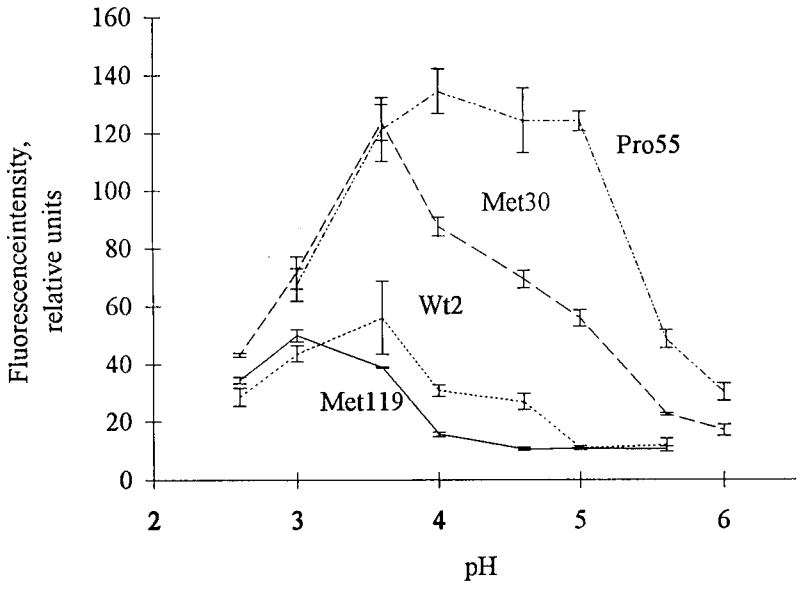


Figure 4 - pH dependence curve of "amyloid-like" fibril formation from TTR. Fluorescence was measured at λex at 436nm and λem at 482nm. Values are reported as the mean of three assays ± standard deviation (in arbitrary units).wt - wild type TTR.

The relative behaviour of these TTR proteins towards amyloid formation can be represented in terms of decreasing susceptibility towards amyloid formation as:

Pro 55 >> Met 30 > wild type > Met 119

This order of amyloidogenicity is further corroborated by examining the kinetics of the amyloid formation process for TTR Pro 55, TTR Met 30 and wild type TTR. In order to be able to quantify the remaining soluble protein, the experiment was carried out in the presence of 50 mM sodium sulphate in acetate buffer pH 4.6, since, as previously observed, fibrils produced in the presence of sulphate can be sedimented at low speed. At the end of the experiment samples were centrifuged, sediments analyzed by fluorometry and the protein in the supernatants quantified. Panel A of figure 5 represents the time course of amyloid formation and in panel B is represented the corresponding percentage of protein remaining soluble in the supernatants.

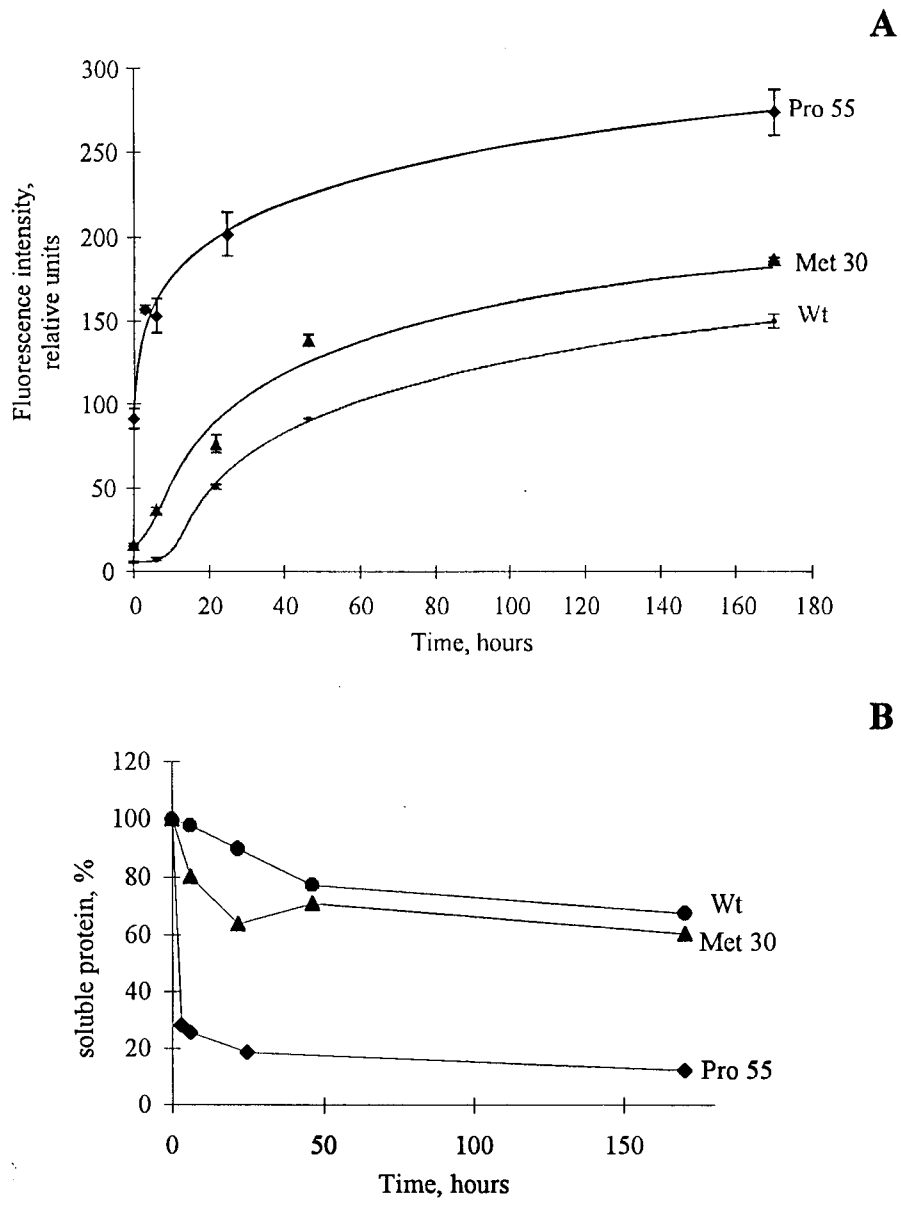


Figure 5 - Amyloid formation from TTR proteins in acetate acetate buffer pH 4.6 with 50 mM Na_2SO_4; samples were centrifuged, sediments analyzed by fluorometry and supernatants used for protein quantification. Wt - Wild type TTR **Panel A** - Time course formation of amyloid like fibrils. Fluorescence measured with λex at 436 nm and λem at 482 nm. Values are reported as the mean of three assays ± standard deviation (in arbitrary units); lines were drawn by hand. **Panel B** - Percentage of protein remaining soluble in supernatants.

Under the same conditions, the TTR Pro 55 variant forms amyloid at higher rates than the TTR Met 30 or the wild type proteins, most of it in the first 30 hours and representing almost the total protein present in the experiment (80-88%). In opposition to TTR Pro 55, both the TTR Met 30 and the wild type proteins, present an initial rate limiting step (lag time); moreover, only 40% of the TTR Met 30, and 33% of the wild type proteins, are precipitated at the end of the experiment, thus confirming the higher propensity of TTR Pro 55 towards amyloid formation comparatively to the other two proteins. When comparing the TTR Met 30 with the wild type protein, the former produces more amyloid, at higher rates, and with a higher percentage of precipitated protein, thereby being more amyloidogenic than the wild type TTR.

In addition, we observed that the suspensions of TTR Pro 55 amyloid-like fibrils were similar to those obtained from the wild type in the presence of sulphate, in that they could be sedimented at low velocities (14,000 rpm), allowing the quantification of the protein present in the supernatants.

We then reasoned that TTR Pro 55 could give us more information on the effect of sulphate in TTR amyloid formation, thus the kinetics of amyloid formation for TTR Pro 55 were analysed in the presence and absence of sulphate (figure 6). As can be observed in panel A, the rate of amyloid formation was the same in the presence or absence of sulphate, but the amount of amyloid formed in the absence of sulphate was higher than in its presence, as expected from the above sulphate studies on wild-type TTR; however, the percentage of protein remaining soluble in the supernatants was the same (figure 6 panel B), indicating that under the conditions tested sulphate favours the formation of amorphous precipitates, counteracting the polymerization of the protein into amyloid.

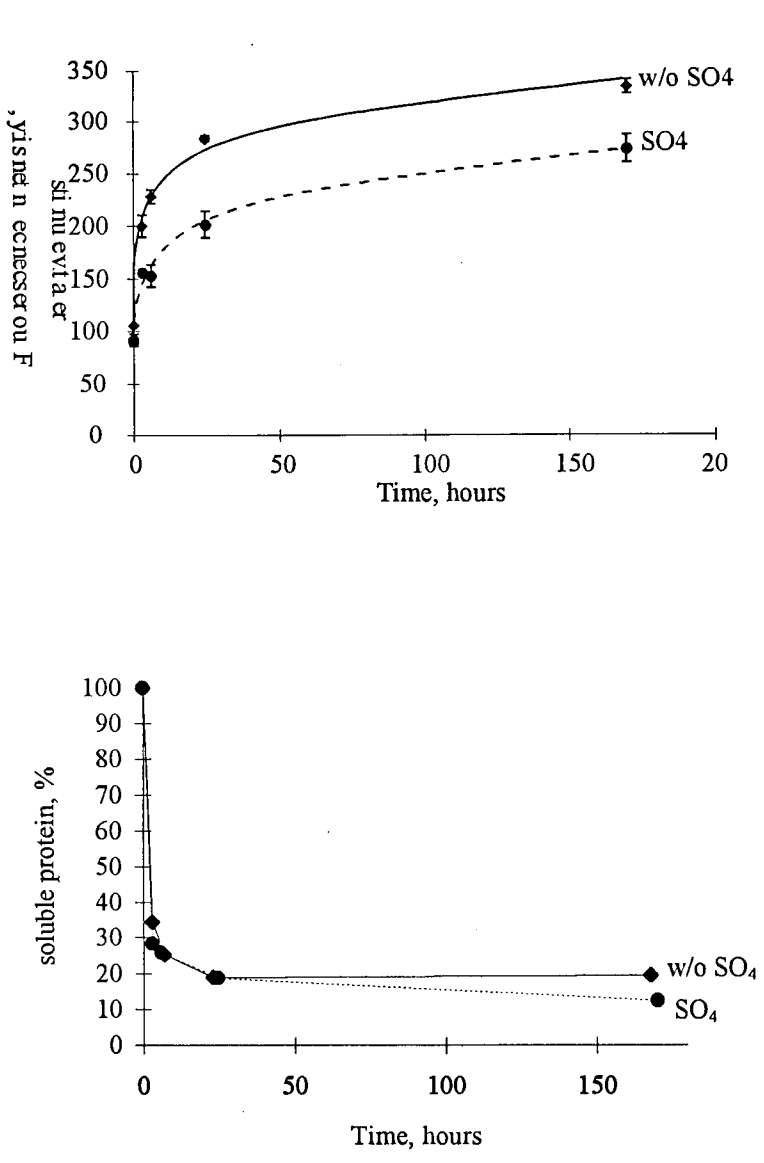


Figure 6 - Amyloid formation from TTR Pro 55 in the presence (SO_4) and absence (w/o SO_4) of Na_2SO_4; samples were centrifuged, sediments analyzed by fluorometry and supernatants used for protein quantification. **Panel A** - Time course formation of amyloid-like fibrils. Fluorescence measured with λex at 436 nm and λem at 482 nm. Values are reported as the mean of three assays ± standard deviation (in arbitrary units); lines were drawn by hand. **Panel B** - Percentage of protein remaining soluble in supernatants.

2.3. Cardiopathic variants

The behaviour of two cardiopathic TTR variants, TTR Met 111 and TTR Ile 122, was also studied in pH dependence assays. Wild type and TTR variants, at a concentration of 2 mg/ml, were incubated in citrate/phosphate buffer, pH range 2.6-6.0, for 96 hours at room temperature. Samples were then used for fluorometry (figure 7).

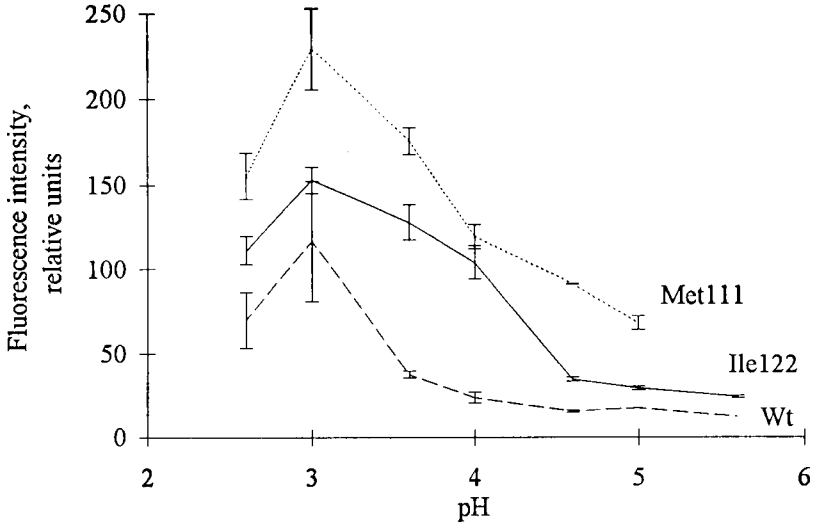


Figure 7 - pH dependence curve of amyloid-like fibril formation from TTR variants. Fluorescence was measured at λex at 436nm and λem at 482nm. Values are reported as the mean of three assays ± standard deviation (in arbitrary units). Wt - Wild type TTR.

It was observed that both the Met 111 and Ile 122 variants formed higher amounts of amyloid and in a wider pH range (2.6 - 4.6 for Ile 122; 2.6 - 5.0 for Met 111) than the wild type protein, thus indicating a higher degree of amyloidogenicity of these variants; among the latter, the Met 111 appears to be more amyloidogenic than the Ile 122, as it forms more amyloid and in a wider pH range. As for the wild type protein, not only did it form amyloid at pH 2.6-4 (instead of 2.6-4.6, as described above) but the peak of amyloid formation was shifted to the more acidic pH 3 instead of 3.6. We cannot exclude that these variations could be due to protein batch heterogeneity, and therefore, as referred to in the previous chapter, caution is necessary when drawing absolute correlations among experiments carried out at different occasions.

In summary, by using pH dependent and kinetic assays we have evaluated the relative amyloidogenicity of 5 TTR variants: two neuropathic - Pro 55, Met 30; two cardiopathic - Ile 122, Met 111; and one non amyloidogenic Met 119, and verified the following order of decreasing susceptibility towards amyloid formation:

Pro 55 >> Met 111, Met 30 > Ile 122 > wild type > Met 119

which is in agreement with the pathological conditions associated with these mutations. We have also observed that serum and recombinant TTR had similar behaviour towards amyloid formation, validating the use of recombinant proteins in *in vitro* assays.

Discussion

TTR is a homotetramer of about 55,000 Da with a well known three dimensional structure. Each monomer, of 127 amino acids, can be described as a β-sheet sandwich formed by two four-stranded β-sheets stacked intramolecularly. The monomers are associated in dimers by extensive hydrogen bonding, and the assembly of two dimers forms a cylinder with a central hydrophobic channel where the thyroid hormones bind (Blake *et al.*, 1978).

The mechanisms by which TTR deposits as amyloid are unknown; however factors such as the amyloidogenic potential of the protein, tissue components and chaperone proteins, appear to contribute to this process (Saraiva and Costa, 1991). Basically, two hypothesis have been raised to explain the amyloidogenic potential of TTR; a proteolytic one, suggesting that proteolysis could trigger fibril formation, by releasing amyloidogenic fragments that would nucleate further polymerization of fragments and monomers (as described in the discussion of the previous chapter) and a conformational one; this latter hypothesis proposes that, through a conformational modification, the formation of an intermediate occurs (amyloidogenic intermediate) capable of self-assembly into fibrils, and that the mutations in the TTR molecule would either facilitate the formation of this species and/or stabilize it. These conformational changes could be induced, *in vivo*, by chaperone proteins, such as SAP, apo E or glycosaminoglycans, tissue components or other still unkown factors.

We have evaluated the relative tendency of some TTR variants towards amyloid formation (amyloidogenicity), using pH dependence and kinetic assays, and measured amyloid formation by thioflavine T fluorometry. The variants TTR Pro 55, TTR Met 30, TTR Ile 122, and TTR Met 111 were chosen since they are associated with different clinical pictures; TTR Met 119 was chosen because it is a non-amyloidogenic variant. It was verified that the TTR Pro 55 variant not only is highly susceptible towards polymerizing as amyloid over a broader pH range, but also forms amyloid at higher rates and in higher amounts, than all the other proteins studied, thus indicating a higher amyloidogenicity of the TTR Pro 55 tetramer. The Met 119 variant, in opposition to the TTR Pro 55, forms amyloid only at very acidic conditions, and in very small amounts, being, therefore, more resistant to amyloid formation than the other TTR proteins; on the light of the "amyloidogenic intermediate" hypothesis this mutation appears to "stabilize" the TTR tetramer as to the conformational change needed to generate the amyloidogenic intermediate. As for the other variants studied, it was verified that both TTR Met 30 and TTR Met 111 polymerized as amyloid in a wider pH range and in higher amounts than the wild type protein, hence being more susceptible towards amyloid formation; and, finally, the TTR Ile 122 variant formed amyloid in a pH range close to the wild type protein but in higher amounts. Since protein batch heterogeneity can lead to variability in the amounts of amyloid formed, as previously referred, it is not possible

to distinguish between the TTR Met 30 and the TTR Met 111 as to which is the more amyloidogenic with the assays performed; time course studies should elucidate these doubtfull cases.

Kelly and coworkers have studied the *in vitro* acid-mediated denaturation pathway of TTR (wild type, TTR Met 30, TTR Pro 55 and TTR Met 119), by spectroscopy, glutaraldehyde cross-linking, and SDS-PAGE analysis, in the presence or absence of Z3-14, a detergent inhibitor of fibril formation (Colón and Kelly, 1992; McCutchen *et al.*, 1993, 1995). It was suggested that at a pH of approximately 4.4 there is an equilibrium of the wild type tetramer to a structured amyloidogenic monomer (this pH was denominated as pHm, meaning midpoint of tetramer to monomer equilibrium), that assembles into amyloid fibrils; upon lowering pH to values below 3.0 this intermediate undergoes further structural rearrangements to an "A-state", lacking the ability to form amyloid; the pHm values for the variants were shown to be 4.7 for the Met 30 tetramer, 5.4 for the Pro 55 tetramer and 3.4 for the Met 119 tetramer, thus implying a higher stability of the Met 119 variant as compared to the wild type and a lower stability of the Met 30 and Pro 55 variants. As for the pH ranges reported (Kelly and Lansbury, 1994) for amyloid formation from TTR proteins (as assessed by turbidimetry analysis), they were similar to the ones obtained by us, as summarized in table 2; however we detect amyloid formation at lower pHs, which is probably due (as already referred in the discussion of the previous chapter) to a higher sensitivity of the thioflavine T fluorometry as compared to the turbidimetry assays.

Table 2 - pH range for TTR amyloid formation

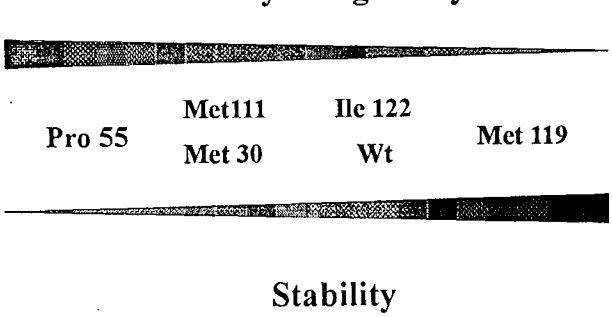
TTR proteins	This work	Kelly and Lansbury, 1994
Met 119	2.6 - 3.6	nr
Wild type	2.6 - 4.0/6	3.5 - 4.8
Ile 122	2.6 - 4.6	nr
Met 111	2.6 - 5.0	nr
Met 30	2.6 - 5.0	3.6 - 4.8
Pro 55	3.0 - 6.0	3.6 - 6.1

nr - not reported

It was further hypothesized that fibril formation could occur in the lysosomes by a mechanism competing with the normal turnover of the protein; *i.e.* through partial acid denaturation and/or degradation, the TTR molecule would structurally rearrange to a monomeric intermediate, that

self-assembles into fibrils. According to this hypothesis, the mutations would either destabilize the tetramer and/or stabilize the amyloidogenic intermediate allowing it to be formed under milder acidic conditions (Kelly and Lansbury, 1994).

We can assume, therefore, that the amyloidogenicity of the variants is inversely correlated with their stability, and thus, the TTR variants we studied can be ordered in terms of decreasing amyloidogenicity and increasing stability as follows:



This correlation amyloidogenicity/stability is further supported by comparative dissociation studies of serum from TTR Met 119 and TTR Met 30 heterozygous carriers and from compound heterozygotes TTR Met 30 - TTR Met 119, by semi-denaturing isoelectric focusing in urea (Alves *et al.*, 1996b). These studies have shown that the TTR tetramers from Met 119 heterozygotes appear to be more stable than those from Met 30 heterozygous, in that they are more resistant to dissociation; as for the compound Met 30 - Met 119 heterozygote, the TTR tetramers have an intermediate stability similar to that of the normal TTR carriers.

Although the amyloidogenicity of the variants studied correlates well with their pathogenicity, it should be kept in mind that these results (ours; Kelly and Lansbury, 1994; Alves *et al.*, 1996b) refer to studies performed under conditions unlikely to occur *in vivo* (acidic or in urea); furthermore it is unlikely that fibril formation occurs *in vivo* in the lysosomal acidic environment for the following reasons: i) there are no descriptions of intracellular amyloid in FAP, FAC or SSA; ii) if fibrils formed intracellularly as a result of the folding or degradation pathways of TTR, they should be identified inside or in close association with the cells responsible for those processes, since TTR is synthesized and catabolized mainly in hepatocytes (Soprano *et al.*, 1985; Makeover *et al.*, 1988), the liver should be a preferential place for amyloid deposition; however in FAP amyloid deposition in liver is seldom observed (Miyazato *et al.*, 1991); in addition, studies with TTR Met 30 transgenic mice have indicated that it is the circulating protein that deposits as amyloid, since there is no direct correlation between the tissues where the transgene is expressed and the tissues where the protein deposits as amyloid (Wakasugi *et al.* 1987).

In summary, other studies are necessary to clarify the stability/amyloidogenicity/pathogenicity correlation of the TTR variants, namely for instances metabolism studies, which should elucidate the relative stability of the different molecules.

Several reports describe the involvement of metals in the aggregation of the β-peptide, namely aluminum (Kawahara *et al.*, 1994; Exley *et al.*, 1995) and zinc (Mantyh *et al.*, 1993; Bush *et al.*, 1994); equimolar amounts of zinc and magnesium were also reported to induce the formation of birefringent congophilic TTR precipitates (Martone and Herbert, 1994); moreover considering that the extensive treatment of native amyloid fibrils with chelators, such as EDTA or dimercaptosuccinic acid, results in partial solubilization of the fibrils, Martone and Herbert (1994) have suggested that metal interactions could play a role in amyloid formation. Nevertheless, in our system none of the ions tested, zinc, magnesium, aluminum or sodium had significant effects on amyloid formation from TTR in any of the concentrations tested (0.5, 1, 10 mM). We cannot exclude in any case, an effect by some of these ions under different experimental conditions; it would be interesting to test the influence of other ion concentrations in time course experiments.

Glycosaminoglycans (GAGs), highly sulfated carbohydrates, are found to be associated with amyloid deposits, regardless of the type of amyloid protein involved; their role in amyloidogenesis is at present unknown. It has been suggested (Snow and Wight, 1989) that GAGs may promote certain protein conformations which induce fibril formation, stabilize preexisting fibrils and/or decrease their susceptibility to proteolysis, or they may influence amyloid deposition to occur at specific anatomical locations. When studying the influence of sulphate ions in the process of amyloid formation in our system, it was observed that sulphate diminished the amounts of amyloid formed from TTR, probably by favouring the formation of amorphous precipitates. Considering that the protein is positively charged at this pH, the neutralization of the protein charge by sulphate, could lead to protein aggregation and precipitation by favouring hydrophobic interactions; however, another phenomenon should also be considered, that is, the presence of sulphate could "stabilize" the TTR tetramer, preventing conformational changes that lead to polymerization into amyloid. It is mentioned by McCutchen *et al.*, (1993), but not shown, that 0.5 M sulphate stabilize TTR, as to the tetramer to monomer transition, and that no inhibitory effect is observed in fibril formation; however, these authors used turbidimetry to assess fibril formation, and this methodology does not distinguish between amorphous precipitates and amyloid fibrils. We have also verified that the amyloid-like fibrils produced in the presence of sulphate were morphologically distinct, since macrostructures containing fibrils were observed by electron microscopy. The latter effect is similar to the sulphate induced macroaggregation of pre-existing β-peptide fibrils (Fraser *et al.*, 1992). This macroaggregation effect could account for an *in vivo* protecting effect of the proteoglycans against proteolytic degradation, by interacting with the amyloid fibrils and promoting their

aggregation. Gupta-Bansal *et al.*, (1995) presented further evidence on the inability of proteases to attack Aβ-proteoglycan complexes. Some reports describe the inhibition of amyloidogenic precursor accumulation either in cell culture by glycosaminglycans (Priola and Caughey, 1994) or *in vivo* by small-molecule anionic sulphonates (Kisilevsky *et al.*, 1995a), and the mechanism proposed for such inhibition is by a competition between the exogenous GAGs/sulphonates and endogenous proteoglycans. *In vitro* studies probing the action of sulphate or sulphonates were described only in relation to pre-existing β amyloid fibrils; Fraser *et al.*, (1992) reported a sulphate-specific tendency of fibrils to undergo extensive lateral and axial growth, and Kisilevsky *et al.*, (1995a) referred partial disassembly of the fibrils by treatment with 1,3-propanediol disulphate. Since our studies were performed *in vitro* at non physiological pHs, it is possible that the inhibitory effect of sulphate in TTR fibril formation is just a result of the conditions of the assay. Also, we have not tested the influence of proteoglycans and/or glycosaminoglycans, and that should be done before drawing any other conclusions.

Chapter 3: Transgenic mice carrying the human TTR Met 30 variant - a strain without amyloid

Aims

- Characterization of transgene expression, and of the human TTR Met 30 protein produced by the transgenic strain MT1-TTR Met 30.
- Search for amyloid deposition in long-term induced animals

Introduction

Several lines of transgenic mice carrying the structural gene for human transthyretin (TTR) Met 30 have been developed as an attempt to create an animal model for FAP.

One of these lines carries the structural gene for the human TTR Met 30 variant under the control of the mouse metalothionein (MT) gene promoter (MT2-TTR Met 30), and was described to have 1.0 to 4.8 mg/dl of the human protein in the circulation (Wakasugi *et al.*, 1987). The transgene was expressed in the liver, heart, brain, skeletal muscle, kidney and lung (Shimada *et al.*, 1989) and amyloid deposits reactive to the human variant and to mouse amyloid P component, were detected as early as 6 months of age in the mucosa of the small intestine. Amyloid deposition increased with the animals age, and occurred predominantly in the intestinal mucosa, renal glomeruli, myocardium, small vascular walls and thyroid; no amyloid deposition was ever found in brain, choroid plexus, peripheral nervous system or hematopoietic tissues. This pattern of amyloid deposition correlates to the one found in human FAP autopsy cases, with the exception of the choroid plexus and the peripheral nervous system which is particularly affected. These animals did not develop neuropathy (Yi *et al.* 1991, Araki *et al.*, 1994).

Sasaki *et al.* (1986) also described transgenic mice carrying the same type of construct, the structural gene for the human TTR Met 30 variant under the control of the mouse MT promoter (MT1-TTR Met 30) that when induced with zinc, had up to 1.4 mg/dl of the human protein in the circulation. The expression of the transgene was found mainly in intestine, testis, heart and brain, and varied among this transgenic strain. Liver and kidney had very low levels of transgene expression, despite the fact that the metalothionein fusion genes are usually highly expressed in these tissues. As the methylation pattern of the promoter did not correlate with expression levels,

this suppression was probably due to the integration of additional plasmid sequences in the fusion construct (Sasaki *et al.*,1989). No amyloid deposits were observed in this transgenic line.

The fact that the MT1-TTR Met 30 transgenic strain develops no amyloid deposition as compared to the MT2-TTR Met 30 strain remains unexplained. Several hypotheses can be raised to explain that fact: i) the human protein levels in the circulation are too low to lead to amyloid deposition; ii) the human protein might be synthesized in a different conformation that prevents its deposition as amyloid; iii) the genetic background of these animals could influence the process of amyloid deposition.

In order to distinguish between these hypotheses, we have characterized the human protein in the circulation of the MT1-TTR Met 30 mice, searched for additional transgene expression tissues, and carried out long term experiments in zinc induced animals to assess the presence of amyloid.

Materials and Methods

MT1-TTR Met30 animals

Animals used in this study, resulted from mattings between mice from transgenic strains MPA551 and MPA552 (provided by Dr. Yoshiuki Sakaki; Sasaki *et al.*, 1986) with Balb C mice, and were considered positive transgenic when meeting the following criteria: presence of the human variant in the circulation, as assessed by ELISA, and presence of the transgene as assessed by DNA analysis. To induce the MT promoter, the drinking water was supplemented with 76 mM $ZnSO_4$ for 3 days to one week prior to sera collection.

Affinity chromatography

The human protein was semipurified from a pool of transgenic mice sera by affinity T_4chromatography with the BA9 monoclonal antibody for human TTR (Regnault *et al.*, 1992). A Pharmacia C-10 column was used containing 1 ml antibody BA9 coupled to Actigel ALD (Sterogene Bioseparations Europe). The gel was washed with 100 mM NaCl, 50 mM Tris pH 8.0 (TBS) and protein was eluted with 0.1 M glycine, 0.5 M NaCl pH 10.5. Fractions collected were neutralized with 1 M Hepes buffer (Gibco) and the ones positive for TTR, as detected by ELISA, were pooled, dialyzed and lyophilized.

IEF

Isoelectric focusing was performed as described in chapter 1.

Immunoblotting

Proteins separated by SDS-PAGE and IEF were transferred to nitrocellulose membranes in 0.192 M glycine, 0.025 M Tris pH 8.3, 20% methanol, and 0.7% acetic acid respectively (Towbin *et al.*, 1979), and probed with anti-human TTR peroxidase labeled antibody (Binding Site).

Gel filtration

Human TTR isolated from transgenic sera was subjected to gel filtration in high performance liquid chromatography (HPLC) using an Ultraspherogel column (Beckman). Elution buffer was 0.1 M in potassium phosphate, 0.1 M in sodium sulphate and 0.05% in sodium azide. Fractions were collected every minute and were analyzed by ELISA.

Thyroxine binding

T_4 binding to plasma TTR was analyzed by electrophoresis in a glycine-acetate buffer system as described (Saraiva *et al.*, 1988). Whole plasma was incubated with thyroxine L-[^{125}I] (NEN-Dupont), with a specific activity of 1300 μCi/μg, and 7.9 mM diphenylhydantoine (Sigma).

ELISA

The human TTR concentration in transgenic mice sera and in conditioned media from primary fibroblasts was determined by sandwich ELISA (Hazenberg *et al.*, 1990). Briefly, the plates were coated with anti-human TTR (DAKO) diluted 1:200 in 0.1 M carbonate/hidrogenocarbonate buffer pH 9.5, and incubated at 37°C for 45 min; 100 μl of cell free conditioned media from primary fibroblasts, or of a 1:200 dilution of transgenic sera, were then added to wells and incubated for 1 h at 37°C; 100 μl of anti-TTR peroxidase labeled (Binding Site) were added to wells and incubated 1 h at room temperature. After each incubation wells were washed with 0.05% Tween 20 in TBS. The substrate solution used for detection was 0.1% ABTS (2,2'azino-di-[3-ethylbenzthiazoline sulphonate] - Sigma), 0.002% H_2O_2 in 0.1 M phosphate/citrate buffer pH 4.3. Absorbance at 405 nm was then measured using a Denley Welscan spectrophotometer. Standard human serum-Behring ORDT 07 (Behringwerke AG, Marburg, Germany) was employed to generate a standard curve (range from 5 to 50 ng/ml).

Radial Immunodiffusion

Radial immunodiffusion (RID) for the quantification of mouse albumin was performed in 2 mm agarose gels containing 4 μl anti mouse albumin antibody (Binding Site) per square centimeter in Veronal buffer pH 8. Albumin standards were prepared with mouse albumin (Calbiochem).

Total RNA isolation

Tissues and cell pellets (about 10^6 cells) were homogenized in in 10-15 ml 150 mM LiCl, 300 mM Urea with a Polytron, and stored overnight at 4°C. Samples were then centrifuged 2 h at

12,000 *g* and the pellets were resuspended in 10 mM TrisCl pH 8, 1 mM EDTA (TE) with 0.1% SDS. After phenol/chloroform extraction and ethanol precipitation, the RNA was resuspended in 100 µl TE and treated with RNase-free DNAse I (BRL) for 1 h at 37°C. After phenol/chloroform extraction and ethanol precipitation, RNA was resuspended in TE.

Reverse transcription and PCR

Reverse transcription to cDNA and PCR were performed using the Gene Amp®RNA kit from Perking Elmer Cetus. Briefly, reverse transcription was carried in a 20 µl total volume reaction containing 5 mM $MgCl_2$, 1 mM each dNTP, 1 U/µl RNase inhibitor, 2.5 µM Oligo $d(T)_{16}$, 2.5 U/µl reverse transcriptase and 0.5-5 µg total RNA, all in 50 mM KCl, 10 mM Tris-HCl. After the following incubations: 15 min at 42°C, 5 min at 95°C and 5 min in ice water, the reaction volume was raised to 100 µl with the appropriate solutions to have a final mixture with 2 mM $MgCl_2$; 2.5 U ampli*Taq* DNA Polymerase and 100 pmol each primer were then added. The primers used for PCR included one set specific for human TTR cDNA (**HCA** - 5' ATG GCT TCT CAT GGT CTG CT 3' and **HCB** - 5' GAA GTC CCT CAT TCC TTG GG 3'), and another set with homology to mouse TTR cDNA (**RCA** - 5' GAT GGC TTC CCT TCG CCT GT 3' — 90% homology, **RCB** - 5' AAG CTA CTG CTT TGG CAA GA 3' — 95% homology). Samples were subjected to 30 cycles of denaturation (95°C, 1 min), annealing (60°C, 1 min), and polymerization (72°C, 1 min) in an automatic DNA thermocycler (Bio-med) and were then analyzed by electrophoresis in 4% NuSieve agarose gels (FMC).

Primary cultures of fibroblasts

The media used for cell culture was Dulbbecco's modified Eagle's medium with low glucose, supplemented with a mixture of 100 U/ml penicillin, 100 µg/ml streptomycin (P/S), and 10% fetal calf serum which we designate as DMEM10P/S.

Fibroblast cultures were prepared from newborn mice. Briefly, mice not older than three days, were killed with anesthetic Fluothane (Zeneca), skin peeled off and incubated overnight in 0.25% Trypsin/EDTA (GIBCO) at 4°C. The other day, dermis was separated from epidermis with the aid of forceps, and put in 0.35% collagenase in DMEM media for 30 min at 37°C. The cell suspensions so obtained were passed through sterile gauze and centrifuged 5 min at 200 *g*. The cell pellet was washed twice with DMEM10P/S and plated on 75 cm^2 flasks. Cells were grown in DMEM10P/S media.

Metabolic labeling and immunoprecipitation

Primary fibroblasts, at a density of about 10^6 cells per 75 cm^2 flask, were washed twice with PBS and incubated with 5 ml Minimal Eagle's Medium (MEM) methionine-free, and 300 mCi (^{35}S)-methionine 1,000 Ci/mmol (Amersham) for 4 h at 37°C. Cell monolayers were rinsed twice

with PBS, scraped off the flasks, centrifuged, resuspended in 1 ml 50 mM K_2HPO_4, 1 mM methionine, 1 mM phenylmethylsulfonyl fluoride (PMSF) pH 7.5, and mechanically disrupted by passing several times through a 25 gauge needle. Cell suspensions and cell free media (1 ml) were added 2 µl normal rabbit serum and incubated 1 h at 4°C; immune complexes were precipitated with 50 µl IgGsorb (Enzyme Center) 60 mg/ml in PBS with 1 mM PMSF. The supernatants were then incubated with anti-human TTR (DAKO) for 1 h at 4°C, and the immune complexes, precipitated as before, were dissociated in 0.1% SDS, 8 M urea. The analysis of the immunoprecipitated proteins was performed in a 15% gel by discontinuous SDS-PAGE. The gel was incubated for 30 min in 10% trichloroacetic acid, 10% acetic acid, 40% methanol, and soaked in Amplify (Amersham) for another 30 min. After drying on a gel dryer the gel was exposed to X-ray film Cronex 4 (NEN Dupont) for 2 weeks.

Preparation of explant cultures

Choroid plexuses, retinas and pieces of skin and nerve were collected immediately after animal death, washed several times in phosphate buffered saline (PBS - 2.7 mM KCl, 1.5 mM KH_2PO_4, 140 mM NaCl, 8.1 mM Na_2HPO_4) and minced into small pieces. These pieces were incubated in 24-well plates, for 5 h at 37°C, in 500 µl DMEM supplemented with 1.2 µM $ZnSO_4$. At the end of the incubation period the tissue pieces were separated from culture media by centrifugation. Tissues were added 1 ml PBS and homogenized in glass Potter-Elvehjem homogenizers. Human TTR was assessed in media and homogenates by ELISA, and total protein was quantified in the media by the BioRad Protein assay.

Tissue and cell fixation

Samples of skin, muscle, intestine, stomach, kidneys, liver, testis/ovary, heart, sciatic nerve and brain were collected, fixed in buffered formalin and embedded in paraffin, following standard procedures.

Primary fibroblasts for immunohistochemistry were grown in Lab®-Tek two-chambers slides, (Nunc), washed with PBS, fixed in 0.2% formaldehyde, 0.2% glutaraldehyde in PBS for 5 min at 4°C, washed with deionized bidestilled water, air dried and processed for histochemistry.

Histochemistry

Paraffin sections (4 µm) were stained with Congo red following standard procedures (Puchtler *et al.*, 1962) and observed under polarized light.

Immunohistochemistry was carried out with a polyclonal anti-human TTR (DAKO) and anti-human TTR peroxidase labelled (Binding-Site) and FAP2, a monoclonal anti-human TTR antibody (Costa *et al.*, 1988). The detection of the antigen-antibody complexes was performed

with either the avidin-biotin-peroxidase (Hsu *et al.*, 1981), alkaline phosphatase, or colloidal gold (Holgate *et al.*, 1983) methods.

Results

1. Characterization of human TTR in transgenic sera

Our first approach for the study of the MT1-TTR Met 30 transgenic mice was the biochemical characterization of the human protein present in the circulation.

Gel filtration in HPLC was carried out to assess whether the human protein was present in transgenic sera in a tetrameric or monomeric form. The column used had previously been shown to have the necessary resolution to separate albumin (M.W. 64,000 Da, retention time - 16 min) from lysozyme (M.W. 14,300 Da, retention time - 21 min). The retention time of human TTR was determined by analyzing the collected fractions by ELISA. In figure 1 is represented the elution profile obtained with transgenic sera; the retention time of the human protein (16-17 min) is very similar to the one of albumin, thus indicating the presence of a tetrameric species. No positive reactions were observed at retention times where the protein would be present as monomers or as higher molecular weight aggregates.

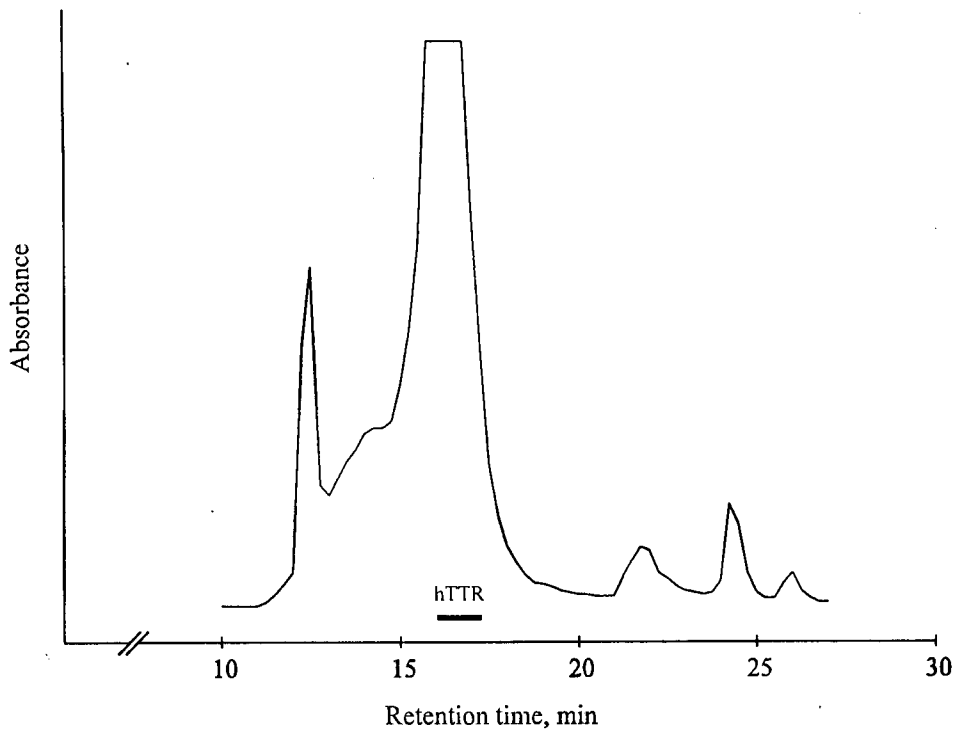


Figure 1- Chromatogram of transgenic mice sera separation by gel filtration HPLC. The bar represents the fractions positive for human TTR (by ELISA).

The human protein was isolated from a pool of transgenic mouse sera, by affinity chromatography with a monoclonal antibody anti-human TTR; however, since the isolated protein was partially contaminated with other sera components, it is referred to as semipurified human TTR. This semipurified protein was then run in semi-denaturing isoelectric focusing (4 M Urea) and the gel was immunoblotted. A specific pattern of bands was observed, similar to the pattern of TTR from human serum (figure 2): a doublet band closer to the cathod (pI ≈ 5.7) corresponds to the normal monomer (although not detected in the human serum sample it is commonly observed in other normal human serum samples - Altland *et al.*, 1981), a band observed at pI ≈ 5.45 corresponds to the oxidized monomer and the bands close to the anode (at pI of 4.2 to 4.7) correspond to undissociated tetramers. Therefore, the isoelectric characteristics of the transgenic protein are identical to the ones of human TTR.

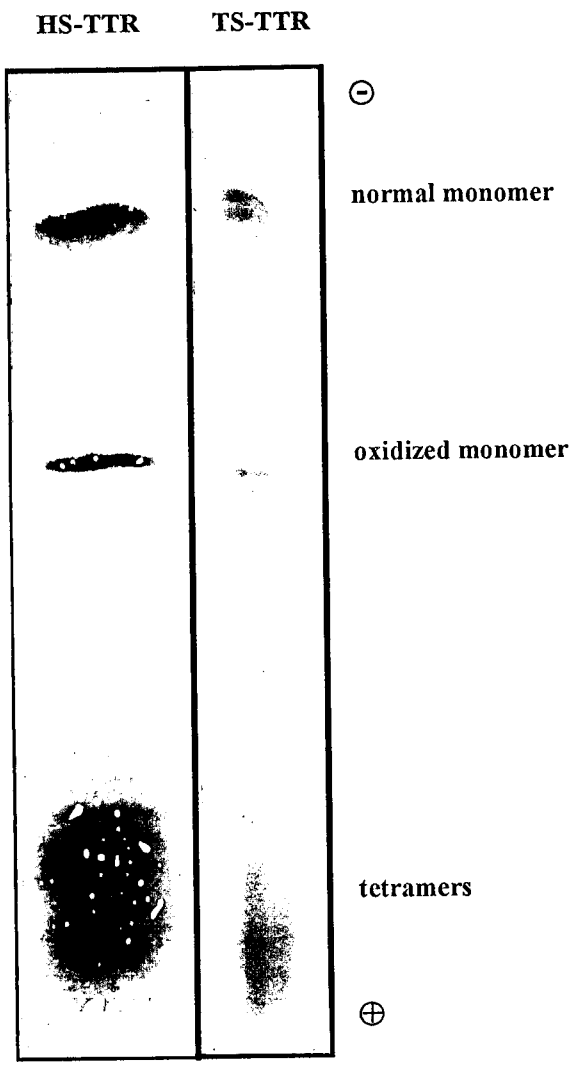


Figure 2 - Transgenic mouse semipurified human TTR characterization by IEF in a 4 M urea gel. HS-TTR - human serum TTR; TS-TTR - semipurified human isolated from transgenic mice.

Studies were next undertaken to determine the functionality of the human protein in mice sera, namely its binding properties to thyroxine (T_4). Since the Met 30 homotetramer has a very low affinity for T_4 (Rosen *et al.* 1993), the binding of T_4 to the mice sera was performed in the presence of diphenylhydantoin, an inhibitor of T_4 binding to thyroxine binding globulin (Wolff *et al.* 1961). The presence of a blur, migrating in the position of the human TTR, in the transgenic mice sera, not observed in the normal mice sera, indicated that the human protein bound T_4 (figure 3). This blur could eventually be due to overload of the gel; in that case however, it should also be present in the normal mice sera. The low intensity of the TTR band is probably due to the low binding affinity of the Met 30 variant for T_4.

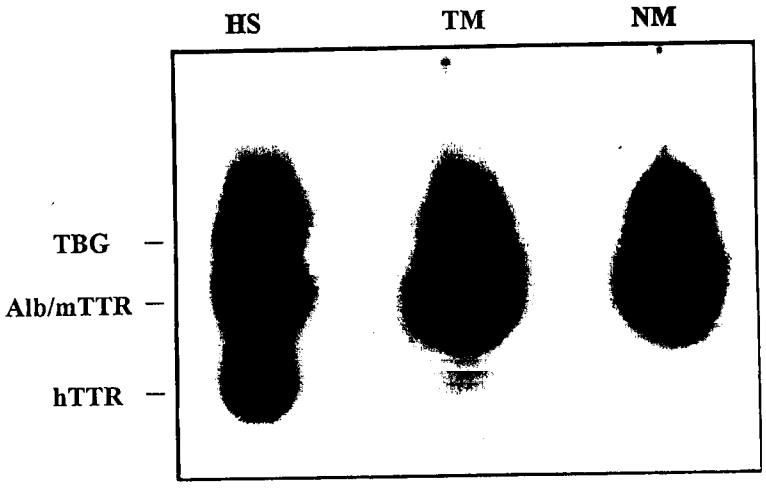


Figure 3 - T4 binding to transgenic mice sera by PAGE. HS - human sera; TM - transgenic mice sera; NM - normal mice sera; TBG - thyroxine binding globulin; Alb/mTTR - Albumin and mouse TTR; hTTR - human TTR.

In another TTR transgenic mice line, MT2-TTR Met 30, the presence of mouse/human TTR hybrid species in the serum was detected by T_4 binding, as a smear between the human and mouse TTR bands (Palha *et al.*, 1994b), since mouse TTR is the main T_4 transporter in mice. In the MT1-TTR Met 30 however, no hybrid forms were detected by this method; that could be due to absence or low synthesis of the human gene in the liver, the main locus of mouse TTR synthesis. The levels of transgene expression in liver had been previously reported to be very low (Sasaki *et al.*, 1989); nevertheless we next investigated the expression of the human TTR gene in these animals.

2. Expression of the human TTR gene in MT1-TTR Met 30 mice

RNA expression was then searched for in liver, skin, nerve, retina and gut by total RNA isolation, followed by cDNA synthesis and PCR with specific human and mouse primers. As shown in figure 4, the approximately 490 bp band corresponding to the human TTR cDNA was evident in the skin, retina, gut and liver. In the nerve only a very faint band was visible. As for the presence of mouse TTR cDNA, it could be detected in liver and retina, as expected.

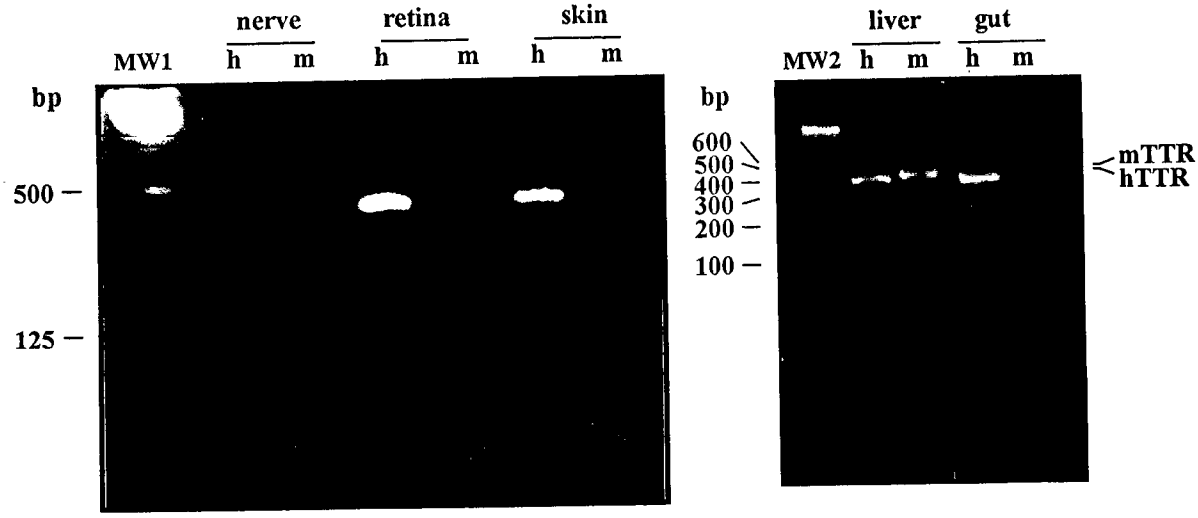


Figure 4 - Transgenic mice human TTR gene expression analysis in a 4% NuSieve agarose gel. Samples were amplified using primers for human TTR (h) and primers for mouse TTR (m); MW1 - λDNA digested with Hind III; MW2 - 100 bp DNA ladder.

The presence of the human TTR mRNA in the skin was unexpected and we further searched protein expression in skin by immunohistochemistry; however it was not possible to detect protein synthesis by any of the detection methods employed, namely the avidin-biotin-peroxidase, alkaline phosphatase or colloidal gold methods. The reason for that could be a low intracellular protein concentration, under the detection limit of the assay used.

3. Detection of human TTR in primary cultures of fibroblasts

Primary cultures of fibroblasts were then established from newborn transgenic mice and analyzed for the presence of human TTR. These cells were shown to express the human transgene as assessed by RNA expression, performed as described above (figure 5A). In figure 5B it is represented an SDS-PAGE of immunoprecipitated proteins, after metabolic labeling of a fibroblast clone (Fib 1). Although faint, a band is evident at about 14,000 Da, which is the expected size for the human TTR monomer.

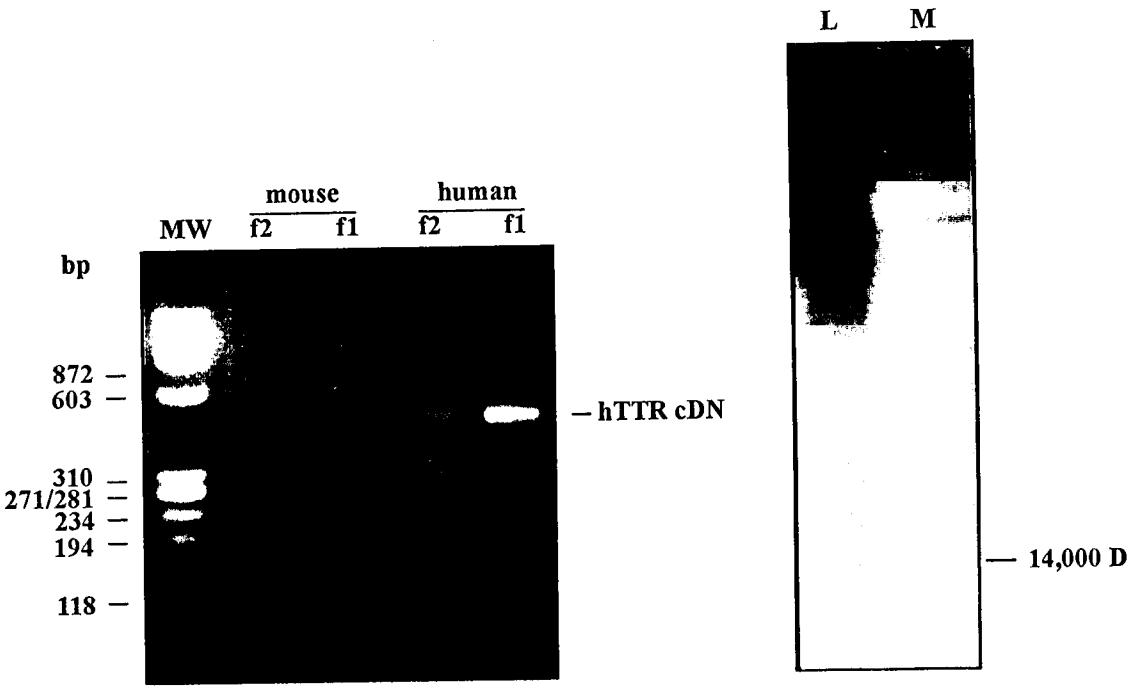


Figure 5 - Human TTR expression by transgenic primary fibroblast cultures. **Panel A:** RNA expression analysis in a 4% NuSieve agarose gel; mouse - samples amplified with mouse primers; human - samples amplified with human primers; f1, f2 - 2 different clones of fibroblasts. **Panel B:** SDS-PAGE analysis of immunoprecipitated proteins, with anti-human TTR, from conditioned media (M) and cell lysate (L) of a fibroblast 1 clone.

The amounts of TTR produced by primary fibroblasts, as determined by ELISA, were highly variable; values as high as 976 ng TTR produced per million cells in 24 hours by one clone (Fib 1) were obtained as opposed to the 25 ng produced by another clone (Fib 4), both established from the same litter. The production of TTR by these 2 clones was also examined by immunohistochemistry using colloidal gold and Hep G2 cells as positive control; Fib 1 clone was unequivocally positively stained (data not shown), although the reaction was not as strong as in Hep G2 cells; Fib 2 clone, however, gave a negative reaction.

4. Synthesis of human TTR by tissue explants

In order to further verify protein synthesis by the human TTR mRNA containing tissues, we made explants from skin, retina, nerve and choroid plexus from three transgenic mice. The presence of the human TTR was analyzed both in media and tissue homogenates by ELISA, and total protein was quantified in media. It was observed that the only tissue producing and secreting significant amounts of TTR was the skin, about 20 ng/100 µg of total protein secreted into the media. The retina also secreted the human protein, but at lower levels, about 3.4 ng/100µg total protein. No protein could be detected on media or homogenates of choroid plexus and nerve. There were no cross reactions with the control tissue explants.

5. Human TTR plasma levels

Quantification of the human protein in the serum of different animals revealed that only the animals induced with $ZnSO_4$, one week prior to sera collection had detectable levels in the circulation. Human TTR serum levels were variable among animals as represented in the histogram of figure 6; although one of the animals had 1.5 mg TTR/dl, most of them had concentrations ranging from 0.2 to 0.5 mg TTR/dl.

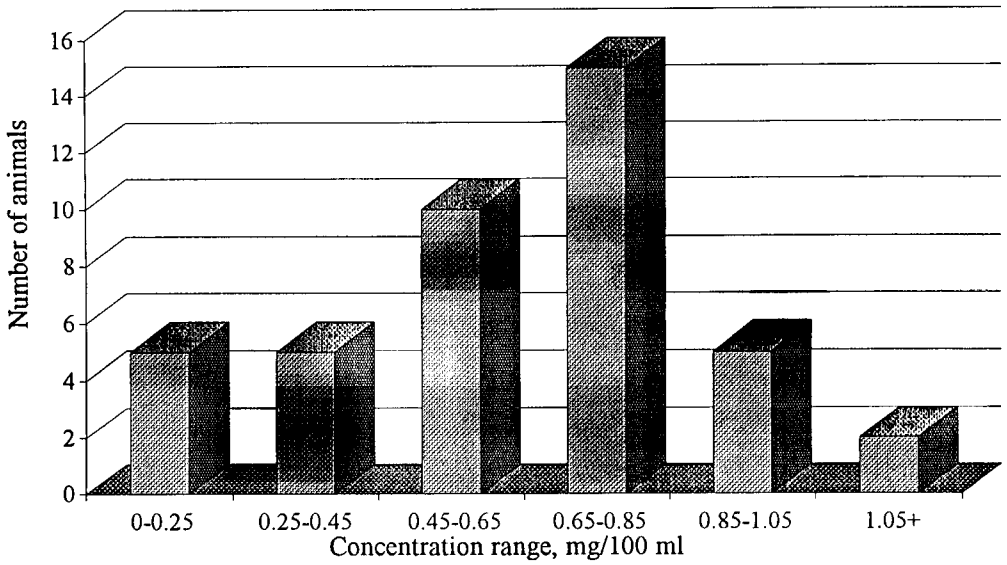


Figure 6 - Distribution of human TTR serum concentration in transgenic mice, as determined by ELISA.

Continuous $ZnSO_4$ induction, for periods up to one year, resulted in persistent higher concentrations of the human protein in the serum, as shown in figure 7 for two transgenic animals that had been exposed to zinc for one year. Some variation of human TTR concentration is observed within the same animal, but this type of variation is also observed for albumin as represented in the same figure.

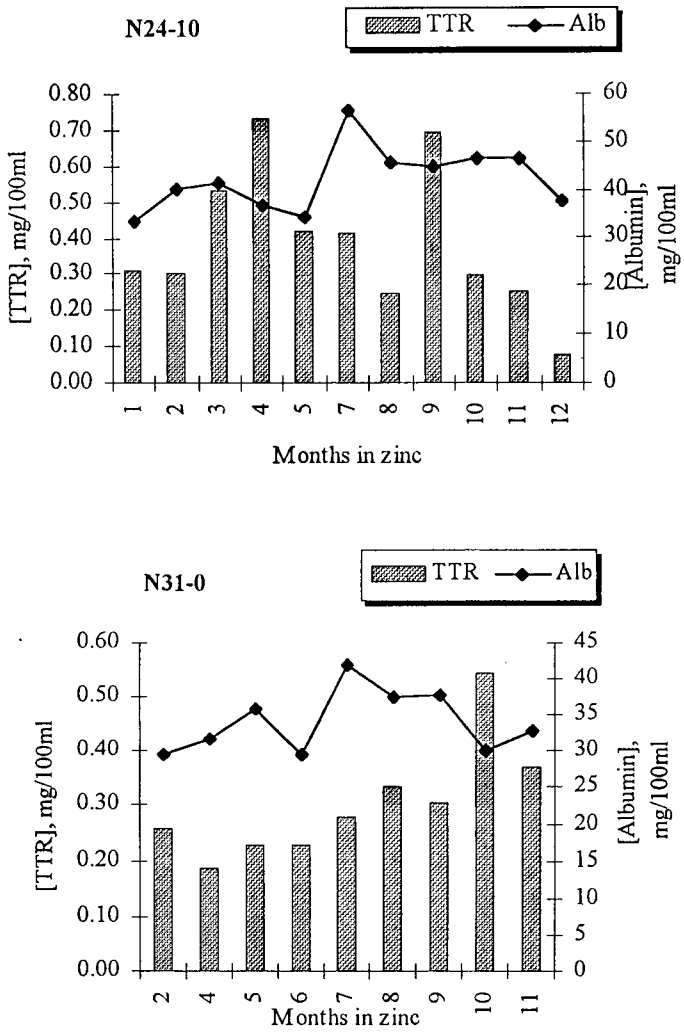


Figure 7 - Human TTR (TTR) and mouse albumin (Alb) serum levels in transgenic mice (N24-10 and N31-0) induced with zinc, as assessed by ELISA and RID respectively .

6. Amyloid search

Among the transgenic mice, five animals with higher human TTR serum concentrations were selected for amyloid search upon one year of zinc exposure, along with five parallel non transgenic animals. Three of the transgenic animals died before reaching the end of the experiment and the other two were sacrificed at the end of the experiment (N24-10 and N31-0 represented in figure 7). Samples of skin, muscle, intestine, stomach, kidneys, liver, testis/ovary, heart, nerve and brain were processed for histochemistry and amyloid was searched by Congo red staining. No amyloid deposits were identified on any of the tissues. Immunohistochemistry with either polyclonal or monoclonal antibodies for human TTR was also performed but always with no sustained positive results.

Discussion

Among the transgenic strains developed as animal models for FAP, MT1-TTR Met 30 and MT2-TTR Met 30 carry the structural gene for human TTR Met 30 under the control of the mouse metalothionein promoter (Wakasugi *et al.*, 1987, Sasaki *et al.*, 1986). Both were described to have the human variant in the circulation but only the MT2-TTR Met 30 strain had deposition of amyloid (Yi *et al.*, 1991).

One of the main differences among these two strains is the structure of the promoter constructs; in the MT2 mice, a fragment of 0.6 kb containing the MT promoter was ligated to the human TTR gene at about 148 nucleotides upstream the transcription start site (thus including the TTR promoter). In the MT1 mice the MT promoter is a fragment of 1.7 kb and was ligated to the TTR gene four nucleotides upstream the transcription start site (Sasaki *et al.*, 1989). The structural differences in the constructs could account for differences in the distribution of transgene expression between both strains, including the fact that, in the MT2 strain, the expression of the transgene is not dependent upon zinc induction; they should not, though, affect the deposition of the human protein as amyloid since this event appears to involve the circulating protein (Wakasugi *et al.*, 1987).

Since the human protein synthesized by the MT1-TTR Met 30 had not been thoroughly characterized, the existence of structural abnormalities was a possibility to explain the lack of amyloid deposition. However, our biochemical characterization of human TTR in this strain indicated the presence of a functional tetramer.

No hybrid tetramers between mouse and human TTR were detected; this is probably due to the stoichiometric ratio between high endogenous TTR versus low transgene expression levels in the liver of these animals, as a suppression of the transgene expression was reported in the liver and kidneys of these animals (Sasaki *et al.* 1989). Shimada *et al.* (1989) suggested that only human homotetramers would deposit as amyloid, and that hybrids mouse/human TTR would not participate in amyloid formation. The authors explained in this way the earlier deposition of amyloid in the MT2-TTR Met 30 mice in relation to another transgenic strain carrying the human structural gene under the control of the human promoter (0.6-hTTR Met 30) and that develops amyloid deposition at 15 months of age (Shimada et al., 1989). However another transgenic mouse strain (6-hMet 30) devoid of endogenous mouse TTR and producing human TTR Met 30 (carries the human structural gene with 6 kb of human TTR regulatory regions) has a latter onset of amyloid deposition than the transgenic strain with mouse endogenous TTR and the same transgene construct (6-hTTR Met 30), *i.e.* 11 *versus* 9 months of age (Yokoi *et al.*, 1996; Nagata *et al.*, 1995).

Human TTR levels in the MT1 strain, only measurable when the transgene was induced with zinc, were usually bellow 1 mg/dl, thus not as high as the ones described for the MT2 mice

(Shimada *et al.*, 1989). A concentration of 2 mg TTR Met 30/dl was reported to be the sufficient to lead to amyloid deposition (Maeda *et al.*, 1996), hence it is not possible to exclude the possibility that the human protein levels might be too low to promote amyloid deposition. However a transgenic strain producing the TTR Pro 55 variant, which is the mutation involved in the most aggressive form of FAP, and a transgenic strain producing the TTR Ser 84 variant were reported to have serum concentrations above 2 mg/dl but no TTR reactive amyloid deposits (Teng *et al.*, 1986; Waits *et al.*, 1996), therefore indicating that the absence of amyloid deposition might be related to other factors, other than the human TTR serum concentration.

The study of transgene expression in the MT1 mice revealed that the message for the human protein was present in retina and skin, in addition to the previously described tissues (Sasaki *et al.*, 1986). Protein expression studies demonstrated that TTR Met 30 was synthesized and secreted by fibroblasts, both in cell culture experiments and in the animals skin. This fact is particularly interesting, since it suggests a significative extracellular pool of TTR Met 30 protein, resulting from both cellular production and from the diffusion from the vascular compartment, at a concentration probably higher than if it resulted merely from serum TTR Met 30. This further emphasizes the peculiarity of the absence of amyloid deposition in these animals. One could hypothesize that the reason/s for this fact reside either in the presence of factors that prevent amyloid deposition or in the absence of factors necessary for the deposition of amyloid; in the different genetic backgrounds of the transgenic strains. There are other cases where the genotype influences the physiological behaviour of the animals in response to a stimulus, for example the development of AA amyloidosis; it is the case of A/J mice that require several months of inflammatory stimuli to develop AA amyloidosis (Wohlgethan and Cathcart, 1980) as opposed to the two weeks required by C57BL/6 or CBA/J mice strains.

In conclusion, the absence of amyloid deposition in the MT1-TTR Met 30 mice is probably due to the genotype of this transgenic strain, although it can not be excluded that the levels of human protein in the serum might also play a role.

From the transgenic lines producing and accumulating human TTR as amyloid (MT2-TTR Met 30, 0.6-hTTR Met 30, 6-hTTR Met 30 and 6-hMet 30), none of them shows amyloid deposition in the peripheral nervous system, and the animals do not develop neuropathy. The reasons for this are unknown and again it must be a characteristic metabolic and/or anatomical feature of the animal. These transgenic strains, although not good models for FAP, are nevertheless very usefull not only for the study of amyloidogenesis, but also as models for the development of therapeutic strategies, either by the inhibition of amyloid formation or by degradation of existent amyloid deposits. As for the development of a neuropathic model, one possible approach not yet tried, would be the production of the human protein in the nerve tissue, by, for example,

the implantation of genetically modified cells producing the human protein; another approach, although considerably more difficult to to perform, would be to use other species to produce transgenic animals. Mice have been the animal of choice to produce models of human genetic diseases, however one should not forget that the different species-specific genetic backgrounds can lead to variable phenotypes, such as the development of spontaneous inflammatory disease observed in transgenic rats carrying the human HLA-B27 an β2- microglobulin, but with no equivalent model generated, when those genes were introduced into the mouse genome (Hammer *et al.*, 1990).

Chapter 4: Genetically modified cells producing human TTR and TTR Met 30 - a potential tool for the study of amyloidogenesis

Aims

- Production of genetically modified cells secreting human TTR and TTR Met 30 by retroviral infection.
- Application of genetically modified cells, in cell culture and as animal grafts, for the study of amyloidogenesis.

Introduction

The process of fibrillogenesis in FAP, as well as in the other amyloid diseases, is highly complex, involving not only the amyloid protein precursors, but also chaperone proteins and other tissue components; in order to study this process it is necessary to have effective experimental models. Ideally, the choice would be to use animal models, that would mimic the human pathological phenotype. Several transgenic mice strains, expressing and synthesizing the human TTR Met 30 variant, have been developed (as described in the previous chapter), and although, in some strains, the human protein deposits as amyloid in several organs and tissues, there is no amyloid deposition in the peripheral nervous system, and the characteristic neuropathy of FAP is not observed. The reasons for this are largely unknown.

An alternative approach towards the study of intervening factors in amyloidogenesis, is to use cell culture systems, whereby genetically modified cells producing the mutant TTRs can be grown in contact with either various extracellular matrixes, or other circulating factors. Cellular models present some advantages over animal models: the technical ease of manipulation and control of exogenous factors, the versatility of factor testing, and the shorter periods of time necessary for the experiments. Limitations exist, as to the study of multiple tissue interactions, and physiological processes such as aging.

In order to develop a cell culture system for the study of FAP, it is necessary to design genetically modified cells secreting the human TTR, since the number of cell lines known to produce TTR is very limited. One widely used system for the introduction of exogenous genetic material into eukaryotic cells, *in culture* and *in vivo*, employs recombinant retroviruses, and is known as retroviral-mediated gene transfer. Recombinant retroviruses present several advantages over other

methods of gene transfer: the efficiency of gene delivery through infection is high, genes are stably integrated into chromosomal DNA and thereby are transmitted to daughter cells; and a large variety of cell types can be infected (Morgan *et al.*, 1993). The basis for this method is the use of defective retroviral vectors. These vectors contain the necessary sequences for packaging, transcription, integration into the host genome and expression of the gene of interest, but lack the information for essential viral proteins for replication and assembly of the viral particles; thus, upon infection, recombinant retrovirus cannot replicate. The recombinant viral stocks are produced by special cell lines, the packaging cell lines, that provide these functions *in trans*, as summarized in figure 1. These cell lines are generally derived from NIH 3T3 fibroblasts, and contain the sequences encoding the necessary proteins for the assembly and packaging of the recombinant vector into virus particles (Mann *et al.*, 1983; Markowitz *et al.*, 1988).

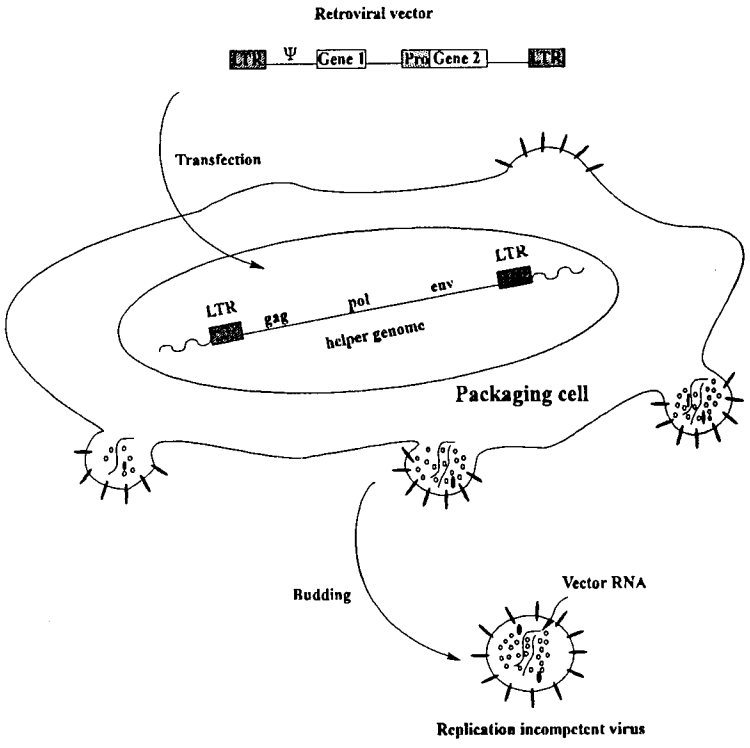


Figure 1 - Diagram of the production of recombinant retrovirus (adapted from Cepko, 1988). The retroviral vector containing the gene of interest (Gene 1) under the control of the virus promoter-enhancer element (LTR) and a selectable marker (Gene 2) under the control of a promoter (Pro), is transfected into a packaging cell line. After integration the vector directs the synthesis of viral RNA that is packaged into viral particles since it contains the sequence of packaging (Ψ). The viral proteins necessary for virus assembly (gag, pol env) are supplied by an helper genome, integrated in the cell genome, that lacks the Ψ region and cannot be packaged.

We have therefore introduced both the variant TTR Met 30 cDNA and the TTR cDNA into retroviral vectors under the control of the strong constitutive LTR promoter of the Moloney murine leukemia virus (MoMLV), and used these vectors to transfect packaging cell lines. The infectious particles obtained were used to infect rodent fibroblasts and hepatoma cells, which were then tested for the production of the human proteins. A cell culture system suitable for the study of amyloid formation was then designed with these genetically modified cells.

Material and Methods

Vector Construction

P4, a plasmid containing the human TTR Met 30 cDNA, was constructed by ligating the 240bp *Xba* I - *Sph* I fragment of pINTR5 (Furuya *et al.*, 1989) to the 3.3 kb *Xba* I-*Sph* I of pHPA27 (Sasaki *et al.*, 1984). The TTR and TTR Met 30 cDNAs were excised from pHPA27 and P4 plasmids, respectively, by digestion with *Pst* I and *Pvu* II. Both cDNAs were then digested with *Ava* II to remove the polyadenylation signal, blunt ended with T4 polymerase and ligated to 10 bp phosphorylated *Bam* HI polylinkers. These modified cDNAs, of approximately 470 bp, were then subcloned into the retroviral vector pLRNL (a kind gift from Dr. M. Rosenberg, Univ. California at San Diego), previously de-phosphorylated with calf intestine phosphatase. The resulting constructs were designated as pLTTR Met30 and pLTTR.

The host cells used for the transformations were *E. coli* DH5α (BRL). The enzymes and polylinkers used were from New England BioLabs.

Plasmid preparation and DNA isolation

Large and small scale plasmid preparations were carried out using the boiling method as described by Sambrock *et al.*, 1989. The isolated plasmids pLTTR Met30 and pLTTR, used for transfection, were further purified by cesium chloride gradient ultracentrifugation.

The DNA fragments excised from plasmids were isolated by low melting agarose gel electrophoresis (1% agarose gels) followed by phenol/chloroform extractions and ethanol precipitation. Isolated fragments were resuspended in 10 mM TrisCl, pH 8, 1 mM EDTA (TE).

Cell lines and culture

The mouse cell lines Y2 (Mann *et al.*, 1983) and NIH 3T3, and the rat cell line H56 (a kind gift from Dr. M. Weiss; Deschatrette *et al.*, 1974) were always maintained in Dulbbecco's modified Eagle's medium with low glucose, supplemented with 100 U/ml penicillin, 100 mg/ml streptomycin, and 10% fetal calf serum (DMEM10P/S), except when mentioned.

Primary fibroblast cultures were established from the dermis of newborn Balb C mice as described in chapter 3.

All culture reagents were obtained from GIBCO.

Transfection of packaging cell lines

Ten micrograms of purified vector DNA were transfected into the ecotropic Ψ2 packaging cell line by the calcium phosphate coprecipitation method (Sambrock *et al.*, 1989). Twenty-four hours later, cells were split 1:20 in medium containing 1 mg/ml of the neomycin analog G418 (GIBCO), and the stably transformed clones were isolated with cloning cylinders (Bellco Glass Inc.) and expanded in mass cultures. Viral stocks (designed LTTR and LMet 30) were obtained by adding 2 ml fresh medium without G418, to a near-confluent monolayer of each producer clone, and by harvesting twenty-four hours later. The media were filtered through a 0.45 μm filter (Schleicher & Schuell) and viral stocks stored at -80°C until further use.

Retroviral infection of cell lines

For infection, approximately $5x10^5$ cells were incubated with 2 ml of viral stocks in the presence of 8 μg/ml Polybrene (Sigma) for twenty-four hours. From that point on, cultures were usually grown in the presence of G418. Viral titers were determined by infecting $5x10^5$ NIH 3T3 cells with dilutions (1:2, 1:4, 1:20, 1:200 and 1:2000) of cell-free conditioned medium from the producer clones in the presence of 8 μg/ml Polybrene (Sigma), passaging cells twenty-four hours later at a 1:5 ratio under G418 selection and scoring surviving colonies 2 weeks later.

The determination of H56 and NIH 3T3 sensitivity to G418 was performed by plating 10^5 cells in six 75 cm^2 flasks and growing cells in DMEM10P/S supplemented with each of the following G418 concentrations: 0.1, 0.25, 0.5, 0.75 and 1 mg/ml. Cell survival was scored after 6 days of culture.

DNA and RNA isolations

DNA was isolated from confluent monolayers of cells following standard procedures (Sambrock *et al.*, 1989). Briefly, cells were resuspended in 6 M guanidine, 0.1 M sodium acetate pH 5.5, incubated 1 h at 37°C, and added 200 μg proteinase K. After an incubation of 1 h at 50 °C, phenol/chloroform extractions were carried out and DNA was precipitated with ethanol.

Total RNA was isolated as described in chapter 3.

Reverse transcription and PCR

Twenty-four micrograms total RNA was reversed transcribed in a 50 μl, total volume, reaction containing 10 μg bovine serum albumin (BSA), 20 units RNAsin (Amersham), 1.6 μg Oligo(dT) (Pharmacia), reverse transcriptase buffer, and reverse transcriptase (BRL). After 1 h at 37 °C,

the reaction was stopped by adding 1/5 vol 0.25 M EDTA and heating at 65°C for 10 min. After phenol/chloroform extraction and ethanol precipitation the cDNA was resuspended in 20 µl TE and amplified by PCR as described in chapter 3, using the primers HCA and HCB. Samples were then analyzed by electrophoresis in 1% agarose gels. RCA and RCB primers were also used when amplifying H56 complementary DNAs.

Metabolic labeling and immunoprecipitation

Cells were plated at a density of about $8x10^6$ cells per 75 cm^2 flask for H56, and $4x10^6$ cells per 75 cm^2 flask, for NIH 3T3. Twenty-four hours later cells were metabolic labeled with 300 µCi (^{35}S)-methionine, 1,000 Ci/mmol (Amersham), and immunoprecipitation carried out essentially as described in chapter 3, with the exception that the samples were subdivided in three aliquots and one aliquot was incubated with rabbit serum (non-immune), another with anti-human TTR (immune), and the other with anti-human TTR plus 2 µg cold TTR (competition).

Chromatography

DEAE cellulose chromatography was performed by incubating 150 mls of conditioned cell media with DEAE cellulose, equilibrated in 50 mM glycine acetate buffer pH 7, for 2 hours at room temperature. The resin was then washed in batch with 700 mls glycine buffer, poured into a column and proteins were eluted with 1 M NaCl in glycine buffer. Fractions containing the human TTR were identified by ELISA, and were dialyzed and lyophilized.

Gel filtration by HPLC was performed as described in chapter 3

Cell culture assays

The determination of the human protein produced by the infected cell clones was performed in 100 µl medium collected in the following manner: one million cells were grown in monolayer in 75 cm^2 flasks, and fed 2 ml fresh media; twenty four hours later, medium was removed and filtered through 0.45 µm membranes (Schleicher & Schuell) and cells counted.

The effect of dexamethasone was tested on TTR production by seeding 10^5 cells in six-well plates, and growing cells in DMEMP/S with (DMEMP/Sdex) or without 50 nM dexamethasone (Sigma). Samples of 100 µl were collected everyday from each well and replaced with same volume of fresh medium. At the end of the experiment all medium was removed from wells. TTR was quantified by ELISA.

ELISA

Sandwich ELISA was carried out essentially as described in chapter 3. In some assays instead of peroxidase labeled anti-human TTR, a biotinylated anti-human TTR available in the laboratory

was used; in this case, an incubation with biotinylated peroxidase streptoavidin complexes (Amersham) was further carried out for 30 min at room temperature.

Cell culture systems for amyloid production

Protein accumulation experiment

About 10^5 cells were plated on the upper wells of six-well collagen coated Transwell-Col plates (Costar) and grown in DMEMP/Sdex, with 1 ml in the upper well and 1.5 ml in the bottom well. The amount of secreted human protein was determined by ELISA on samples of 100 µl. The presence of amyloid was assessed by thioflavine T fluorometry of media, and Congo red staining of cells and cellular debris.

Protein supplementation experiments

Six-well Transwell-Col plates were plated in duplicate about 10^5 cells of each H56, H56 Met 21-3 and H56 TTR 1 cells. Twenty-four hours later medium was replaced by fresh DMEMP/Sdex, with 1 ml in upper wells and 1.5 ml in bottom wells. Recombinant TTR/TTR Met 30, to a final concentration of 0.1 mg/ml, was added to half of the samples (both uninfected and infected cells). Cells were grown until detaching, at which time upper and bottom media were collected, cells and cellular debris were pelleted from upper well media by centrifugation (in microfuge) and put in a slide for Congo red staining. The supernatants and bottom well media were used for fluorometry.

Coating experiments

These experiments were carried out in Lab®-Tek two-chamber slides, (Nunc); for each coating experiment two slides were prepared, one with H56 and H56 TTR cells and the other with H56 and H56 Met 30 cells. About 10^4 cells were seeded in each chamber; cells were grown in DMEMP/Sdex. The slides were coated with the following matrixes:

a) 30 µl of a 1:10 dilution of thyroid amyloid fibril suspension (same preparation described in chapter 1) were put in the chambers of 2 slides, and spread with the pipette tip. The chambers were kept overnight under UV light for sterilization, and the other day cells were plated.

b) 300 µl of the above suspension added 700 µl formic acid (final concentration 70%), incubated overnight at room temperature and extensively dialyzed with PBS; this suspension was used to coat 2 chamber slides as described above (100 µl for each chamber) and in the same way H56, H56 TTR and H56 Met 30 cells plated.

c) Suspensions of heart and thyroid material, obtained by submitting heart tissue and thyroid tissue from individuals without amyloidosis to the amyloid fibril extraction procedure, were also used to coat slides as described in a).

d) The saline washes resulting from the vitrectomy of an FAP individual with amyloid deposition in the vitreous; 100 µl of a 1:20 dilution of this suspension was used to coat each cell chamber as described in a).

At the end of a six day incubation without medium exchange, media were used for fluorometry and cells fixed and used for Congo red staining.

Fluorometric assays

Fluorometry assays were performed as described in chapter 1 with 3 µM thioflavine T, in 1 ml conditioned medium, in place of the glycine buffer.

Implantation experiments

The cellular monolayers (about 10^4 - 10^5 cells) used for the implantation experiments were incubated with trypsin/EDTA, dispensed in media and pelleted by centrifugation for 5 min at 1000 *g*. Cells were resuspended in 100 µl PBS and maintained at 4 °C until injection.

Wistar rats were anesthetized with an intraperitoneal injection of sodium pentobarbital. The surgical manipulations were carried out under the dissecting scope. The injections were made with a 10 µl Hamilton syringe with a blunt 30 gauge needle. A cut was made on the sclera with a scalpel, and the needle introduced in the cut. The cell suspension was injected into the posterior chamber, and the cut stitched with surgical thread.

Tissue and cell fixation

Rats were sacrificed using Fluothane (Zeneca) and cervical dislocation. The eyes were fixed in buffered formalin and embedded in paraffin.

Cells for immunohistochemistry and Congo red staining were fixed as described for primary fibroblasts in chapter 3.

Histochemistry

Congo red staining was performed on cell monolayers by incubating fixed cells in 0.5% Congo red in 50% ethanol, followed by Meyer's hematoxylin counterstaining and observation under polarized light.

Immunohistochemistry was carried as described in chapter 3.

Results

1 . Generation of genetically modified cells producing human TTR

1.1. Construction of recombinant retroviruses

We have introduced the full length cDNAs for TTR and TTR Met 30, into the parental plasmid pLRNL, producing two recombinant plasmids, respectively pLTTR and pLTTR Met 30 represented in figure 2.

The human cDNAs are transcribed under the control of Moloney Murine Leukemia Virus (MoMLV) promoter-enhancer element contained in the long terminal repeat sequences (LTR). These recombinant vectors contain in addition the sequences required for packaging and integration originating from MoMLV (Ψ), the neomycin-resistance (neo^R) gene from the transposon Tn5, driven by the Rous sarcoma virus promoter, the colE1 bacterial origin of replication and the bacterial ampicillin resistance gene (Dr. Michael B. Rosenberg, personal communication).

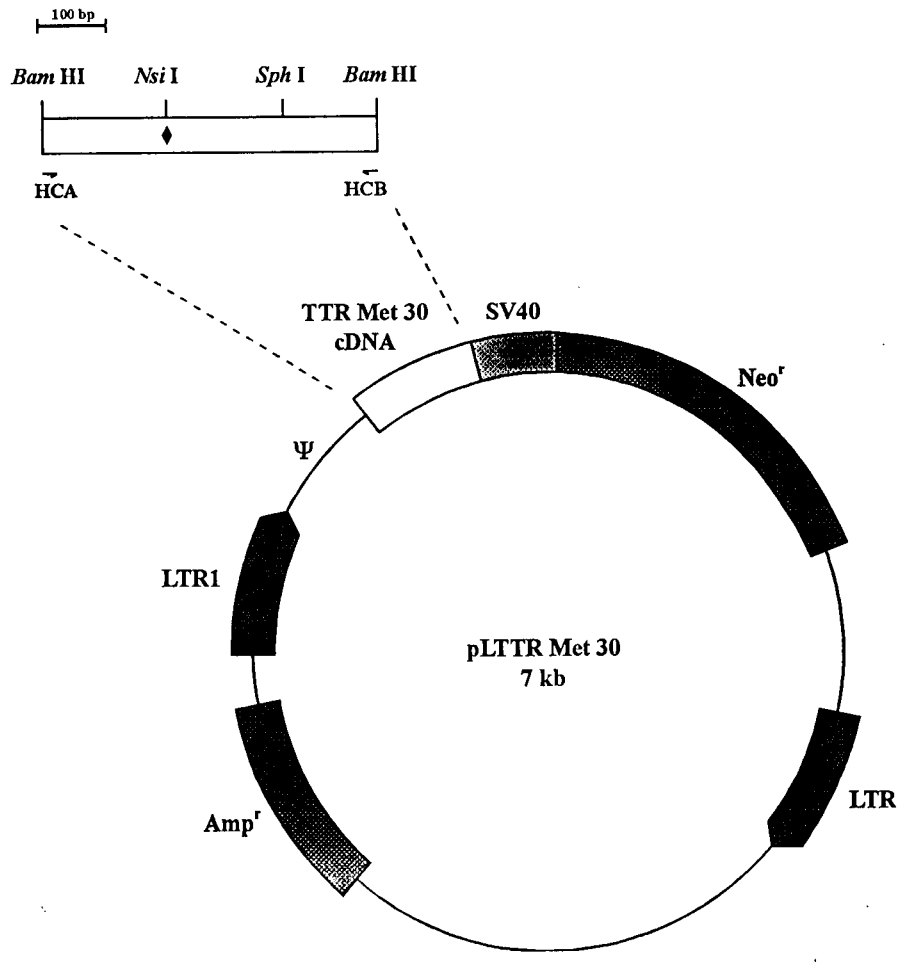


Figure 2 - Diagram of the structure of the retroviral vector pLTTR Met 30 and partial restriction map of TTR Met 30 cDNA. LTR1 - long terminal repeat from MoMLV; Ψ - packaging sequence from MoMLV; TTR Met 30 cDNA - human mutant transthyretin cDNA; RSV - Rous sarcoma virus promoter; Neo^R - neomycin resistance gene; LTR2 - long terminal repeat from Moloney murine sarcoma virus; Amp^R - ampicillin resistance gene. The diamond represents the codon for Met 30. pLTTR is identical, with the exception that it contains the human cDNA for normal TTR, and therefore has no restriction site for *Nsi* I.

The recombinant pLTTR and pLTTR Met 30 were then transfected into the producer cell line Ψ2, which is an ecotropic packaging cell line, *i.e.* the retrovirus particles produced are able to infect only murine cells. Sixteen stable transformant clones of Ψ2TTR were isolated as well as fourteen clones of Ψ2Met30.

In order to determine the integrity of the cDNAs for TTR and TTR Met 30 in the cellular genomes, DNA from some of the clones was isolated and amplified by PCR. The amplified DNA appeared as a 490 bp band in both Ψ2TTR and Ψ2Met 30 clones (figure 3A), which is the expected size for the human TTR cDNA. To confirm that no deletions or other rearrangements had occurred, the amplified DNA was then digested with the restriction enzymes *Sph* I and *Nsi* I. Since *Sph* I has one restriction site in the normal TTR and TTR Met30 cDNAs, and *Nsi* I cuts specifically in the Met 30 codon, the Ψ2TTR clones should present 2 bands of 350 and 140 bp respectively, and the Ψ2Met 30 clones 3 bands of 180, 170 and 140 bp; this was the pattern obtained as can be observed in figure 3B.

As the producer clones appeared to have the human cDNAs correctly integrated into the cellular genomes, we next analyzed them for viral titers by infecting NIH 3T3 cells and scoring colonies surviving to G418 selection. We obtained viral titers ranging from 10^3 to 10^5 cfu/ml, which are similar to the ones described using the same vector (Ezzedine *et al.*, 1991) corroborating the observation that viral titers are determined by the vector (Gilboa *et al.*, 1986).

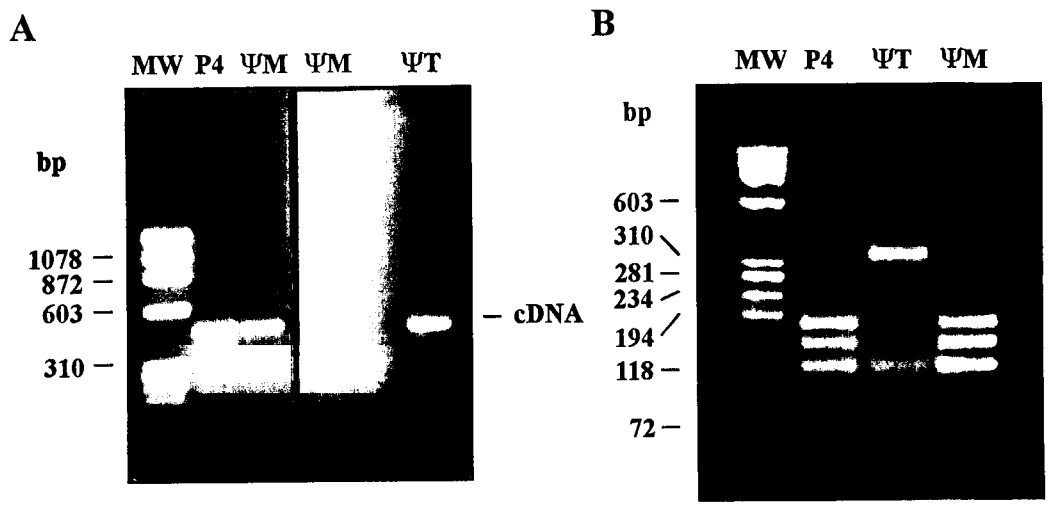


Figure 3 - Analysis of isolated DNA from producer clones. **Panel A:** Two percent agarose gel of amplified DNA; **Panel B:** Restriction analysis of amplified DNA in a 4% Nusieve agarose gel; all samples digested with *Sph* I and *Nsi* I. MW - DNA of phage ϕX174 digested with *Hae* III P4 - plasmid P4 as positive control; ΨT - DNA isolated from Ψ2TTR cells; ΨM - DNA isolated from Ψ2Met 30 clones.

1.2. TTR expression by infected cells

1.2.1. Mouse NIH3T3 fibroblasts

We next investigated whether infected NIH 3T3 cells, with either LTTR or LMet 30 virus, secreted the TTR proteins into the media. Infected clones, grown under G418 selection, were metabolic labeled with ^{35}S-methionine, conditioned media and cell lysates immunoprecipitated with anti-human TTR antibodies and proteins were resolved in a SDS-PAGE gel together with iodinated isolated human TTR as a control. Under conditions where TTR migrates as a single monomeric band of about 14,300 Da, no corresponding TTR band was observed in the conditioned media; in the cell lysates it was not possible to distinguish the presence or absence of the TTR monomer due to a high level of cross reactions with other proteins. ELISA of conditioned media from infected cells was also negative for the human TTR. The lack of protein expression is surprising, since the infected NIH 3T3 cells survived G418 selection, demonstrating that the neo^R gene was expressed.

To verify whether the human DNA was transcribed into mRNA, we isolated total RNA from infected as well as uninfected NIH 3T3 cells, treated it with DNase, reverse transcribed it into cDNA and performed PCR with specific primers for the human TTR mRNA.

As it is evident from figure 4, in uninfected NIH 3T3 cells no human TTR cDNA was detected, but in most of the NIH 3T3 clones, infected either with LTTR or LMet 30, the 490 bp fragment corresponding to TTR cDNA was observed. Thus the construct is being transcribed, and either the cells lack factors for the proper synthesis of the protein, or the protein is being produced in very low amounts, below the detection limit of the immunoassays used.

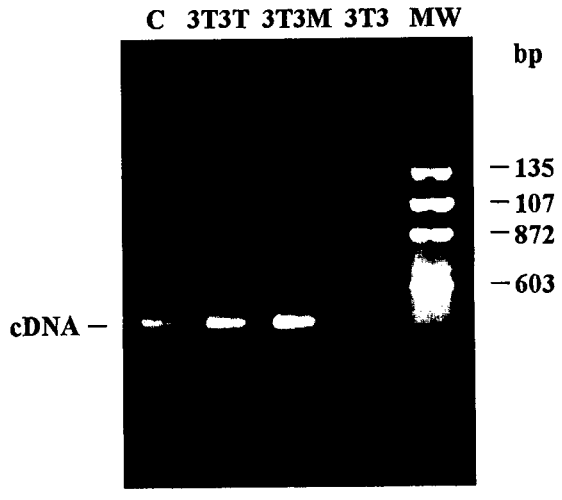


Figure 4 - Analysis of amplified cDNA from infected and uninfected NIH 3T3 cells in a 1% agarose gel. C - control TTR cDNA; 3T3T - cDNA from 3T3TTR cells; 3T3M - cDNA from 3T3Met 30 cells; 3T3 - cDNA from uninfected NIH 3T3 cells; MW - DNA from phage ϕX174 digested with Hae III.

We decided then to test a cell line established from an organ known to produce TTR *in vivo*.

1.2.2. Rat H56 hepatomas

The liver is one of the major locations of TTR synthesis *in vivo,* and therefore we chosed the rat hepatoma H56 cell line, which is a "dedifferentiated" line, meaning that many liver-specific genes are not expressed (Deschatrette *et al.*, 1979).

The sensitivity of these cells to different concentrations (0.1, 0.25, 0.5, 0.75 and 1 mg/ml) of the antibiotic G418 was determined in comparison to the NIH 3T3 cells; and after six days of incubation in the antibiotic it was verified that no cells survived 1 mg/ml of G418; at 0.75 mg/ml some NIH 3T3 and H56 cells were still alive. H56 cells can, therefore, be selected at an antibiotic concentration of 1 mg/ml.

The H56 cells were then infected with the available LTTR and LMet 30 viral stocks, and grown under G418 selection. Total RNA was isolated from some H56 Met 30 and H56 TTR clones, as well as from uninfected cells, reverse transcribed and amplified. Similarly to the NIH 3T3 cells, the infected H56 cells, but not the uninfected cells, presented the 490 bp TTR cDNA band (data not shown). When performing PCR on the uninfected cells cDNA, with rat TTR primers, we obtained no amplification, thus indicating that the H56 cells do not synthesize rat TTR. Cross reactions (mouse/human) are, therefore, avoided; moreover, no formation of TTR hybrid species will occur in the infected cells. The production of the protein was assessed in H56 TTR (clones 17 and 18) and H56 Met 30 (clones 8 and 9) by metabolic labeling, immunoprecipitation and SDS-PAGE analysis, as described for the NIH 3T3 cells. H56 uninfected cells were used as a negative control. As exemplified in figure 5A with the H56 Met 9 clone (clones derived from LMet 30 are referred to as H56 Met plus the number of the clone), in the conditioned media incubated with anti-human TTR (lane I) a weak band migrating in the position of the TTR monomer is visible. This band is not detected when the medium was incubated with rabbit serum (lane N) and is diminished in the presence of an excess of cold TTR (lane C), thus indicating the presence of the human TTR monomer. This band is not the product of cross-reactions of the antibody with endogenous proteins since no signal was detected in the conditioned media from uninfected cells (figure 5B). In the cell lysates, both from uninfected cells as well as from infected clones, a large number of proteins is present, not being possible to discriminate the TTR monomer in the infected clones.

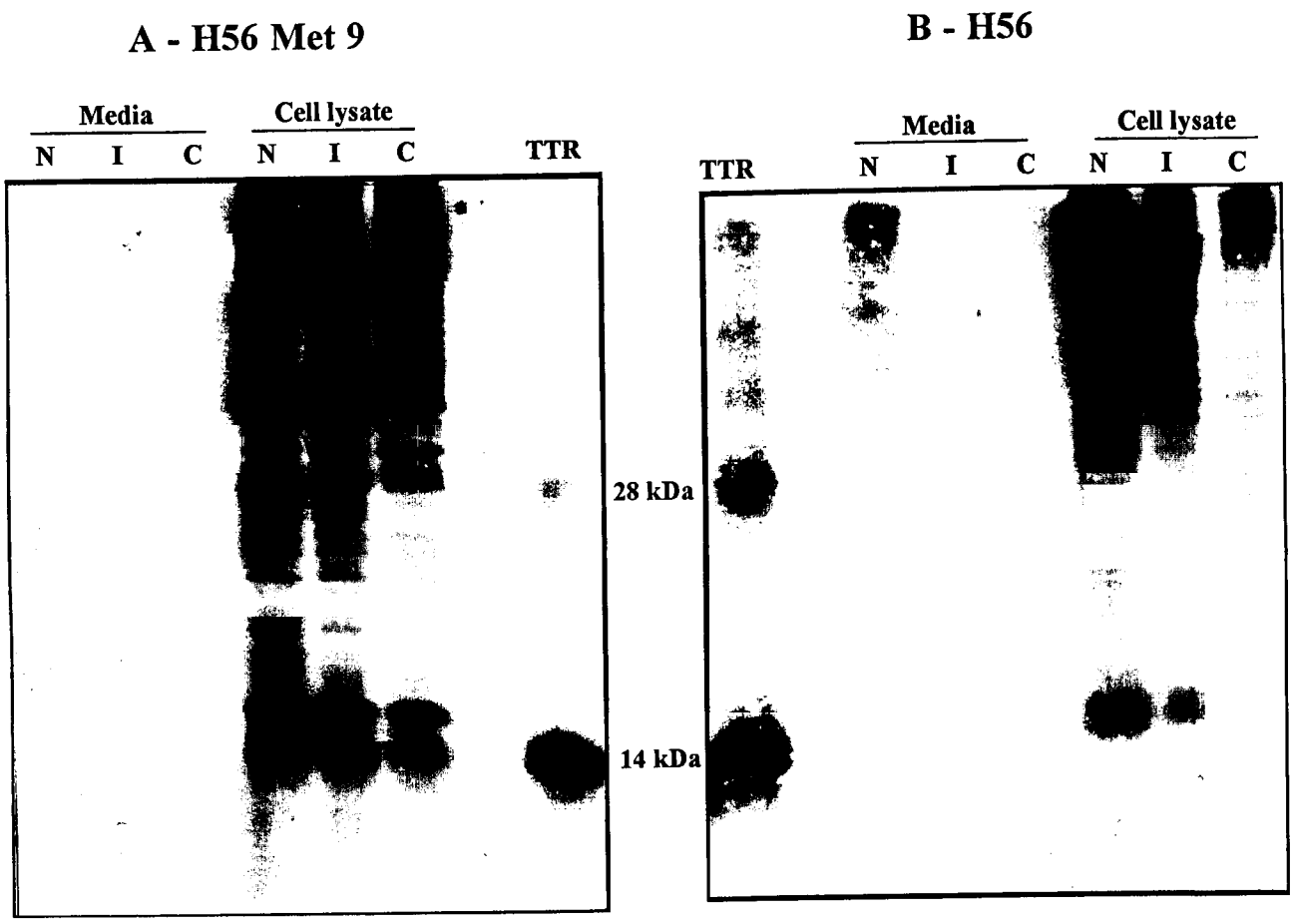


Figure 5 - Secretion of human TTR by H56 Met 8 cells. SDS-PAGE analysis of immunoprecipitated proteins from conditioned media and cell lysates. **Panel A:** H56 Met 8 cells. **Panel B:** Uninfected H56 cells. N - precipitation with normal non-immune rabbit serum, I - precipitation with anti-human TTR; C - precipitation with anti-human TTR in the presence of excess cold TTR; hTTR - iodinated human TTR; rTTR - iodinated rat TTR.

The amounts of protein secreted into the media by infected cells were determined by ELISA. Both conditioned media from uninfected H56 cells and DMEM10P/S media were used as controls, and no cross reactions were ever observed with these samples. In table 1 are represented TTR concentration values obtained for infected clones. As can be observed, the amounts of secreted TTR were usually low, particularly for the Met 30 clones. With some clones, several independent determinations were performed and some variations in the protein secreted was noticed, however the differences were not significant.

Table 1

Infected H56 clones	TTR secreted into media, ng/10^6 cells/24 h
TTR1	20
TTR3	78
TTR4	37.5
TTR6	53
TTR10	18.4
TTR12	46
TTR17	72
TTR18	60.7
TTR19	5.2
Met1	19.2
Met7	27.2
Met8	45
Met9	10
Met20	3.2
Met21-3	29
Met22	28
Met22D4	31.2

Since dexamethasone is described to increase synthesis of proteins expressed under the control of LTRs, we have studied whether treatment of our cultures with 50 nM dexamethasone would increase the production of TTR by infected cells. H56 Met 21-3 cells were, therefore, seeded in six-well plates with 2 mls media supplemented with dexamethasone. Samples of media were taken at day 2, 3 and 4 after which cells died. As observed in figure 6, dexamethasone does increases the amounts of TTR produced, albeit not at high levels.

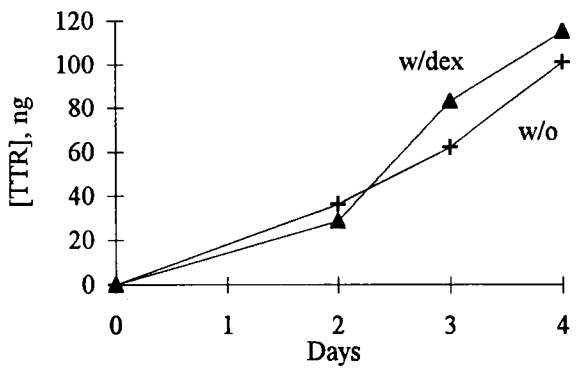


Figure 6 - Effect of dexamethasone on TTR secretion by H56 Met 21-3 cells. w/dex - medium supplemented with 50 nM dexamethasone; w/o - DMEMP/S media without supplementation.

Regardless the low increase in TTR production, we chose to supplement culture media with dexamethasone in subsequent amyloidogenesis experiments, where maximum production of protein is desirable.

Immunohistochemistry using colloidal gold was performed on the higher producing clone of H56 TTR and H56 Met 30 and in uninfected H56 cells; Hep G2 cells were used as positive control. No positive staining was found in any of the infected cell clones, in opposition of the positive reaction observed with the Hep G2 cells. Further experiments using alkaline phosphatase as detection method have corroborated this result. It is possible that TTR is very rapidly secreted by the cells and thus the intracellular amounts of protein are below the detection sensitivity of the techniques used.

A pool of H56 Met 21-3 conditioned media (about 150 ml) was partially purified by DEAE cellulose chromatography. Fractions containing the human TTR were identified by ELISA, pooled, dialyzed and lyophilized. After resuspension in 200 µl ddH_2O, this material was submited to HPLC preparative gel filtration. A calibration curve previously performed indicated that albumin (M.W. 64,000 Da) had a retention time of 16 min and lysozyme (M.W. 14,300 Da) had a retention time of 20-21 min. TTR eluted at 17 min, as assessed by ELISA on the collected fractions. Thus the human protein is secreted by the infected cells as a tetramer.

These studies showed that infected hepatoma cells were able to synthesize and secrete the human proteins, making the TTR retroviral expression system useful for the creation of genetically modified mammalian cells expressing mutant TTR proteins, that could be used to develop cell culture models for the study of amyloidogenesis.

1.2. 3. Mouse primary fibroblasts

We further aimed at establishing whether primary cell cultures were also capable to be infected and to synthesize the human protein. Primary cultures of mouse dermal fibroblasts were prepared and about 10^6 cells were infected with two LMet 30 viral stocks. Fourty eight hours after infection, cells were fed fresh media and twenty-four hours later media was removed, and TTR quantified in the media by ELISA. In one of the clones 72 ng of TTR Met 30 were secreted, indicating that these cells are also suitable for infection with the retroviral vector system.

1.3. TTR expression by HepG2 cells

We had available in the laboratory a human hepatoma cell line described as not synthesizing TTR (Knowles *et al.*, 1980). However, the presence of the TTR message in these cells had been observed (Dr. W. Blaner personal communication). Therefore we searched for the expression of TTR by this cell line by metabolic labeling and immunoprecipitation, the same way as described for infected NIH 3T3 and H56 cells. The results of this experiment are shown in figure 7.

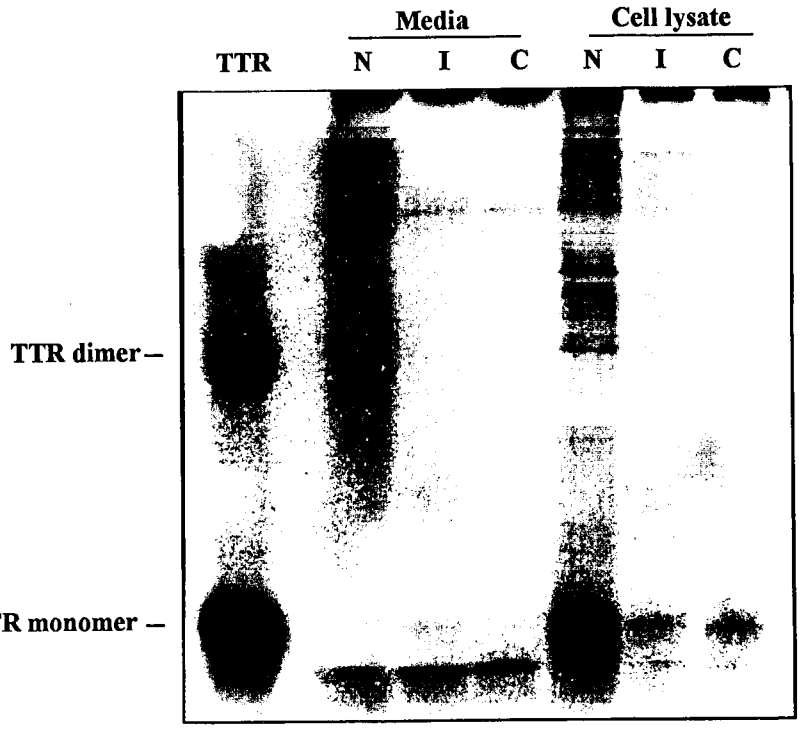


Figure 7 - Secretion of TTR by Hep G2 30 cells. SDS-PAGE analysis of immunoprecipitated proteins from conditioned media and cell lysates. N - precipitation with normal non-immune rabbit serum, I - precipitation with anti-human TTR; C - precipitation with anti-human TTR in the presence of excess cold TTR; TTR - iodinated human TTR as control.

The presence of the TTR monomer in the conditioned media precipitated with anti-human TTR is evident (lane C). A band at a lower molecular weight is also observed in the conditioned media (lanes N, I and C), but, since it occurs in all lanes with the same intensity it is probably a highly secreted product of the cells. Similarly to the observed with the H56 cells, in the cell lysates it is not possible to distinguish TTR due to the high background. The protein secreted into the media was further quantified by specific radioimmunoassay and shown to be approximately 1 $\mu g/10^6$ cells in 24 hours (Blaner *et al.*, 1991).

This cell line can therefore be used not only to study TTR secretory pathways and interactions with retinol-binding protein (as described by Bellovino *et al.*, 1996), but also for the study of amyloidogenesis, although very limited by the fact that it secretes only normal TTR.

Having obtained cell lines expressing and secreting the human TTR proteins, both the normal and the Met 30 variant, we then aimed at the development of a cell culture model for the study of amyloidogenesis.

2. Cell culture models for the study of amyloidogenesis

In order to develop a cell culture system to study amyloidogenesis, both TTR and the Met 30 variant should be used since they are both able to form amyloid in human pathological conditions (SSA and FAP) and in *in vitro* assays as previously described (chapters 1 and 2). Therefore in all the experiments described below we used clones H56 TTR12 and H56 Met 21-3, as well as uninfected H56 cells as control.

2.1. Accumulation and aging experiments

To study whether it was possible to obtain amyloid formation from either TTR or TTR Met 30 by aging, through cell mediated events, we chosed to test a transwell system, represented in figure 8. In this system, cells can be grown on the microporous membrane (0.4 μm) that physically separates the upper from the bottom compartment.

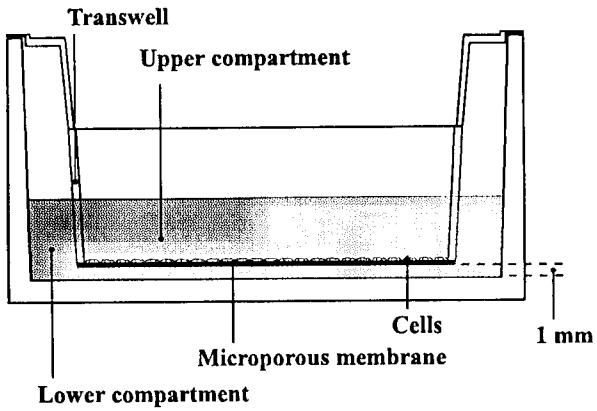


Figure 8 - Transwells culture system.

We reasoned that: a) since the survival of our cells is limited by surface size, the time period of culture might not be enough for protein aging (which could or could not lead to amyloid formation); therefore replacing the upper compartment with new cells would allow further aging of protein in the lower compartment; b) the lower compartment media is free of cellular debris, resulting from cell death, that could somehow interfere with the assays for amyloid assessment. The transwells used were collagen coated because in these transwells the membranes are translucent and thus, it is possible to examine the cells under the microscope.

We started by testing if there was preferential accumulation of secreted TTR in any of the compartments; for that purpose, six-well transwell plates were used. About one hundred thousand H56 Met 21-3 cells and H56 cells were seeded in four upper wells of a transwell. Samples of both upper and bottom wells media were collected on days 3, 4 and 5, at which time cells detached

and died. As observed in figure 9, the TTR Met 30 protein is evenly distributed in the bottom and upper wells, although a higher accumulation tends to occur in the lower well; these results demonstrate, in addition, that the rate of protein synthesis exceeds the uptake and degradation of the protein by the cells during the time period of the experiment. As for the conditioned media of H56 cells, they were negative for TTR, as expected.

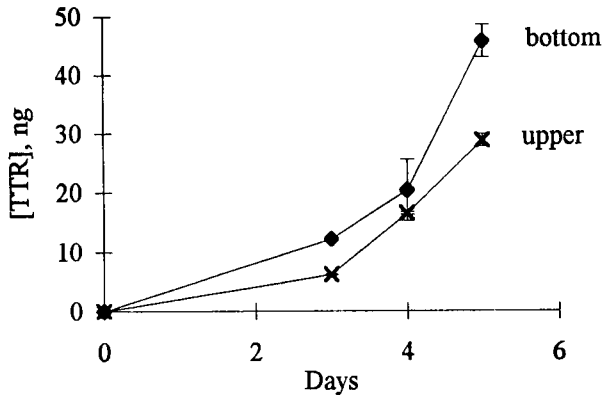


Figure 9 - Time course accumulation of secreted TTR Met 30 by H56 Met 21-3 in transwell plate; upper - upper well; bottom - bottom well. Samples of media were collected every day and were quantified by ELISA.

To determine whether the aging of the protein in the media during this period of time (basically a three day period) led to amyloid formation, the upper and bottom well conditioned media of both H56 and H56 Met 21-3 cells, were assayed with thioflavine T fluorometry. To the resulting spectra were subtracted the spectra of unconditioned media. Under these conditions no differences were observed in the thioflavine T spectra of the samples. It is possible that the time period of the experiment was not long enough; a longer period might be necessary for protein aging and amyloid formation; likewise, it is also possible that a critical protein concentration, higher than the one achieved, is needed to trigger amyloid formation. In order to test the first hypothesis we plated both H56 and H56 Met 21-3 cells, in duplicate, in transwells with DMEM10P/Sdex medium (1.5 ml in the upper compartment and 2 ml in the bottom compartment); eight days later cells detached, and the upper compartments were replaced with new ones containing freshly plated H56 and H56 Met 21-3 cells and 1 ml fresh media. 5 days later cells from the upper compartment and media from both compartments were collected; the media were used for protein quantification by ELISA and for fluorometry. The cells and cellular debris were stained with Congo red and observed under polarized light. About 400 ng TTR Met 30 accumulated at the end of the experiment, *i.e.* 13 days; however, no amyloid was detected with any of the methods used.

To establish if the protein concentration is not high enough for amyloid formation, we raised the concentration of human TTR in media of H56 TTR 12 and H56 Met 21-3 cells by adding recombinant isolated TTR and TTR Met 30, respectively, to a final concentration of 0.1 mg/ml. After an incubation of 8 days cells detached, and both upper and bottom wells media were assayed by fluorometry, as well as the H56 media controls. Again no differences were observed in the thioflavine T spectra of the samples *versus* the controls. Therefore, other factors, besides protein concentration and protein aging, are determinant for amyloid formation. Since amyloid fibrils are found *in vivo* associated with particular tissues and organs, we decided next to grow cells in contact with particular matrixes.

2.2. Coating experiments

An assay was designed towards the investigation of the induction of amyloid formation by specific matrixes: 10^4 infected and uninfected cells are grown in separate chambers of Lab-Tek cell chambers slides, in the presence of the factors to test, and at the end of the experiment amyloid was searched for, in the conditioned media by fluorometry, and in cell monolayers by Congo red staining and polarization microscopy.

Using this assay, and H56, H56 TTR and H56 Met 30 cells in DMEMP/Sdex, we have tested the following conditions:

- chambers coated with amyloid fibrils isolated from an FAP thyroid.
- chambers coated with formic acid treated thyroid amyloid fibrils.
- chambers coated with a sediment from a normal heart (extracted following the protocol of amyloid fibril isolation).
- chambers coated with a sediment from a normal thyroid (extracted following the protocol of amyloid fibril isolation).
- chambers coated with amyloid containing human vitreous.

Under polarized light the Congo red stained cells did not show any apple green birefringence, with the exception of the cells growing in contact with the thyroid amyloid fibrils and vitreous amyloid fibrils; however, only very tiny patches of birefringence were observed, also seen in the negative control with H56 cells, and therefore we adscribed it to the coated fibrils. Using 3 μM thioflavine T, samples were assayed by fluorometry, but in all cases the spectra overlapped with the negative control (unconditioned medium).

In summary, none of the experimental conditions tested in cell culture, *i.e.* protein aging, concentration, and the influence of some matrixes, triggered amyloid formation from either TTR or TTR Met 30; however, many other factors need to be tested using the above described system.

3. *In vivo* experiments

Another application of cells producing the mutant TTR is the possibility to perform implants in particular tissues or organs in animals, and to study the production and interactions of the secreted protein with tissue factors. We have performed preliminary experiments with this goal, and in figure 10 is represented the overall scheme followed.

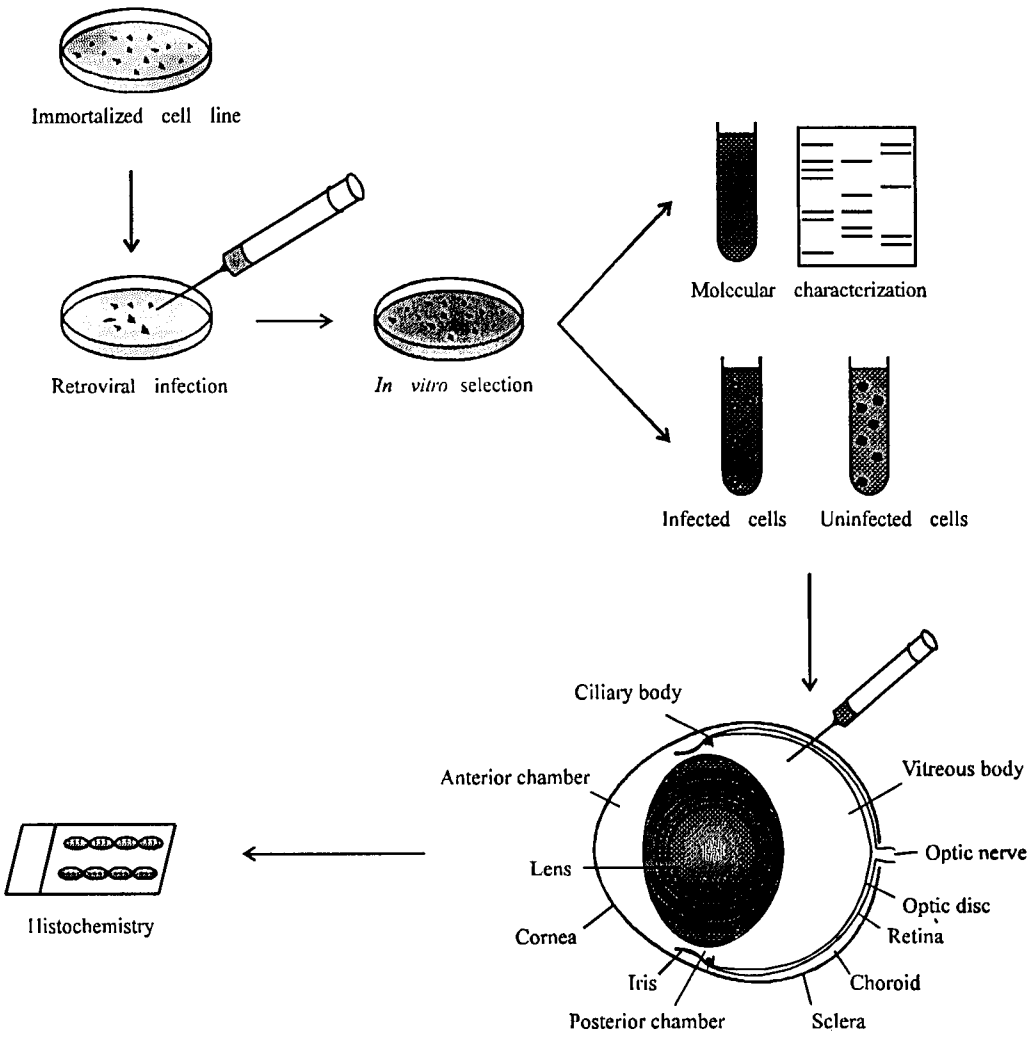


Figure 10 - Engrafting genetically modified cells producing TTR Met 30. The H56 cell line is infected with LMet 30 virus and grown under G418 selection; after molecular characterization of gene expression and of the secreted protein, the infected cells can be injected into the vitreous body of a rat eye. The eye is then processed for histological examination.

We chose as a first approach the eye as target for cell implantation, since it is of relatively easy access, the immune response to the graft is not very rapid due to the limited vascularization, and it is one of the locations were amyloid deposits are observed in FAP patients.

To implant the cells we used an Hamilton syringe and injected 10 µl of a cell suspension (about 10^3 - 10^4 cells in PBS) into the posterior chamber of one of the animal eyes. For easier technical manipulation the animals chosen were rats.

In the first experiments we used H56 Met 22 cells, and the animals were sacrificed four days after the implantation procedure. In figure 11A and 11B are represented two hematoxylin/eosin stained sections of the eye; in panel A it is observed that the cells grew into a tumour, between the retina and the lens, and that the center of the tumour is necrotic due to the lack of blood vessels; in panel B is shown the local of the injection where some cells also grew; Congo red staining of the sections did not show any green birefringence, and by immunohistochemistry with anti-human TTR it was not possible to detect the human protein, neither in the cells or as aggregates; since previously performed immunohistochemistry of the cells failed to reveal the human protein, indicating that the amounts of the human protein inside the cells were under the detection limit of the assay, we decided to implant cells known to produce high levels of TTR Met 30.

The choice was one of the transgenic primary fibroblast cultures, the Fib 1 cells that had been shown to produce the human protein by several assays, including immunohistochemistry. In this experiment the animal was sacrificed eight days after implantation. In figure 12 is represented a hematoxylin/eosin stained section of the rat eye containing the injected cells, present between the retina and the lens; inflammatory cells can also be observed. As with the H56 Met 22 cells, Congo red staining of these sections failed to reveal any amyloid deposits; as for immunostaining with anti-human TTR, cross reactions were observed with the sclera and other structures in the eye, but it was not possible to distinguish any positive signal in the injected cells.

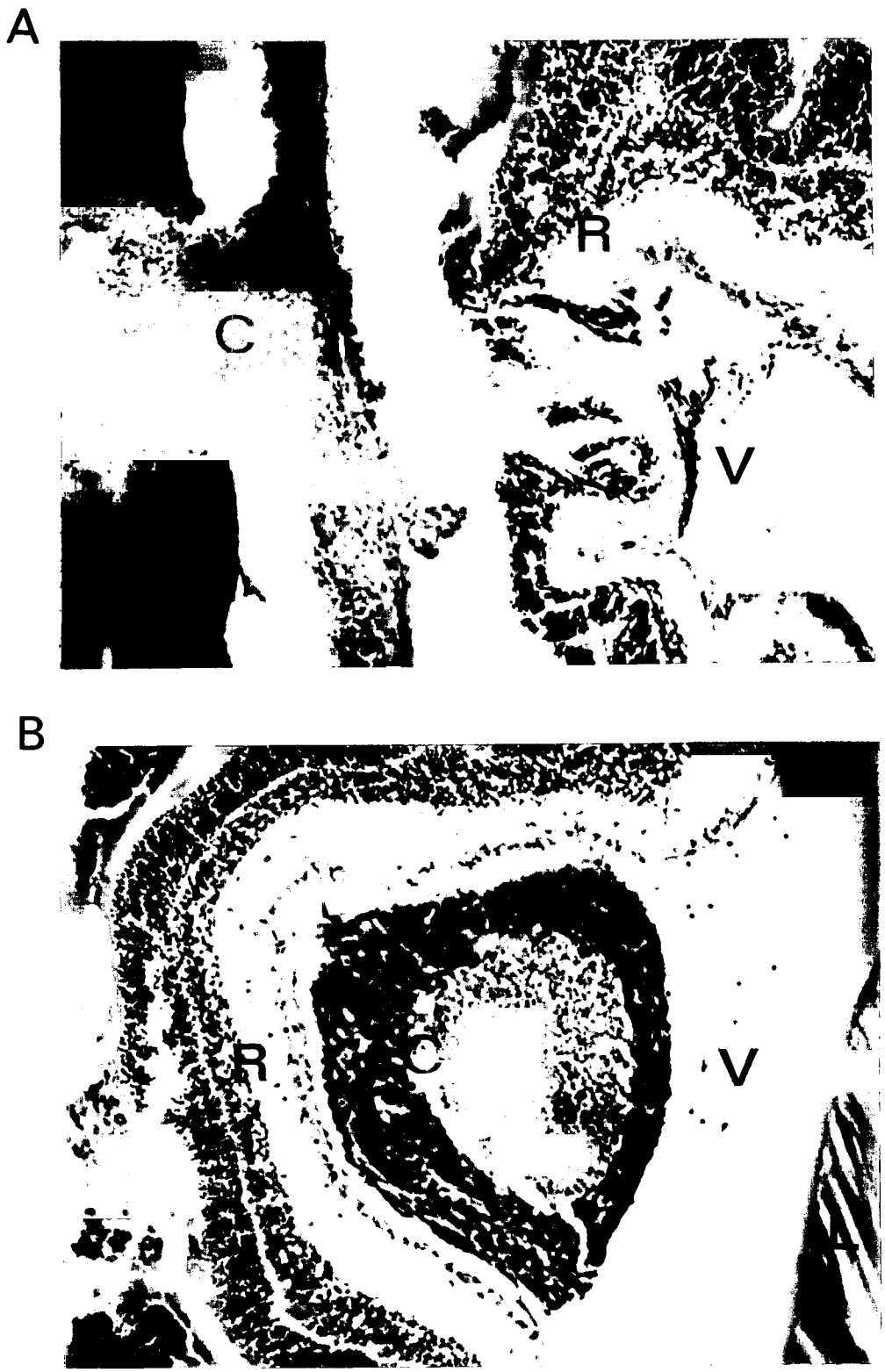


Figure 11 - Hematoxylin-eosin stained sections of a H56 Met 22 four day implant. **Panel A:** Local of injection; **Panel B:** implant localization in the eye; magnification 125×. C - implanted cells; L - lens; R - retina; V - vitreous.

Figure 12 - Hematoxylin-eosin stained section of transgenic fibroblasts implant; magnification of 125×. C - implanted cells; L - lens; R - retina; V - vitreous.

The reason for not detecting the human protein is not known; several hypotheses can be considered; it is possible that a decrease, or even a shutdown, of the expression of some proteins, including TTR, could be induced either by the implantation of the cells in the *in vivo* environment, or as a result of the immune reaction to the cells; it is also possible that the problem lies in the technical manipulations of the tissue. Further experiments are needed to explore the potentialities of the implantation assays, for instance, testing longer periods of time.

Discussion

Cell culture systems for the study of amyloidogenesis, whereby the production of the amyloid precursor protein leads to intracellular and/or extracellular amyloid deposition, have been described for Aβ (Maruyama *et al.*, 1990), and AIAPP (O'Brien *et al.*, 1995, Koning *et al.*, 1994). To develop one such system for the ATTR study, two conditions need to be fulfilled: one is to have cell lines expressing and secreting the variant TTRs, and the other is to achieve amyloid formation in this system.

The diversity of existent cell lines producing the human protein is limited, since very few tissues synthesize TTR *in vivo*; significant TTR synthesis is limited to hepatocytes, choroid plexus epithelium, and retinal pigment epithelium. In addition, the few cell lines that might express TTR

will produce the normal protein and not the amyloidogenic variants, as is the case of the human Hep G2 cell line. We have verified that Hep G2 cells express and secrete TTR in amounts similar to primary cultures of rat hepatocytes, that is, about 1 μg per million cells over 24 hours (Dixon and Goodman, 1987). Although this cell line can be used for the study of TTR metabolism and interactions with natural ligands, such as RBP, it is not as useful for the study of amyloidogenesis.

As previously referred to, it is possible to engineer genetically modified cells for the production of variant TTRs by gene transfer technology. The most frequently applied methodology for the introduction of foreign genes into mammalian genomes, and the most efficient one, is based on the use of viral vectors, such as: retroviral (Morgan, 1993), adenoviral (Levrero *et al.*, 1991), adenovirus-associated (McLaughlin *et al.*, 1988) and herpes-based vectors (Latchman, 1994). Non viral gene transfer techniques have also been described, namely: lipofection (Felgner *et al.*, 1987), electroporation (Keating and Toneguzzo, 1990) and direct gene injection (Acsadi *et al.*, 1991).

We have chosen the retroviral vector system to introduce the human TTR and TTR Met 30 cDNAs into mammalian cells, since it not only presents several advantages over the other methods of gene transfer (as previously mentioned), but also their main disadvantages (the incapability of retroviral vectors to integrate in non diving cells, and the size limit of viral nucleic acid) were not of particular concern to our objective. Therefore we constructed two recombinant retroviral vectors, containing the full length human cDNA for TTR and for TTR Met 30. The plasmids were transfected into an ecotropic producer cell line and the viral stocks were used to infect different types of cell lines, namely the mouse fibroblast line NIH 3T3, the rat hepatoma cell line H56, and mouse primary fibroblasts.

The hepatoma clones of TTR and TTR Met 30 were shown to possess the messenger RNA for the human protein and to correctly translate the mRNA into tetrameric TTR, as assessed by gel filtration analysis and by immunoprecipitation of metabolic labeled protein. The amounts of protein secreted into the media were low, up to 78 ng of TTR and 32 ng of TTR Met 30 by a million cells over twenty four hours, which is 10 fold less than primary cultures of rat hepatocytes (Dixon and Godman, 1987). Since dexamethasone had been reported to increase gene expression from murine leukemia retrovirus promoters, we tested also the effect of this glucocorticoid on the expression of TTR by infected clones and, although the increase in TTR production was not prominant, we supplemented cultures with dexamethasone thereafter.

Concerning the NIH 3T3 fibroblasts, although they have been used to express exogenous genes successfully (Lo *et al.*, 1988; Krauss *et al.*, 1991; Garver *et al.*, 1987), they failed to produce detectable levels of either TTR or TTR Met 30. Two possibilities must be considered; either the amounts of the protein produced are very low, under the detection limit of the immunoassay used,

or the cells are unable to translate the TTR mRNAs. The latter hypothesis seems unlikely, since TTR appears not to have any other post-translational modification besides cleavage of the signal peptide, and it is secreted as a biological active tetramer in an *E.coli* expression system (Furuya *et al.*, 1991). Furthermore, infected primary mouse skin fibroblasts were shown to produce and secrete the mutant TTR at the levels of the hepatoma cells, i.e. 72 ng TTR Met 30 per million cells over 24 hours, indicating that fibroblasts do not require any specific factors for the production and/or secretion of these proteins. One cannot exclude also the possibility of expression shutdown resulting from LTR methylation or other still unclear mechanisms (Xu *et al.*, 1989; Hoeben *et al.*, 1991).

In summary, although the retroviral-mediated gene transfer of TTR appeared not to work with the NIH 3T3 cell line, it was successful with the hepatoma H56 cell line and also primary cell cultures. Thus the first condition for the development of a cell culture system for the study of ATTR amyloidogenesis was achieved.

We then used these genetically modified cells to design cell culture models for the study of amyloidogenesis. We started by studying the accumulation of the human protein secreted into medium in a time course experiment using a transwell system; exponential accumulation of protein in the medium was observed over the five days experiment, indicating that the rate of protein synthesis overcomes protein degradation. It is therefore possible to use this system to investigate whether cells secrete some factor that, with time, might induce amyloid formation from accumulated protein in the media, and/or if the aging of the protein can trigger its deposition as amyloid.

The conditioned media from H56, H56 TTR and H56 Met 30 different time period cultures (five and 13 days) were investigated for the presence of amyloid by thioflavine T fluorometry; however, amyloid was not detected under these experimental conditions. Since a critical concentration of the amyloid precursor might be needed for amyloid formation, and the amounts of TTR Met 30 in the conditioned media could be below that value, we have also studied the effect of the addition of isolated TTR to H56 TTR cultures, or TTR Met 30 to H56 Met 30 cultures, over 8 day experiments. Again, no amyloid was detected by fluorometry of media or by Congo red staining of pelleted cells. Several hypothesis can be raised to explain this fact; first, the final amount of protein accumulated in media might still be under the hypothetical necessary critical concentration. Second, it is possible that a longer period of culture time is needed for triggering amyloid formation. Last, one should not forget that other factors such as chaperone proteins and tissue components might be necessary for induction of amyloid formation *in vivo* or in cell culture. Although amyloid formation is obtained *in vitro* from most precursors alone, it usually needs the use of strong acidic conditions, rather non-physiological. It cannot be excluded also that if very little amounts of amyloid were deposited on the cells, they might be under the detection limit of

Congo red staining. One could also try thioflavine T fluorometry of a cellular suspension or otherwise detection with labelled SAP.

We then performed some very preliminary experiments to test whether growing TTR or TTR Met 30 secreting cells in contact with either different tissue matrix components or fibril preparations would induce or nucleate amyloid formation. Tissue components from normal heart and thyroid were used, as well as amyloid containing vitreous and thyroid fibril preparations; however, in none of the assays was amyloid identified, either in the media or in the cell monolayers. This system needs to be optimized, particularly when using preparations containing amyloid fibrils, to discriminate *de novo* amyloid formation.

With our system we have not yet achieved amyloid formation in cell culture models, however many other experimental conditions need to be tested, for example: growing the cells in contact with true extracellular matrixes, and not just isolated components; test the addition of chaperone factors such as serum amyloid P component and apolipoprotein E; to experiment stress conditions such as oxidations; and, in addition, to experiment combinations of the above examples.

We have also used the genetically modified cells with another purpose, which was to the study the TTR Met 30 production in particular tissues and organs in animals. This objective is based on our thoughts on the absence of amyloid deposition in the peripheral nervous system in the TTR Met 30 transgenic mice that develop amyloidosis. We hypothesize that either the human protein produced in these animals is not available to the nerves, or particular components of the nervous tissue, that are species-specific, are missing in these mice strains. Therefore, by implanting cells producing the human variant in nerve tissue, it should be possible to determine whether or not the protein is reaching the nerves in sufficient amounts. On the other hand by performing implants in other tissues, and also in other species, it is possible to verify whether the problem lies in particular matrix components.

We have performed two successful implant experiments, one with H56 Met 30 cells and another with primary transgenic fibroblasts producing high levels of the human variant. Since we were developing the feasibility of this kind of experiments, we used the eye as a target organ, and the rat as the experimental animal. In both cases, the implanted cells were identified by histochemistry, and although a large number of inflammatory cells was identified in the fibroblast implant (which was expected as it is a xenograft), the cells appeared to be in good condition. We were not able, however, to detect the human protein by immunohistochemistry, and it remains to be determined whether that was due to the low sensitivity of the immunochemical technique, or whether the cells had inactivated the gene. In the case of the H56 Met 30 cells, it is probable that the amounts of human protein secreted are under the detection limit of the immunoassay technique, since even in culture it was not possible to detect the human protein by immunohistochemistry.

In the transgenic fibroblasts, although the protein was detected by immunohistochemistry in cell culture, since the immunohistochemistry technique in sections is less sensitive than in cultured cells, that could also be the case. Further experiments are necessary to elucidate these aspects, and the detection assays need to be improved and optimized; one possibility would be to study the expression of the human gene by *in situ* hybridization. Having detected the human protein, it will be then possible to perform implants in other locations and elucidate the role of tissue matrixes in amyloidogenesis.

In conclusion, we have developed a cell culture system, using cells genetically modified by retroviral-mediated gene transfer, now available not only to study experimental conditions for amyloid formation, but also to study the interactions of mutant TTRs with extracellular matrixes and other factors. We have also used these cells for grafting experiments in animals, as an attempt to create an efficient animal model for FAP.

Part III

Synopsis and Perspectives

Synopsis and Perspectives

It is clear that the polymerization of a normally soluble protein, such as TTR, into amyloid fibrils, is a complex process involving several factors, namely the amyloidogenic potential of the protein, chaperone molecules, and other still unknown factors. The main purpose of our study was to elucidate the role of possible intervening factors in TTR amyloidogenesis; to achieve that objective, we have used available experimental models and developed new tools.

➤ ***Study the effect of factors influencing the process of amyloid formation from TTR "in vitro" (chapters 1, 2)***

To study the influence of modulating factors in the formation of amyloid fibrils *in vitro*, it was necessary to analyze first the components of native amyloid fibrils; we have used Met 30 kidney and thyroid amyloid fibril preparations, and during their characterization we noticed the presence of lower amounts of TTR fragments in kidney fibrils as compared to a previously described variant, TTR Ile 122, isolated from an FAC heart. This fact led us to conclude that fragmentation is not a major characteristic of TTR Met 30 amyloid fibrils. It should not be excluded, however, the importance of proteolysis resulting in the fragmentation observed in some TTR amyloid fibrils, namely in FAC TTR Ile 122 fibrils and in the SSA cases, where normal TTR deposits. Proteolysis as the general mechanism of amyloid formation in the amyloidoses has been loosing importance, with the finding of whole molecules in the amyloid fibrils and with the *in vitro* production of amyloid-like fibrils from most precursors; the latter, however, makes use of generally non-physiological conditions. Besides, it cannot be ignored that, at least in the majority of the AL, AA and AGel cases, the amyloid fibrils are constituted by fragments, and not whole precursor molecules; it is possible that an initial proteolytic event could trigger the formation of an amyloid subunit, and polymerization of intact molecules could then occur.

Thioflavine T fluorometry was tested as an amyloid quantification method and the assay conditions for TTR amyloid were standardized; we confirmed that this procedure is very sensitive and specific for amyloid detection *in vitro*, not recognizing amyloid precursors neither in a non-aggregated form nor as non-organized aggregates. The production of amyloid-like fibrils from the whole TTR molecule was then achieved in acetate buffer pH 4, and the experimental conditions for *in vitro* amyloid formation were optimized by assessing the effect of incubation buffers, TTR concentration and pH dependence, and the time stability of the amyloid-like fibrils.

Using the above described assay, we have also evaluated the propensity of two neuropathic variants, TTR Met 30 and TTR Pro 55, two cardiopathic variants, TTR Ile 122 and TTR Met 111, and the nonamyloidogenic TTR Met 119, towards *in vitro* amyloid formation. By using kinetic and pH dependent assays we demonstrated that these variants could be ordered in terms of decreasing amyloidogenicity as follows: TTR Pro 55 >> TTR Met 30, TTR Met 111 > TTR, TTR Ile 122 > TTR Met 119, which correlates to the clinical phenotypes associated with these variants. These results are consistent with the conformational hypotheses put forward to explain TTR amyloidogenesis, that suggest that it is a conformationally different form of the TTR molecule (amyloidogenic intermediate) that assembles into amyloid fibrils. What leads to the formation of this intermediate is not known, and is highly debatable. Some authors (Kelly and Lansbury, 1994) propose that it is formed under the acidic environment of lysosomes, competing with the acid mediated degradation pathway of the protein, and that the amyloidogenic mutations would either decrease the molecule acid stability and/or alter the degradation pathway. The results obtained by us confirm that the amyloidogenic mutations originate a molecule more prone to acid denaturation, which can also be interpreted as a higher tendency of the tetramer towards dissociation, and thus *in vitro* amyloid formation is favoured. That the non amyloidogenic Met 119 mutation stabilizes the TTR tetramer, and the amyloidogenic Met 30 mutation has the opposite effect, is also suggested by studies carried out with other denaturing agents; isoelectric focusing of heterozygous sera, both TTR Met 119 and TTR Met 30, in semi-denaturing gels, showed that the Met 30 tetramers appear less resistant to urea dissociation and, on the contrary, the Met 119 tetramers are highly resistant to dissociation (Alves *et al.*, 1996). It is, therefore, conceivable to hypothesize that the amyloidogenic mutations somehow destabilize the TTR tetramer, facilitating dissociation, and either the dimers or the monomers would then associate into amyloid fibrils.

A more structural interpretation of the "amyloidogenic intermediate" hypothesis was carried out by Serpell and colleagues (Serpell *et al.*, 1996); they suggested that the mutations in the TTR molecule promote the formation and/or stabilization of this intermediate by leading to structural changes at the edge of the β-sheets that form the framework of the TTR molecule.

The three dimensional structures, determined so far, of amyloidogenic variants (Met 30, Ile 122, Ser 84) show no major alterations in the folding of the proteins as compared to the normal TTR, however some relevant aspects were noticed: in the Met 30 variant, there is a higher spacing between monomer sheets to accommodate the Met 30 residue, which leads to a movement of the outer sheet towards the solvent, these movements giving rise to a more elliptical central channel and thus explain the low affinity of Met 30 tetramers for thyroxine. A higher distance between dimers is also observed in this variant (Hamilton, 1992), as well as an exposure of residue 10 to the solvent due to movement of strand A (Terry *et al.*, 1993). Concerning the Ile 122 variant,

it was observed a decrease in the dimers contact, resulting from an increase in the length of the hydrogen bonds between dimers, and within dimers (Damas *et al.*, 1996). The three dimensional structure of TTR Pro 55 is not yet known, however recent preliminary data indicate that there is a dissociation of strands D and A; furthermore the asymmetric cell unit contains eight monomers, in contrast to the normal protein with only one dimer. One interesting fact is the observation that the crystallographic packing of this variant is different from that of the normal protein (Sebastião *et al.*, 1996), and thus it was hypothesized that this variant could resemble an amyloidogenic intermediate (Saraiva *et al.*, 1996).

That the Pro 55 variant might exist in a more amyloidogenic conformation was also suggested by our study of the amyloid formation kinetics from TTR. We observed that amyloid formation from the normal protein had an initial rate determining step, not diminished in the presence of preformed fibrils, which indicates, apparently, a nucleation independent process; this lag time could be associated with the conformational change of the protein into a more amyloidogenic species; since the kinetics of amyloid formation from the TTR Pro 55 variant showed the absence of this lag time, this could suggest that this protein already exists in an amyloidogenic form, aggregating immediately as amyloid under the conditions tested.

In summary, major structural alterations appear not to take place in the amyloidogenic TTR variants, although conformational modifications do occur, indicated also by the different thyroxine binding affinities of the TTR variants (Almeida and Saraiva, 1996), possibly leading to a destabilization of the tetramer in favour of dimers and/or monomers. Recent electron microscopy structural studies of native amyloid fibrils, indicated that fibrils are formed by four protofilaments arranged in the corners of a square with a hollow center, with the TTR molecule fitting into the protofilament dimensions (Serpell *et al.*, 1995). Further biophysical studies are, therefore, necessary, to establish the structure of the amyloidogenic intermediate. As for the elucidation of the relative stability of the TTR variants, such as the TTR Met 119, it can be investigated by *in vivo* metabolic experiments.

Explaining amyloidogenesis merely in the view of "amyloidogenic intermediates" or proteolysis is, however, an oversimplification. Although one general mechanism for amyloid formation implies a modification of the amyloid precursor protein, that is converted into an amyloid subunit, what triggers this modification must be dependent on other factors. That the deposition of the protein precursor as amyloid must be dependent on other factors/conditions is also indicated, in the case of FAP, by the phenotype variability observed within the same TTR mutations, such as in the case of TTR Met 30, where we have patients with an age of onset that goes from 17 to 78 years old, and by the fact that penetrance is incomplete. Moreover, although TTR mutations appear to have preferential places for deposition, there are some mutations that give rise to different patterns

of deposition, such as TTR Ile 50, that is associated with neuropathy in one kindred and with cardiomyopathy in another.

Putative amyloidogenesis modulating factors include: chaperone proteins, circulating and/or tissue factors; they might intervene in the triggering of the amyloid subunit formation and/or contribute to the persistence of the fibrillary deposits *in vivo*. We have studied the effect of metal ions in TTR amyloidogenesis, since metal ions have been implicated in the process of Aβ amyloid formation, and are also suggested to be involved in TTR fibrillogenesis; none of the metal ions tested, however, significantly diminished or augmented the amounts of amyloid formed. Moreover treatment of native amyloid fibrils with chelators did not result in significant solubilization. These facts raise some questions on the effectiveness of chelator therapy in FAP, as suggested by some authors (Martone and Herbert, 1994). Further experiments with other metal ions, and in other concentrations, should be carried out to elucidate this point.

The role of sulphate ions in this process was also investigated by us, trying to mimic the sulphate moiety present *in vivo* in proteoglycans and glycosaminoglycans; we concluded that sulphate ions not only favour the formation of amorphous precipitates *versus* oriented polymerization into amyloid fibrils, but also promote aggregation of the fibrils formed. This latter effect could be related to an *in vivo* role of the glycosaminoglycan sulphate moieties; however to determine whether this is so, glycosaminoglycans should also be tested in these assays.

The results obtained from *in vitro* amyloid formation studies should always be regarded with some care, not only because they are usually carried out in conditions, most probably, not corresponding to the *in vivo* situation, but also because the sample preparation methods and sample history might affect the results obtained. The latter is illustrated, for instance, by the variability of effects observed when testing chaperone proteins in β-peptide aggregation; opposite effects have been reported at least for SAP, apoE and α_1-antichymotrypsin.

In summary, *in vitro* assays are very important for the study of conditions where amyloid can be formed, and to study possible intervening factors, but to determine the mechanisms of amyloidogenesis it is essential to carry out *in vivo* studies.

- ***Investigate the transgenic strain MT1-TTR Met 30 lacking amyloid deposition and determine possible intervening factors (chapter 3)***

Analysis of animal models for FAP should further elucidate possible mechanisms of amyloidogenesis *in vivo*. One of the transgenic mice strains carrying the human TTR Met 30 gene was described not to have amyloid deposits, in opposition to a strain carrying a similar DNA construct. We characterized the human protein present in the serum, having verified that the protein

was a functional tetramer, and concluded therefore that the absence of amyloid deposition was not due to structural abnormalities in TTR Met 30. Moreover, we demonstrated that the human protein was secreted *in vivo* in the skin. The amounts of human protein produced in these animals, only detectable when animals had been induced with zinc, were rather low, and we have never been able to detect amyloid deposition or extracellular TTR aggregates, even when under zinc induction for long periods of time.

We raised essentially two hypotheses to explain this fact: the first hypothesis considers that the genetic background of this mouse strain determines the deposition of the human protein as amyloid, that is, either there are genetically determined factors that prevent the deposition of the protein or, in the other hand, necessary factors are lacking in these animals. One possible strategy to test this hypothesis would be to perform crossings of these animals with other strains known to have TTR Met 30 amyloid deposits; another would be to generate transgenics in other animal species, ideally primates; however, due to evident limitations, smaller animals should be considered.

The second hypothesis relates to the fact that the amounts of human protein present in these animals might be below a specific critical concentration, needed for amyloid formation; however, other transgenic mice studies have revealed that TTR concentration is most probably not the key condition for amyloid deposition, as mice carrying either TTR Pro 55 or TTR Ser 84 did not develop amyloidosis, although having serum TTR concentrations similar to the one stated to be sufficient for amyloid formation in the amyloidosis developing TTR Met 30 mice (Waits *et al.*, 1996; Teng *et al.*, 1996; Maeda *et al.*, 1996). Furthermore it is known that high concentrations of amyloid precursors appear to be a necessary condition for the development of some amyloidoses, namely the PHD amyloids, Aβ2M and AA, although it is also clear that this is not a sufficient condition. In Met 30 FAP, however, both asymptomatic carriers and patients have lower serum TTR concentrations than normal individuals, and no correlation is observed between the levels of mutant protein in patients and asymptomatic carriers (Saraiva et al., 1985). In addition, since the amounts of TTR present in the extravascular fluids should be related to the vascular fluid concentrations (with the exception of CSF), it could be hypothesized that high protein concentrations are not necessary for amyloid deposition in vivo. It cannot be excluded, however, the formation of microenvironments with relatively high protein concentrations.

None of the transgenic mice strains carrying the human TTR mutant gene produced so far, can mimic the human FAP phenotype; in some of the strains, systemic amyloid deposition is observed, but the peripheral nervous system is spared, and the animals do not develop neuropathy.

Transgenic mice as models for other amyloidoses have also been developed, namely for Alzheimer's disease and AIAPP; however, as with ATTR mice, in none of the cases is the human phenotype completely represented. The reasons for that are not known; multigenicity could be the

cause, at least for Alzheimer's disease, and that could be established by generating mice with simultaneous genotypes associated with AD. It is also possible that interference with endogenous genes affects the resulting phenotype; knock-out animals should elucidate this point, although in the case of ATTR mice bearing the Met 30 gene and without endogenous mouse TTR no significant differences have been observed. Null mutations in other genes, such as SAP, could also be very useful to determine the role of those products in amyloidogenesis.

➤ ***Production of genetically modified cells producing TTR Met 30 and development of models for the study of amyloidogenesis (chapter 4)***

The above described facts, together with the advantages presented by cell culture over animal models, including the technical ease of manipulation and the shorter time duration of the experiments, led us to develop a cell culture experimental model for the study of amyloidogenesis.

We have produced genetically modified cells secreting either the human TTR or TTR Met 30 proteins by infecting hepatoma cells with recombinant retrovirus. The amounts of human protein produced varied among clones and were not high, approximately 10 fold less than primary cultures of hepatocytes. After confirming that the secreted human protein accumulated in medium exponentially with time, thus indicating a higher secretion over degradation rate, we seeked for conditions that could lead to amyloid formation. We tested whether the accumulated protein in medium would aggregate as amyloid, as a result of aging and/or by influence of some cellular factors; no amyloid deposits were detected in experiments carried out with different incubation times, and by increasing the protein concentration in medium with the addition of isolated recombinant TTR or TTR Met 30. We concluded, therefore, that aging of the protein, and protein concentration, are not sufficient for amyloid formation under the conditions tested.

A few preliminary experiments were carried out where the infected cells, producing TTR or TTR Met 30, were grown in contact with normal tissue extracts, or amyloid fibril extracts; amyloid formation was not, however, detected under the conditions tested. Amyloid fibril extracts were used since amyloid fibrils are known to contain amyloid enhancing factor activity (AEF), and when administered intraperitoneally were shown to reduce the lag phase in the induction of experimental AA amyloidosis (Ganowiak et al., 1994). Furthermore it was recently reported that both rat and human mesangial cells were capable of processing amyloidogenic light-chains added to the cultures only if in presence of AEF (Tagouri et al., 1996). The fact that we have not detected amyloid formation in the presence of amyloid fibril extracts, might be attributed to the loss of components during the extraction procedures and/or to the need of a specific concentration of the putative enhancing factor. Native fibril staining might also mask *de novo* fibril formation, thus it is necessary

either to optimize the assay conditions to discriminate both types of amyloid, i.e. native and *de novo*, or use other nucleating factors such as other AEF preparations.

The role of tissue components must not be underestimated when studying amyloidogenesis; in the ATTR amyloidosis amyloid deposition is not observed in liver, thus it is possible that either the hepatocyte extracellular matrix lacks the necessary "anchoring" factors or, on the other hand, has components preventing the deposition of the protein as amyloid. The same is probably occurring in the TTR Met 30 transgenic mice that develop systemic amyloidosis, except in the peripheral nervous system, which might lack essential tissue components present in the human counterpart nerves.

These preliminary assays of amyloid formation in cell culture, at any rate, pave the way to future experiments designed at elucidating the complex questions of critical precursor concentration, triggering of amyloid formation and enhancing factors, namely: a) to perform comparative experiments with other cell lines, such as mesenquimal cell lines, since they are usually in close association with amyloid deposits. Retrovirus could be used to infect fibroblast lines, other than the NIH 3T3, and Schwann cells to study the importance of tissue components. b) To study the reasons why particular mutations in the TTR make the molecule more prone towards amyloid deposition in particular organs or tissues, but not in others. This could be approached by producing recombinant retrovirus with other TTR variant cDNAs and, after infecting appropriate cell lines, study the interactions of these proteins with various extracellular matrixes. c) The study of interactions with circulating factors, such as SAP, apolipoprotein E, glycosaminoglycans and, as previously referred to, other AEF preparations, by the addition of these components to cellular cultures secreting the human proteins. d) Application of the TTR producing cells in in vivo studies, by implanting these cells in particular locations in animals, and investigation of the interactions of the tissue matrixes with the secreted proteins, and the effect of other local environment factors; the latter being the main advantage of the implant experiments over the cell culture experiments. We have performed some trial experiments; however, optimization of the experimental conditions is still necessary.

In summary, the cell culture system developed can be used for a wide variety of experiments, with the objective of elucidating the mechanisms of amyloid formation.

We think that it is clear, from the above exposed, that the process of amyloid formation is very complex involving a multiplicity of factors, and that although amyloid is the unifying characteristic of the amyloidoses, the mechanisms by which it is formed are probably different in the various syndromes, and therefore direct correlations should not be drawn. Furthermore, only by integrating the results obtained from several experimental models (in vitro, in vivo and in cell culture) can we truly elucidate the mechanisms of such devastating diseases and develop therapeutic strategies.

Final Remarks

Main contributions of this work for the study of amyloidogenesis:

- Development of thioflavine T based fluorometry as a highly sensitive and specific method of TTR amyloid detection and quantification.
- Development of an in vitro system, by using TTR amyloid-like fibril formation, for the study of intervening factors in the process of TTR fibrillogenesis.
- Demonstration that amyloidogenicity of the TTR variants studied correlates with the clinical phenotypes.
- Evidence that the phenotype of TTR Met 30 transgenic mice is dependent upon mice strains.
- Development of a cell culture system for the study of amyloidogenesis by using genetically modified cells producing the mutant TTR.

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