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Transcriptional Regulation of the Human ß-Amyloid Precursor Protein Gene

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-À ma famille-

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Publications

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List of Abbreviations

AD	Alzheimer Disease
ApoE	Apolipoprotein E
APLP	Amyloid Precursor-Like Protein
APP	β-Amyloid Precursor Protein
APPL	Amyloid Precursor Protein-Like
bFGF	basic Fibroblast Growth Factor
bHLH	basic Helix-Loop-Helix
CAT	Chloramphenicol Acetyl Transferase
CBP	CREB Binding Protein
CMV	Cytomegalovirus
CRE	cAMP Response Element
CREB	cAMP Response Element Binding Protein
dbcAMP	Dibutyryl Cyclic Adenosine Monophosphate
DS	Down Syndrome
EGF	Epidermal Growth Factor
EMSA	Electrophoretic Mobility Shift Analysis
FAD	Familial Alzheimer Disease
HDL	Hight Density Lipoprotein
HSE	Heat Shock Response Element
HSV	Herpes Simplex Virus
hTAF	human Transcription Factor II D Associated Factors
IL-1	Interleukin-1
JNK	JUN N-Terminal Kinase
KPI	Kunitz-like Protease Inhibitor Domain
LDL	Low Density Lipoprotein
LTR	Long Terminal Repeat
LZ	Leucine Zipper
NFT	Neurofibrillary Tangles

NFT	Neurofibrillary Tangles
NGF	Nerve Growth Factor
PHF	Paired Helical Filaments
PPA	Protéine Précurseur de la ß-Amyloïde
PS	Presenilin
RSV	Rous Sarcoma Virus
SAD	Sporadic Alzheimer Disease
SAPK	Stress Activated Protein Kinase
sAPP	secreted ß-Amyloid Precursor Protein
TBP	TATA Box Binding Protein
TFIID	Transcription Factor II D
TGFβ	Transforming Growth Factor ß
TK	Thymidine Kinase
VLDL	Very Low Density Lipoprotein

Abstract

The ß-amyloid precursor protein (APP) is an extensively processed membrane protein with a single membrane spanning domain. One of the natural proteolytic products of APP is a peptide of 39 to 43 amino acids in length, ß-amyloid, that has been identified as a major constituent of senile plaques, a neuropathological hallmark of Alzheimer disease (AD). In Down syndrome (DS) (trisomy 21), overexpression of the *APP* gene, located on chromosome 21, is thought to be responsible for the early formation of senile plaques in the brain of DS patients. We investigated the transcriptional regulation of the *APP* gene in order to identify the *cis*-elements located in its promoter and the *trans*-acting factors recognizing these elements. Identification of the factors regulating APP gene expression could help define conditions leading to the development of AD. The *APP* promoter possesses multiple transcriptional start sites, has a high percentage of GC and lacks a consensus TATA box. We have characterized, using electrophoretic mobility shift analysis (EMSA), DNase I footprinting, site-directed mutagenesis and transient transfection studies, three elements located in close proximity of the major transcriptional start sites of *APP*. We present evidence that two of the elements are recognized by the ubiquitous transcription factors Sp1 and USF respectively. Both the Sp1 and USF elements are essential for full activity of the *APP* promoter, regulate the *APP* promoter in an additive manner and synergize with the factor(s) recognizing a GC-rich element located upstream in transient transfection assays in NG108-15 and HepG2 cell lines. We also show that treatment by cyclic AMP of NG108-15 cells which had been transiently transfected with reporter constructs driven by different portions of the *APP* promoter caused an increase in reporter gene activity mediated by sequences located between -2991 to -488 and -303 to -204, even though the upstream sequences of *APP* do not contain a canonical cyclic AMP response element. Co-transfection assays carried out in HepG2 cells with AP-2, a cAMP-regulated transcription factor, failed to activate the *APP* promoter through the AP-2 consensus sequence (GCCNNNCGG) located at position -205.

Résumé

La protein précurseur de la ß-amyloïde (PPA) est une protéine possédant un domaine transmembranaire et soumise à de nombreuses modifications post-traductionnelles. La PPA est clivée par des protéases cellulaires pour générer un peptide de 39 à 42 acides aminés nommé ß-amyloïde. La ß-amyloïde a été identifiée comme la principale composante des plaques séniles observées dans le cerveau des individus atteints de la maladie d'Alzheimer. Chez les personnes souffrant de Syndrome de Down (trisomy 21), surexpression du gène de la PPA, causée par la troisième copie située sur le chromosome 21, est soupconnée être responsable de la formation de plaques seniles très tôt chez les individus atteints. Nous avons entrepris de caractériser le promoteur du gène de la PPA dans le but d'identifier les séquences régulatrices (éléments) presentes en amont du site d'initiation de la transcription ainsi que les protéines les reconnaissant. Nous avons caractérisé le rôle de trois éléments du promoteur de la PPA situés entre les positions -96 et -30 relativement au site principal d'initiation de la transcription. Nous démontrons que deux de ces éléments sont reconnus par les facteurs de transcription Sp1 et USF. Les élément reconnus par Sp1 et USF sont essentiels au bon fonctionnement du promoteur de la PPA et partiticipent de manière additive à l'activité du promoteur en essais transitoires de transfection dans les lignées cellulaires NG108-15 and HepG2. De plus, nous avons observé que le protéines reconnaissant les éléments Sp1 et USF agissent de manière synergique avec le troisième élément, situé en amont, pour conférer sa pleine activité au promoteur de la PPA. Dans une autre série d'expériences, nous démontrons qu'en essais transitoire de transfection, le traitement de la lignée cellulaire NG108-15 avec de l'AMP cyclique mène à l'activation d'un gène rapporteur sous le contrôle de diverses portions du promoteur de la PPA, bien que le promoteur de la PPA ne possède pas de séquences consensus reconnues par le facteur de transcripton CREB (cyclic AMP response element binding protein). Les regions étant responsables de cette activation sont situées entre les positions -303 et -204, et -2991 et -488. En essais transitoires de tranfection dans des cellules HepG2, la co-transfection du facteur de transcription AP-2, qui est activé par l'AMP cyclique, et du promoteur de la PPA

ne résultat pas en une augmentation de l'activité du promoteur par la séquence consensus de AP-2 (GCCNNNCGG) présente à la position -205.

Statement of Originality

As stated in the guidelines concerning thesis preparation, Faculty of Graduate Studies and Research, McGill University: "Elements in the thesis that are to be considered as contribution to original knowledge must be clearly indicated in the preface or by a separate statement at the beginning or at the end of the thesis. This requirement is mandatory for Ph.D. thesis.

We have characterized DNA elements which are involved in basal and inducible expression of the *APP* gene:

1. We have shown that an E box (CANNTG) in the proximal *APP* promoter is essential for *APP* transcription. We have further determined that the transcription factor USF transactivates the *APP* promoter in a specific manner through its recognition of this E box.
2. We have shown that *in vitro* the transcription factor Sp1 recognizes an essential Sp1 element of the *APP* promoter.
3. We have shown that protein(s) recognizing the GC-element, located upstream of Sp1 and USF binding sites, act in a synergistic manner with Sp1 and USF to confer full activity to the *APP* promoter.
4. We determined that cAMP treatment of NG108-15 cells activated the *APP* promoter through two different regions but that this activation did not occur through a putative AP-2 binding site in the *APP* promoter.

Chapter 1

Introduction

One of the neuropathological hallmarks of Alzheimer disease (AD) is the presence of extracellular protein deposits in the cerebral cortex and blood vessels of the brain. The major constituent of these extracellular deposits called senile plaques, is a small peptide of 39-42 amino acids called ß-amyloid (Glenner and Wong, 1984). Senile plaques are also present in the brain of individuals affected by Down syndrome (DS) and to a certain extent in normal aging (Masters et al., 1985). In senile plaques, ß-amyloid is present as insoluble filaments that form fibrils of 7-9 nm in width. Other constituents of senile plaques are alpha-1 antichymotrypsin (Abraham et al., 1988), apolipoprotein E (ApoE) (Namba et al., 1991), lysosomal proteases (Cataldo et al., 1990), antioxidant enzymes (Pappolla et al., 1992) and heparan sulfate proteoglycans (Snow et al., 1990). Besides senile plaques, other neuropathological characteristics of AD include intracellular neurofibrillary tangles, dystrophic neurites and neuronal cell loss towards the end of the disease. In AD, neurofibrillary tangles (NFT) are present in dystrophic neurites that surround the senile plaques. Intracellular NFT are made of paired helical filaments (PHF). The major constituent of the PHF is the abnormally hyperphosphorylated TAU protein (Delacourte and Defossez, 1986). TAU has been characterized as being predominantly a microtubule associated protein which plays a role in microtubule stabilization. In the central nervous system, TAU is mostly present in neurons. It is located in axons (Binder et al, 1985) although it can also be observed in dendrites (Migheli et al., 1988). The cytoskeleton of dystrophic neurites is severely destabilized (Schmidt et al., 1991) and the structure of synapses is lost (Masliah et al., 1989).

The ß-amyloid precursor protein gene.

Isolation of and purification of ß-amyloid from senile plaques led to the cloning of a cDNA encoding a protein of 695 amino acids that was named the ß-amyloid precursor protein (APP) (Goldgaber et al., 1987; Kang et al., 1987; Robakis et al., 1987; Tanzi et al., 1987). *APP* is located on the long arm of chromosome 21, spans more than 200 kilobases and is constituted of at least 16 exons (Lemaire et al., 1989). Alternative splicing gives rise to at least five different transcripts, the major ones encoding for proteins of 695, 751 and 770 amino acids (Kitaguchi et al., 1988; Ponte et al., 1988; Tanzi et al., 1988; de Sauvage and Octave, 1989; Konig et al., 1993). *APP* is expressed ubiquitously with higher levels found in the brain and kidneys (Neve et al., 1988).

Expression of *APP* in the mouse is first detected in 9.5 day embryos in motor neurons of the hind brain and spinal cord, and is initially restricted to the developing central and peripheral nervous systems (Fisher et al., 1991; Salbaum and Ruddle 1994). At this stage, APP may be involved in axogenesis since it is expressed only on differentiated neurons at a time when they grow neurites and generate axons (Salbaum and Ruddle, 1994). By day 11.5, APP protein is detected on only subsets of differentiated neurons (Salbaum and Ruddle,1994). In the rat, levels of total *APP* mRNA are highest in the second postnatal week (Sherman and Higgins, 1992) which is the time of brain maturation suggesting that at this later period *APP* expression may be associated with synaptogenesis (Sherman and Higgins, 1992; Ohta et al., 1993). This pattern of expression implies that there is both developmental and cell-type specific regulation of the *APP* gene.

In the adult, *APP* is expressed in many tissues (Tanzi et al., 1987) and accordingly, the 5' upstream region of the *APP* gene resembles that of the promoters of housekeeping genes with no CAAT or TATA boxes but with several GC boxes and multiple transcriptional start sites (Salbaum et al., 1988; LaFauci et al., 1989). There is a high level of conservation of sequence in the 500 base pairs upstream of the major transcriptional start site between humans and rodents (Izumi et al. 1992; Chernak, 1993). In humans, homology with consensus sequences

for AP-1, at positions -350 and -45, presence of six GC boxes (putative Sp1 binding sites) between -300 and -200 as well as a heat shock response element (HSE) (-310) have been reported (Salbaum et al., 1988). The region located between positions -96 to +100 relative to the major transcriptional start site has been shown to be fully functional as a basal promoter suggesting that sequences upstream of -96 are not essential for basal transcription (Pollwein et al., 1992; Quitschke and Goldgaber, 1992).

Growth factors have been shown to increase the expression levels of the *APP* gene. Treatment of endothelial cells and cultured astrocytes with interleukin-1ß (IL-1) (Goldgaber et al., 1989; Gray and Patel, 1993a), transforming growth factor ß (TGFß) (Gray and Patel, 1993a) and basic fibroblast growth factor (bFGF) (Gray and Patel, 1993b) led to an increase in *APP* mRNA levels. The observed increases varied between 1.6 and 2.0 fold except for bFGF which caused a 4.5 fold increase in *APP* expression. Lahiri and Nall (1995) observed an increase in activity of a reporter gene under the control of either 489 or 416 bases of upstream regulatory sequences from the *APP* promoter in transiently transfected PC12 cells treated with nerve growth factor (NGF), bFGF, phorbol 12-myristate 13-acetate (PMA), IL-1 and retinoic acid. The activation was only observed when the cells were treated for a period of four days with the agents before transfections were performed, suggesting that this was not due to an immediate effect but rather to slow changes induced by the agents.

Further investigation of the effects of IL-1 on *APP* expression led to the identification of a region of the promoter located between -485 and -305 responsible for the increase in *APP* activity (Donnelly et al., 1990). This region contains an AP-1 consensus sequence and the HSE originally described by Salbaum and co-workers (1988). A direct role for the AP-1 element located at position -350 of the *APP* promoter was later demonstrated (Trejo et al. 1994). Treatment of 132N1 and HeLa cells with PMA, which activates AP-1 activity through protein kinase C, increased more than 4 fold the activity of a reporter gene under the control of 488 bases of upstream sequences from the *APP* promoter. These workers demonstrated that *in vitro*, c-Jun, Jun B and Fra 1 but not c-Fos, Jun D or Fra 2 were part of a complex formed with an oligonucleotide containing the *APP* AP-1 element. In co-transfection studies, c-Jun,

but not c-Fos, activated the *APP* promoter. These results suggest a direct role for c-Jun in the regulation of the *APP* promoter. c-Jun is activated by growth factors, such as EGF (epidermal growth factor), NGF (Minden et al., 1994) and cellular stress through a family of protein kinases called stress activated protein kinase (SAPK) (Kyriakis et al., 1994) or Jun N-terminal kinase (JNK) (Hibi et al., 1993). Interestingly, c-Jun kinase has been shown to be activated in HepG2 cells treated with IL-1α (Bird et al., 1994) which would agree with previous results reporting that IL-1 increased *APP* promoter activity through a region containing an AP-1 element (Donnelly et al., 1990).

Cellular stress induced by heat shock treatments, ethanol and sodium arsenite on the neuroblastoma NT2 and HeLa cell lines led to the increase in activity of a luciferase reporter gene under the control of 488 base pairs of upstream regulatory sequences of the *APP* promoter (Dewji et al. 1995). The largest increase (10 fold) was observed following heat shock treament whereas ethanol and sodium arsenite caused a 2- and 3- fold increase respectively. Heat shock treatment of NT2 cells induced recognition of the HSE present in the *APP* promoter in a specific manner, suggesting that heat shock proteins could be involved in regulation of *APP* expression following cellular stress. Heat shock had previously been reported to increase *APP* mRNA levels 1.6 fold in lymphoblastoid cell lines (Abe et al., 1991).

A role for the NF-κB family of transcription factors has also been proposed. The introduction of two oligonucleotides (-2257 to -2234 and -1844 to -1821 of the *APP* promoter) upstream of an heterologous promoter led to an increase of reporter gene activity when co-transfected with expression vectors for p50, p50/p65 and p50/c-Rel (Grilli et al., 1995). In another series of experiments it was demonstrated that treatment of primary neuronal cultures with IL-1ß and glutamate increased the recognition of the NF-κB elements from the *APP* promoter and again activated an heterologous promoter carrying *APP* NF-κB binding sites (Grilli et al., 1996). However, direct activation of the *APP* promoter by NF-κB has not been reported. These studies suggest that signal transduction pathways leading to an activation of the NF-κB family of transcription factors could result in an increase in expression of the *APP* promoter.

Structure and processing of ß-amyloid precursor protein.

The deduced amino acid sequence from the initial cloning of the cDNA encoding APP predicted a protein of 695 amino acids with a single transmembrane domain, a short intracellular C-terminal domain and large extracellular N-terminal domain reminiscent of the structure of a receptor protein (Goldgaber et al., 1987; Kang et al., 1987; Robakis et al., 1987; Tanzi et al., 1987). Alternative splicing of *APP* primary transcripts give rise to three major proteins of 695, 751 and 770 amino acids. The 751 and 770 forms of the protein contain a domain sharing homology with the Kunitz-type serine protease inhibitor family (Kitaguchi et al., 1988; Ponte et al., 1988; Tanzi et al., 1988). APP has been shown to be subject to N-linked and O-linked glycosylation in the extracellular domain as well as phosphorylation at two different sites in the intracellular domain (reviewed in Selkoe, 1994). The cytoplasmic end of APP contains a consensus motif for reinternalization through clathrin-coated vesicles and consequent targeting to the endosomal lysosomal pathways (Chen et al., 1990). The purification of late endosomes/lysomes has demonstrated the presence of both full length APP and short C-terminal fragments containing ß-amyloid (Haass et al., 1992a), suggesting that ß-amyloid was generated through the endosome/lysosome pathway.

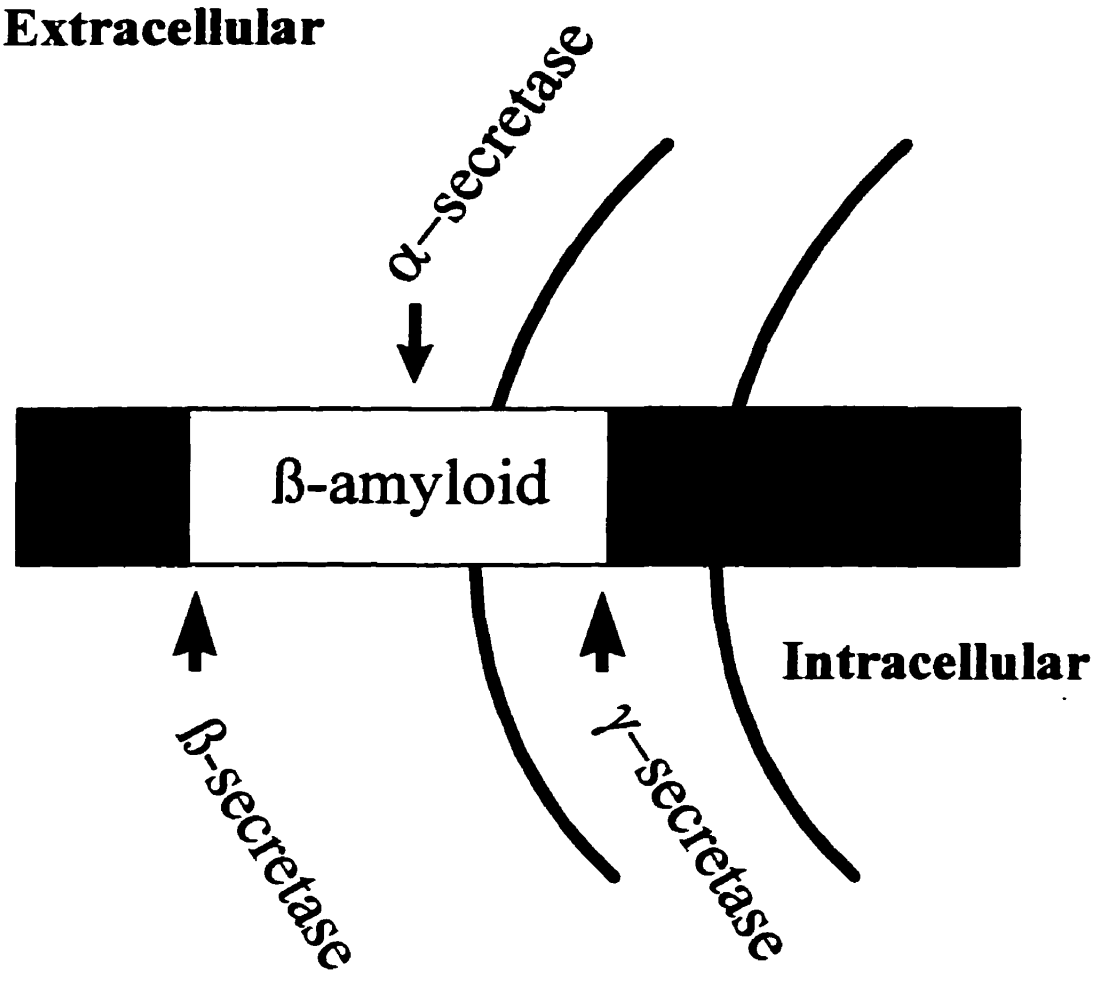
The C-terminal end of the ß-amyloid peptide is located inside the transmembrane domain of APP whereas its N-terminal end faces the extracellular milieu (see Fig. 1.1). APP is processed by proteases to give rise to a secreted form (sAPP). This cleavage which involves the α-secretase cleaves the ß-amyloid in two (Esch et al., 1990). In order to generate ß-amyloid peptides, two independent proteolytic events are thought to occur. First, the ß-secretase cleaves at the N-terminal end of ß-amyloid followed by γ-secretase cleavage at the C-terminal end to liberate ß-amyloid (Haass et al., 1992b; Seubert et al., 1992). The γ-secretase activity is responsible for the heterogeneity in the length of ß-amyloid (39-42 amino acids). So far, none of the secretases have been identified.

The β-amyloid precursor protein family.

APP is a member of a larger gene family encoding single spanning membrane proteins. An homologue of APP, called amyloid precursor protein-like (APPL), was identified in *Drosophila melanogaster* (Rosen et al., 1989). APPL immunoreactivity is first detected in developing neurons and remains associated with them after differentiation. In *Drosophila*, *APPL* expression is restricted to the peripheral and central nervous systems suggesting an evolutionarily conserved function associated with the nervous systems (Luo et al., 1990). *Drosophila melanogaster* flies deficient for *APPL* show behavioral deficits that can be compensated by introduction of human *APP* (Luo et al., 1992). *Caenorhabditis elegans* has also been reported to express an homologue of APP (Daigle and Li, 1993). Apl-1, for amyloid precursor protein-like, has only been partly characterized and whether it plays a role in development of *Caenorhabditis elegans* nervous sytems has not been determined. In humans, two genes have been identified that code for proteins related to APP. The predicted amino acid sequence deduced from the cDNA of the amyloid precursor-like protein-1 (APLP-1) shows 64% similarity to APP and shares most of its structural characteristics except for the presence of a ß-amyloid domain (Wasco et al., 1992). *APLP-1* is ubiquitously expressed with highest levels observed in the brain. Using immunohistochemistry, APLP-1 was localized to the post-synaptic density in rat as opposed to APP which is associated with synaptic membranes (Kim et al., 1997). The second gene, *APLP-2*, codes for a protein predicted to have 71% similarity (52 % identical) at the amino acid level with APP (Wasco et al., 1993). In contrast to APLP-1, both the expression pattern and cellular localization of APLP-2 are similar to that of APP (Slunt et al., 1994). Of all the members of this family of single spanning membrane proteins, only APP possesses the ß-amyloid domain found in senile plaques. Whether APLP-1 and APLP-2 play a role in the development of AD through a direct or indirect mechanism has not been determined.

Fig. 1.1 Localization of ß-amyloid in APP.

Schematic representation of the localization of β-amyloid in APP. The cleavage sites of the various secretases are indicated by arrows. Only the transmembrane region of APP is represented.


Extracellular
α–secretase
ß-amyloid
Intracellular
ß-secretase
γ–secretase

Functions of APP and ß-amyloid.

The functional role of APP has not been clearly defined yet. The protein is ubiquitously expressed and extensively processed. These reasons have greatly contributed to slowing down determination of the exact function of APP. Moreover, it is still not clear if the normal function(s) of the various forms of APP are involved in the development of AD.

The transmembrane form of APP was proposed to play a role in neurite extension. In an elegant series of experiments, Qiu et al., (1995) have shown that primary rat hippocampal neurons grown on a monolayer of CHO cells, which had been transfected with various forms of full length *APP*, had enhanced neurite outgrowth as compared to when they were grown on non-transfected CHO cells. The soluble forms of APP present in conditioned media had no significant effect on neurite outgrowth. These data suggested that the transmembrane form of APP could interact with proteins on the surface of neurons to promote the extension of neurites. The putative receptor of membrane-bound APP has not been identified yet. Another function proposed for the transmembrane form of APP is its possible role as a receptor itself, based on homology with the receptor for the insulin-like growth factor II. Nishimoto et al. (1993) proposed that the short intracellular C-terminus portion of APP could interact with G proteins. They went on to show that the amino acids located between position 657 and 676 (APP-695 numbering) interacted *in vitro* with the heterotrimeric GTP-binding protein G_o but not with other G proteins. G_o proteins have been proposed to play a role in the regulation of cellular calcium levels through voltage sensitive ion channels. It is still not clear if this putative interaction plays a role, *in vivo*, in the development of AD.

The secreted forms of APP (sAPP) were the first reported to have a putative physiological function (Oltersdorf et al., 1989; Van Nostrand et al., 1989; Sinha, et al., 1990). The alternative splicing of the *APP* mRNA leads to the presence of a Kunitz-like protease inhibitor domain (KPI) in APP770 and APP751 or its absence in APP695. The KPI domains are involved in inhibitory processes of serine type proteases, like trypsin and chymotrypsin. In fact, the secreted forms of APP containing the KPI-like domain have been shown to be protease

nexin II (Oltersdorf et al., 1989; Van Nostrand et al., 1989), a protease inhibitor involved in the clotting cascade by complexing with factor XIa (Smith et al., 1990). Another function proposed for sAPP is a role in promoting cell growth (Saitoh et al., 1989). Experiments performed by Saitoh's group identified an essential region of five amino acids (RERMS: arg-glu-arg-met-ser), which is present in all forms of sAPP, located just beyond the point of insertion of the KPI domain (Ninomiya et al., 1993). Synthetic peptides containing this sequence were able to promote the growth of neuroblastoma cells, PC12 cells as well as fibroblasts. The putative receptor involved in the growth promoting functions of sAPP molecules has not been identified.

Also, APP has been found in growing neurites during rat development (Masliah et al., 1992), associated with the cytoskeleton of embryonic cortical neurons (Allinquant et al., 1994) and sorted to axons, and to a lesser extent to dendrites, in transfected primary cultures of hippocampal cells (Tienari et al., 1996). These observations have led to the proposal that APP could be involved in growth and maintenance of synapses. The molecular details of this putative function have not been worked out.

Interactions of APP with extracellular matrix (ECM) proteins was also proposed to be one of the possible mechanisms of how APP could be involved in synaptogenesis. APP can interact physically with members of the ECM proteins. Schubert et al., (1989) demonstrated that APP could bind an analog of heparan sulfate, heparin. APP can also recognize proteins of the basement membrane such as the proteoglycan perlican (Narindrasorasak et al., 1991). Other proteins from the ECM which have been shown capable of interacting with APP are laminin (Narindrasorasak et al., 1992), entactin (Narindrasorasak et al., 1995) and collagen (Beher et al., 1996).

The secreted forms of APP added exogenously to primary cultures of hippocampal neurons have been shown to lower internal calcium concentration (Mattson et al., 1993) and increase cGMP levels (Barger et al., 1995). The effect could be reproduced with a membrane permeable analogue of cGMP. The sAPP effect is through a membrane-associated guanylate

cyclase (Barger and Mattson, 1995). cGMP has been shown to protect neurons from glutamate toxicity and consequently one of the roles of sAPP could be to protect neurons from glutamate toxicity. These observations suggested that sAPP could play a role in signal transduction. Evidence has been published proposing that one of the possible mechanisms involved in the development of AD is abnormal signal transduction in early phases of the disease (reviewed in Saitoh et al., 1991). Whether this putative abnormal transduction of signal(s) directly involves APP has not been demonstrated.

The ß-amyloid peptide is produced as a normal consequence of the processing of the precursor protein (Haass et al., 1992b; Seubert et al., 1992 and Shoji et al., 1992). It is present in the conditioned media of cultured cells, in the plasma and cerebral spinal fluid of humans and lower mammals. These findings suggest that ß-amyloid has a normal function in cell homeostasis. Soluble ß-amyloid peptides promote neurite outgrowth and survival of hippocampal neurons (Whitson et al., 1989). The neuroprotective characteristics of ß-amyloid have been shown to be dependent on its soluble state (Yankner et al., 1990). However, ß-amyloid peptides have a natural tendency to aggregate and form insoluble protein deposits (Pike et al., 1991). In tissue culture, aggregated ß-amyloid peptides were shown to be neurotoxic whereas non-aggregated forms of ß-amyloid were not (Pike et al., 1991; reviewed in Yankner, 1996).

Inactivation of the *APP* gene in mice did not yield any clues as what its normal physiological function might be (Zheng et al., 1995). Lack of expression of APP during gestation in homozygous mutant mice did not affect their normal development. The authors observed a decrease in overall body mass of 15% to 20% that correlated with a reduced food and water intake. They also observed a reduction in locomotor activity in the mutant mice when compared to wild type litter mates but did not determine the exact cause of this affliction. No abnormal pathologies were detected in APP-null mice except for diffuse reactive gliosis in certain but not all lines of mutant mice. These results suggest that APP is not necessary for normal development and/or that other members of its family (APLP-1 and 2) can compensate in part for its absence.

Alzheimer disease.

Alois Alzheimer was a psychiatrist born in Marktbreit, Germany, on June 14, 1864 (reviewed in Bick, 1994). Early in his career, he proposed that the many forms of dementias reported in textbooks of his time should be further characterized and subdivided into smaller groups. In 1906, at the meeting of southwest Germany psychiatrists held in Tübinguen, he reported the case of a 51 year old woman who had suffered from a form of pre-senile dementia. The combined psychological and histological descriptions of this first historical case of pre-senile dementia laid the foundations for the assignment of Alzheimer's name to similar cases by his colleague Emil Kraepelin, in 1910.

Today AD is recognized as the leading cause of dementia in the aging population. The age of onset of AD varies greatly. Individuals have been reported to be affected as early as the age of 35 (Martin et al., 1991). The prevalence of AD rises exponentially between the ages of 65 and 85 from 1% to 15%-25% respectively, depending on the population studied (reviewed in Katzman et al., 1994). Due to the wide distribution in the ages of onset, AD has been divided in two types: early and late onset. The age of 65 has become, for historical more than scientific reasons, the age that separates early from late onset cases of AD. Another level of distinction between forms of AD also exists: Familial Alzheimer Disease (FAD) versus Sporadic Alzheimer Disease (SAD). The distinction between FAD and SAD does not necessarily mean that one gene is involved but suggests that there may be a combination of genetic and/or environmental factors. Cases of AD that do not fall under the category of FAD are classified as SAD. These still constitute close to 75% of all the AD cases. The genetic and environmental factors that lead to the development AD are not all identified yet. The distribution of the AD cases between FAD and SAD will change as more risk factors are identified.

In the first years of the disease, individuals remain fairly independent with relatively intact judgement. With time, they become increasingly forgetful. As the disease progresses, supervision is needed and affected individuals can rarely be left alone. They start experiencing

difficulty expressing themselves, ask repetitive questions followed by progressive disorientation. In the final stages of AD, constant supervision is required. Patients develop abnormal reflexes, for example sucking or grasping. In the end, death is usually a consequence of pneumonia, sepsis or other illnesses that are frequent in the elderly (Berg and Morris, 1994). In FAD, life expectancy after the onset of the disease is between 7 to 10 years (Bird et al., 1989) but can show great variability (from 1 to 20 years). There are no significant differences between the duration of FAD and SAD.

Risk factors for Alzheimer disease.

Epidemiological studies have allowed the identification of some of the risk factors associated with AD. The most important risk factor is age (reviewed in Jorm et al., 1987). The risk of developing AD after the age of 65 doubles every 5 years. The absence of data on the population over the age 85 does not allow one to predict if this exponential progression holds after the age of 85.

Family history is another important risk factor associated with AD. The presence of a first degree relative (mother, father, brother or sister) affected by AD increases the probabilities of developing the disease later on in life (van Duijn et al., 1991). This increase is caused by the probable presence of genetic markers linked to the disease. As it was observed with age, the importance of this risk factor after the age of 80 is not known. So far, four genetic loci, located on chromosomes 1, 14, 19 and 21, have been linked to AD. The putative role of the products of the genes linked to these loci will be discussed in the section Genetics of Alzheimer Disease. Individuals who have suffered severe or repetitive head injuries have twice the risk of developing AD (reviewed in Mortimer et al., 1991). A study carried out in China (Zhang et al., 1990) determined that individuals over the age 75 who had low educational levels were twice at risk of developing AD as individuals of the same age who had completed grade eight in school. This first study was later confirmed by other groups (Snowdon et al., 1989; Katzman, 1993). In these studies, it has been put forth that increased risk of developing AD

may be associated with a reduced neuron reserve and consequently with a smaller synaptic reserve (Katzman, 1993). Gender was also proposed to be a risk factor in AD. In some studies, women were found to be at greater risk than men (Zhang et al., 1990; Heyman et al., 1991; Bachman et al., 1993). Differences in hormonal levels and education were proposed as possible explanation for these cases. So far, the risk factors associated with AD are either of a genetic nature or a combination of genetic and environmental factors.

Down Syndrome and Alzheimer disease.

Individuals affected with DS carry an extra copy or additional portions of chromosome 21. By the age of 35, these individuals develop neuropathologic characteristics that are similar to those of patients suffering from AD (Burger et al., 1973), that is formation of senile plaques and neurofibrillary tangles in similar areas of the brain. Progression of the dementia associated with DS is very similar with the one observed in AD (reviewed in Berg and Morris, 1994). 15% to 30% of DS patients will be affected by dementia by the age of 50 to 70 (Wisniewski and Rabe, 1986). The symptoms associated with DS dementia, for example increases in memory loss, decreased verbal output and increased disorientation, are all reminiscent of AD. In DS, the presence of diffuse deposits of ß-amyloid, as early as the ages of 15 to 25, precedes the formation of senile plaques and appearance of dystrophic neurites (Giaccone et al., 1989). In normal aging and in AD, diffuse ß-amyloid plaques can also be detected (Joachim et al., 1989). These observations led to the proposal that some of these diffuse deposits could, over long periods of time, become insoluble by, for example, a local increase in concentration of ß-amyloid or other proteins found in senile plaques (reviewed in Selkoe, 1994). So far, this putative course of events has not been directly demonstrated. However, overexpression of *APP*, located on chromosome 21, through its third copy, and consequently overall increase in production of ß-amyloid peptides, are thought to be the cause of all the similarities between individuals affected with DS and AD.

Genetics of Alzheimer disease.

Four genes have now been linked to Alzheimer disease. The *APP* gene on chromosome 21, the *ApoE* gene on chromosome 19 and two other recently identified genes called presenilin 1 and 2 (*PS-1* and *PS-2*) located on chromosomes 14 and 1 respectively.

β-Amyloid precursor protein.

Mutations in the *APP* gene were the first to be linked to AD. So far six mutations have been identified that segregate with the disease (Levy et al., 1990; Chartier-Harlin et al., 1991; Goate et al., 1991; Murrel et al., 1991; Hendriks et al., 1992; Mullan et al., 1992). They account for 2% to 3% of all published cases of FAD and are all missense mutations located within or in close proximity of the putative cleavage sites that give rise to the ß-amyloid peptide. These mutations are thought to alter the processing and/or the trafficking of APP. For example, mutations in amino acid 670 and 671 of APP (Swedish mutations) are located near the ß-secretase cleavage site and increase proteolytic processing which results in a 5- to 8-fold increase in production of all forms of ß-amyloid (Cai et al., 1993). Mutations around the α- and γ-secretase cleavage sites lead to an increased production of ß-amyloid (1-42), the more amyloidogenic form of ß-amyloid, when compared to ß-amyloid (1-39/40) (reviewed in Hardy, 1997). These observations suggest that an overall increase in production or an altered ratio of the various forms of ß-amyloid peptides predisposes to the development AD.

Apolipoprotein E.

The *ApoE* gene is so far the only gene that has been linked to late onset FAD and that is associated with SAD (Corder et al., 1993; Poirier et al., 1993; Saunders et al., 1993) The gene is located on the long arm of chromosome 19, contains four exons and gives rise to a 299 amino acid protein. It is the major lipoprotein expressed in the brain (Dierich et al., 1991). The mRNA is present only in glial cells although the protein can be found in neurons. Three alleles of the *ApoE* gene are found in the population, *ApoE*-ε2, *ApoE*-ε3 and *ApoE*-ε4 with respective frequencies in the human population of 7%, 78% and 15% (reviewed in Roses, 1996). These proportions vary between racial and ethnic groups, especially for *ApoE*-ε2 and *ApoE*-ε4. The

proteins differ from one an other by one or two amino acids. ApoE4 has arginine residues at positions 112 and 158 of the protein, ApoE3 has a cysteine residue at position 112 and an arginine residue at position 158 whereas ApoE2 has two cysteines residues at those same positions. These changes are sufficient to change the overall charge of the molecules, where ApoE4 is the more positively charged and ApoE2 the less positively charged (reviewed in Poirier, 1994). ApoE is a component of the very low density lipoprotein (VLDL) and high density lipoprotein (HDL) complexes involved in metabolism of cholesterol (Mahley, 1988). It has been proposed that its role in the peripheral and central nervous systems, after neuronal injury, is to scavenge and sequester cholesterol (reviewed in Poirier, 1994). The ApoE-lipoprotein complexes are then taken up by neurons through the low density lipoprotein (LDL) receptor and intracellular free cholesterol is released and presumably transported to nerve terminals where it participates in neurite outgrowth and synaptogenesis. (Poirier et al., 1991). The *ApoE* gene as a risk factor for AD was reported at the same time by Corder et al., (1993) and Poirier et al., (1993). They observed that the frequency of the *ApoE*-ε4 allele was increased in AD affected individuals over the age of 60 when compared to a control population. In their studies, the frequency of *ApoE*-ε4 in controls was 0.16 whereas in AD cases it was 0.40. This large increase was only observed for *ApoE*-ε4 and not for the other two alleles. It was also observed that the *ApoE*-ε2 allele could have a protective effect in the development of AD when compared to the other two alleles (Corder et al., 1994). Moreover, two copies of the *ApoE*-ε4 decreases the age of onset of both FAD and SAD when compared to the age of onset of individuals carrying only one copy: ε4/ε4 < ε4/ε3 < ε4/ε2 (Poirier et al., 1993; Corder et al., 1994).

In vitro, ApoE proteins interact physically with soluble ß-amyloid peptides (Strittmatter et al., 1993) and promote the formation of insoluble fibrils (Ma et al., 1994). The formation of ß-amyloid fibrils is dependent on the ApoE isoform. ApoE4 promotes the formation of insoluble ß-amyloid more potently than ApoE3 and in turn ApoE3 more than ApoE2. *In vivo,* after neuropathological examination of AD patients, individuals carrying the ApoE-ε4 allele showed a significant increase in the number of senile plaques (Schmechel et al., 1993). These observations, combined with the presence of ApoE in senile plaques, suggested that the mode

of action of ApoE in AD could be to promote the formation of soluble ß-amyloid into senile plaques in an isoform and age dependent manner. Another mechanism for ApoE in AD involving TAU has been proposed. ApoE binds *in vitro* to non-phosphorylated TAU (Strittmatter et al., 1994). ApoE3 binds more avidly to TAU then ApoE4. *In vivo*, ApoE can be found in the cytoplasm of neurons and is associated with NFT in AD (Namba et al., 1991). One of the roles of ApoE in neurons would be to protect TAU from hyperphosporylation which promotes the formation of PHF. Individuals with ApoE4 would be incapable of properly protecting TAU from this abnormal phosphorylation. So far no *in vivo* interactions between TAU and ApoE have been reported (reviewed in Yanker, 1996).

Presenilins.

The presenilin genes are responsible for the majority (~60%) of early onset FAD cases (Sherrington et al., 1996). *PS-1*, originally known as *S182* (Sherrington et al., 1995) was identified using a positional cloning strategy against a putative region located on chromosome 14 (Schellenberg et al., 1992; St George-Hyslop et al., 1992; van Broeckhoven et al., 1992). The *PS-2* gene (also known as *STM2*) was isolated based on its homology with *PS-1* (Levy-Lehad et al., 1995; Li et al. 1995; Rogaev et al., 1995) and is responsible for FAD in Volga-German families. Like *APP*, *PS-1 PS-2* are ubiquitously expressed. In rat brain, *PS-1* is mostly expressed in neurons, with some low levels observed in astrocytes and oligodendrocytes of the white matter. The highest levels of *PS-1* have been observed in the rat hippocampus, cerebellar granule cells and the choroid plexus (Kovacs et al., 1996). In humans similar results have been observed with *PS-1* and *PS-2* (Page et al., 1996; Takami et al., 1997). Results on the expression levels of the presenilins in AD cases versus controls are still controversial. Page et al. (1996) reported no differences in levels of expression of *PS-1* whereas a second report (Takami et al., 1997) observed lower RNA levels of both presenilins in the hippocampus but not in the cerebellum. Presenilins are expressed at higher levels in areas of the brain that are vulnerable to AD neuropathology, but their distribution is not sufficient to account for all the selective vulnerability observed in AD (reviewed in Tanzi et al., 1996).

The *PS-1* and *PS-2* genes encode proteins of 463 and 448 amino acids respectively. PS-1 and PS-2 share 67% homology at the amino acid level suggesting a similarity in function. The deduced amino acid sequence predicts serpentine proteins of 6 to 9 transmembrane domains (Sherrington et al., 1995; Levy-Lehad et al., 1995) with both the C- and N-terminus of the protein present in the cytoplasm (Doan et al., 1996). In 1996, 33 mutations in *PS-1* had been identified in more than 60 kindreds and families and only 2 mutations had been found in *PS-2* in two unrelated families (reviewed in Tanzi et al., 1996). The majority of mutations identified in *PS-1* and *PS-2* are localized near or within putative transmembrane domains of the proteins (for a recent compilation see Hardy, 1997).

The role of the presenilin proteins in normal cells is still being defined. Some clues about their putative functions came from their homology with *Caenorhabditis elegans* proteins SEL-12 (~50%) and SPE-4 (25 %). The SEL-12 was identified as a protein that facilitated signaling in the Notch/LIN-12 pathway that plays a role in developmental regulation of *C. elegans* (Levitan and Greenwald, 1995). Wild type *PS-1* and *PS-2* can rescue *C. elegans sel-12* mutants whereas *PS-1* carrying some of the FAD mutations can only partially rescue the mutant phenotype (Levitan et al., 1996). Mice where *PS-1* had been inactivated by homologous recombination die during embryogenesis with defects in somitogenesis (Wong et al., 1997; Shen et al., 1997) similar to the one observed in *Notch1* knock-out mice (Conlon et al., 1995). These results suggest that PS-1 could participate in Notch signaling during development but how partial inactivation or gain of function of the PS-1 protein in humans could lead to the neuropathological features and dementia associated with AD is still not known. Like the presenilins, SPE-4 is a transmembrane protein with seven hydrophobic regions. It is involved in trafficking of membrane proteins from specialized Golgi-derived organelles essential for spermatogenesis (L'Hernault and Arduengo, 1992). These observations about the intracellular localization of SPE-4 are similar to what was observed for PS-1 and PS-2. Both presenilin proteins have been localized to the endoplasmic reticulum, nuclear envelope and to a lesser extent the Golgi compartment (Kovacs et al., 1996). Both mature proteins are rapidly proteolytically processed to give rise to two fragments. PS-1 is found as two major polypeptides of 27 kDa (N-terminal fragment) and 18 kDa (C-terminal fragment) (Thinakaran

et al., 1996) whereas processing of PS-2 gives rise to a 34 kDa N-terminal fragment and a 20 kDa C-terminal fragment (Kim et al., 1997). The resulting fragments of PS-1 accumulate at a 1:1 stoichiometry (Thinakaran et al., 1996), suggesting that they are the functional units. It is still not clear whether they physically interact once processed and/or if they are part of a larger heteromeric functional complex.

Due to their predicted structure, homology to the *C. elegans* proteins SEL-12 and SPE-4 and cellular distribution, it was originally proposed that the presenilins could act as receptors, ion channels or in protein processing and/or trafficking (Sherrington et al., 1995,; Levy-Lahad et al., 1995; Kovacs et al., 1996). Evidence is now accumulating that in AD, the altered function of the presenilins is related to protein trafficking and/or processing. Fibroblast cells and plasma from patients carrying FAD mutations in *PS-1* and *PS-2* contain an increased amount of the more amyloidogenic peptide ß-amyloid (1-42/43) (Scheuner et al., 1996). Transfection of mutant presenilins in cell lines leads to a 2 to 3 fold increase in the ratio of ß-amyloid (1-42/43) over ß-amyloid (1-40) (Borchelt et al., 1996; Citron et al., 1997; Tomita et al., 1997; Xia et al., 1997). Transgenic mice expressing presenilin mutants also produce an altered ratio in favor of the longer amyloidogenic form of ß-amyloid (1-42/43) (Borchelt et al., 1996; Citron et al., 1997; Duff et al. 1996). These *in vitro* and *in vivo* results are in accordance with the observation that FAD patients carrying mutations in the presenilin genes have abundant deposits of ß-amyloid (1-42/43) (Lemere et al., 1996; GomezIsla et al., 1997) How exactly mutations in these transmembrane proteins alter the processing of APP and ß-amyloid is still unclear but the common thread of all the mutations on the presenilin genes so far studied is the marked increase in production of ß-amyloid (1-42/43).

Recently, Vito et al., (1996) identified 6 cDNA clones that modulated apoptosis in T cells. One of these cDNA coded for 103 amino acids from the C-terminus of PS-2 and protected T cells from apoptosis. Using transiently transfected nerve growth factor (NGF) differentiated PC12 cells, where apoptosis was induced by growth factor withdrawal, they observed that the C-terminus of PS-2 and antisense *PS-2* reduced apoptosis whereas full length *PS-2* and a mutant *PS-2* increased apoptosis. Pretreatment of differentiated PC12 cells with ß-amyloid (1-42)

potentiated the effect of PS-2. The effect of PS-2 on apoptosis could be blocked using pertussis toxin, an inhibitor of G_i and G_o suggesting heterotrimeric G proteins play a role in PS-2 functions. Similar results were obtained with stably transfected PC12 cells and mutant *PS-1* (Guo et al., 1997). These workers observed that the induced expression of a mutant *PS-1* led to an increase in reactive oxygen species and $[Ca^{++}]_i$ in PC12. The extent of apoptosis could be blocked by antioxidants and calcium channel blockers. They proposed that one of the consequences of the mutations present in PS-1 would be an increase in $[Ca^{++}]_i$. This change in calcium homeostasis could in turn lead to an increase in production of ß-amyloid (1-42/43) since changes in calcium concentration have been shown to affect ß-amyloid production in cultured cells (Querfurth and Selkoe, 1994).

All genes so far linked to FAD (*APP, ApoE, PS-1, PS-2*) have been found to interact with APP or ß-amyloid whether by increasing the overall production of ß-amyloid peptides, like in DS and the Swedish *APP* mutation, by changing the ratio of ß-amyloid in favor of the more amyloidogenic ß-amyloid (1-42/43) or by promoting the formation of insoluble fibrils of ß-amyloid. These observations suggest that APP and consequently ß-amyloid play a central role in the development of AD.

The amyloid hypothesis.

The observation that senile plaques are mainly composed of insoluble ß-amyloid led to the hypothesis that the neuronal degeneration observed in AD was a consequence of the presence of ß-amyloid fibrils (Yankner et al., 1989). One possible course of events happening in AD would be first the formation of diffuse plaques containing non-aggregated ß-amyloid. In DS, diffuse ß-amyloid deposits are observed well before mature senile plaques. This seeding of ß-amyloid would lead to the formation of fibrils over time and neuronal degeneration (Jarret and Landsbury, 1993). The initial formation of insoluble ß-amyloid could be triggered by the increased presence of the more amyloidogenic fragment of 42-43 amino acids of ß-amyloid (Barrow and Zargorski, 1991; Iwatsubo et al., 1994; Suzuki et al., 1994), ApoE (Strittmatter

et al, 1993), heparan sulfate proteoglycan (Fraser et al., 1992; Snow et al., 1994), heavy metals (Mantyh et al., 1993; Bush et al., 1994) or oxidation (Dyrks et al., 1992).

How does ß-amyloid act to cause neuronal damage? It has been shown that ß-amyloid is capable of inducing oxidative stress in different culture models (reviewed in Mattson, 1997) and that this sustained stress may ultimately lead to cell death. This effect of ß-amyloid seems to function through the production and accumulation of H_2O_2 (Behl et al., 1994). Interestingly, H_2O_2 has been shown to activate the NK-κB family of transcription factors (Behl et al. 1994) as well as c-Jun activity through JNK activation (Lo et al., 1996). Moreover, ß-amyloid itself has been shown to activate NF-kB (Kaltschmidt et al., 1997) and c-Jun (Anderson et al., 1995) directly. The presence of these elements in the *APP* promoter could lead to an increase in *APP* levels of expression and contribute to a positive feedback loop to increase the production of ß-amyloid, accelerating the deposition of ß-amyloid. *In vitro* experiments have shown that the levels of toxicity associated with ß-amyloid are dependent on its local concentration as well as its physical state. Overexpression of *APP* and/or overproduction of ß-amyloid could lead to an increase in the local concentration of ß-amyloid peptides and influence the rate of formation of extracellular protein deposits. Whether ß-amyloid itself initiates this chain of events has not been determined.

Rationale.

Individuals affected with DS develop the same neuropathological characteristics as in AD but at a much earlier age. These similarities are thought to arise from the third copy of the *APP* gene located on chromosome 21. Moreover, it has been shown that the mRNA levels of *APP* are 4-5 times higher in DS than in normal individuals (Neve et al., 1988) suggesting that overexpression of the APP gene may be responsible for the development of the neuropathologies observed. Increases of *APP* mRNA levels by cellular stress (IL-1 and heat shock) and by growth factors (EGF and bFGF) suggest that *APP* expression can be modulated in response to certain conditions. More recently, the development of mouse models overexpressing mutants of APP (Games et al., 1995, Hsiao et al., 1996; Moechars et al., 1996) or a C-terminal fragment containing the ß-amyloid domain (Nalbantoglu et al., 1997) have also contributed to the hypothesis that overexpression of *APP* can lead to the neurpathologies observed in AD.

These observations led us to investigate the transcriptional regulation of the *APP* gene in order to identify the DNA elements present in its promoter and the proteins recognizing them. Identification of these factors could yield information on the possible implication of transcriptional mechanisms in the development of AD.

Chapter 2

Materials and Methods

Cell culture.

The cell lines NG108-15 (Nelson et al., 1976), SK-N-SH (Biedler et al. 1973) and HepG2 (Aden et al., 1979) were maintained in Dulbecco's modified Eagle's medium (DMEM, Gibco-BRL) supplemented with 10% foetal calf serum (FCS), 2 mM glutamine, 50 U/ml penicillin and 5 μg/ml streptomycin (Gibco-BRL). PC12 cells (Greene and Tischler, 1976) were grown on collagen treated tissue culture dishes with DMEM supplemented with 10% horse serum and 5% FCS. Cells lines were maintained at 37°C in 5% CO_2 incubator. *Drosophila* S2 cells (a gift from Dr. Stefano Stifani, McGill University, Montreal) were cultivated in Shields and Sang M3 insect medium (Sigma-Aldrich) supplemented with 10% FCS and grown at room temperature in regular atmospheric conditions (on the bench).

Differentiation of NG108-15 cells.

NG108-15 cells were plated at a density of 1 x 10^6 per 100 mm tissue culture dish in DMEM supplemented with 5% FCS and 1 mM dibutyryl cyclic AMP (dbcAMP; Sigma-Aldrich). After four days, the medium was changed to DMEM supplemented with 1% FCS and 1 mM dbcAMP. Throughout the differentiation process, the medium was replaced every 48 hours with fresh medium containing 1 mM dbcAMP.

Plasmids.

The plasmid pKCXB contains an *APP* promoter/reporter chloramphenicol acetyl transferase (CAT) construct generated by introduction of an Xma III / BamH I DNA fragment from the *APP* promoter (-203 to +104) into the Sma I / BamH I sites of pKK232-8 (Bourbonnière and Nalbantoglu, 1993), a promoterless CAT vector distributed by Pharmacia Biotech Inc. To further characterize the *APP* promoter, a 3.8 kilobase (kb) BamH I fragment of genomic DNA, taken out from the plasmid pUC18BamHI3.8, containing a large portion of *APP* upstream sequences (-3699 to +100) (Salbaum et al., 1988), was introduced into the BamH I site of pBLCAT6, a high copy number, promoterless, CAT based reporter plasmid (Boshart et al., 1992), creating pAPPCAT-3699. Internal deletions in the *APP* promoter were made from pAPPCAT-3699 by single or double digestions with the following restriction enzymes: Sal I and Nsi I, pAPPCAT-3343; Sal I and EcoR V, pAPPCAT-2991; HinD III, pAPPCAT-488; Sal I and Stu I, pAPPCAT-364; Xba I, pAPPCAT-303; Sal I and Eag I, pAPPCAT-204; Sal I and Nar I, pAPPCAT-96. pAPPCAT-77 and pAPPCAT-45 were created by digesting pAPPCAT-488 with Sal I followed by a partial digestion with Bsa I and Pvu II respectively. After complete digestion of the plasmids, the ends were filled with the Klenow fragment of DNA polymerase I when necessary and ligated with T4 DNA ligase. The plasmid pBLCAT5 contains the promoter (-105 to +51) of the herpes simplex virus (HSV) thymidine kinase (TK) gene introduced in the BamH I / Bgl II sites of pBLCAT6, upstream of the CAT reporter. The plasmids pBCAT, pA2BCAT, pRSVAP2, pPac0, and pPacSp1 were gracious gifts from Dr. Robert Tjian (University of California at Berkeley). pBCAT contains the TATA box from the E1B gene of adenovirus introduced upstream of the CAT gene (Lillie and Green, 1989). pA2BCAT is a derivative of pBCAT with two AP-2 elements introduced upstream of the E1B TATA box (Williams and Tjian, 1991a). pRSVAP2 contains the cDNA sequence of the transcriptional activator AP-2 (Williams and Tjian, 1991a) introduced downstream of the Rous Sarcoma Virus long terminal repeat (RSV LTR) of pRSV, an eukaryotic expression vector. pPac0 is a vector designed for expression of cloned genes in *Drosophila* cell lines. Whereas pPac0 does not carry any eukaryotic open reading frames, pPacSp1 contains the cDNA sequences coding

for the human transcription factor Sp1 introduced downstream of the *Drosophila melanogaster* actin 5C promoter. The cDNA clone encoding the 43 kDa human USF-1 introduced into pBKS$^-$, pΔi2, was a generous gift from Dr. Michèle Sawadogo of the M.D. Anderson Cancer Centre, Houston, TX (Gregor et al., 1990). This clone was used to produce recombinant USF-1 in a coupled transcription/translation assay in combination with T7 RNA polymerase. The eukaryotic expression plasmid pCXUSF, containing a cDNA coding for USF-1 driven by the cytomegalovirus (CMV) promoter, was kindly provided by Dr Robert G. Roeder of the Rockefeller University, New York, NY (Du et al., 1993). In another series of experiments, the cDNA coding for USF-1 was removed from pΔi2 by Xho I / EcoR I restriction digest and introduced in the Xho I / EcoR I sites of the expression vector pcDNAI/amp (Invitrogen) creating the expression vector pCMVUSF-1. Plasmid p2XAdMLTF/TKCAT, kindly supplied by Dr. Howard C. Towle from University of Minnesota, Minneapolis, contains 2 recognition elements for USF from the adenovirus major late promoter (-69 to -49) introduced upstream of the minimal promoter from the TK gene of HSV (Shih and Towle, 1994). The vector pUT535, expressing the ß-galactosidase gene under the control of a CMV enhancer/promoter, was a generous gift from Dr. Ken Hastings (McGill University, Montreal).

Oligonucleotides.

Single stranded oligonucleotides were synthesized by either Dalton Chemical Laboratories Inc. (North York, Ont., Canada), General Synthesis and Diagnostics (Toronto, Ont., Canada) or the Sheldon Biotechnology Center (Montreal, Que., Canada). The double stranded oligonucleotides AP-1, AP-2 and Sp1 were purchased from Promega. Only the sequences from the coding strand are indicated for oligonucleotides used in EMSA and UV crosslinking experiments. The underlined regions mark the core binding site of the various transcription factors. Positions relative to the major transcriptional start site of the APP gene are indicated in parentheses. Mutations introduced in the *APP* promoter elements are in ***bold italics***

AP-1: 5'-CGCTTGATGAGTTCAGCCGGAA-3';

AP-2: 5'-GATCGAACTGACCGCCCGCGGCCCGT-3';

SP1: 5'-ATTCGATCGGGGCGGGGCGAGC-3' ;

Ad-USF: 5'-TGTAGGCCACGTGACCGGGT-3';

APP-GC1 (-97 to -78): 5'-CGGCGCCGCTAGGGGTCTCT-3';

APP-E1 (-56 to -37): 5'-GCCGGATCAGCTGACTCGCC-3';

APPSP1 (-71 to -50): 5'-TGCCGAGCGGGGTGGGCCGGAT-3';

SP1-USF (-71 to -37): 5'-TGCCGAGCGGGGTGGGCCGGATCAGCTGACTCGCC-3';

APP-AP-2 (-222 to -195): 5'-CGAAGCCCAGGTGGCCGTCGGCCGGGGA-3';

APPUSF-M1-1 (-59 to -36): 5'-TGGGCCGGATC***GAT***TGACTCGCCT-3';

APPUSF-M1-2 (-36 to-59): 5'-AGGCGAGTCA***ATC***GATCCGGCCCA-3';

APPUSF-M2-1 (-59 to -36): 5'-***CA***GGCCGGATC***GAT***TGACTCGCCT-3';

APPUSF-M2-2 (-36 to -59): 5'-AGGCGAGTCA***ATC***GATCCGGCC***TG***-3';

APPSP1-M1-1 (-71 to -48): 5'-TGCCGAGCGG***AACA***GGCCGGATCA-3';

APPSP1-M1-2 (-48 to -71): 5'-TGATCCGGCC***TGTT***CCGCTCGGCA-3';

APP-395FPst1 (-403 to -385): 5'-CACCCTAGC***T***GCAGTCCTT -3';

APPRBamH1 (+93 to +111): 5'-AGATCTGGATCCGCCGCGT-3';

ASAMY (+71 to +100): 5'-CGCCGCGTCCTTGCTCTGCCCGCGCCGCCA-3'

Recombinant PCR mutagenesis.

PCR buffer composition (final concentration): 50 mM KCl, 10 mM TRIS.HCl (pH 8.3 at room temperature), 1.5 mM $MgCl_2$, 0.2 mM dNTP, 0.01% (w/v) gelatin, 10% formamide and 50 pmol of each primer. PCR mixtures were denatured for 10 minutes at 94°C before the thermostable polymerase was added. Taq polymerase was purified according to Pluthero, 1993. The amount of units of the crude preparation of Taq polymerase was estimated by comparing with commercial preparations of the enzyme. Typically, each PCR reaction contained 4 units/100 µl of reaction volume. The reactions were amplified for 24 cycles in the following conditions, 94°C 1 minute, 55°C 1 minute and 72°C 1

minute. Recombinant PCR is a two step process (Horton, 1993). In the first step, two independent PCR reactions are performed using the same template. The reverse primer of reaction number one is complementary to the forward primer of the second reaction, creating an overlap. In the second step, the overlap between the two PCR products allows one to combine them in presence of the forward primer of reaction number one and the reverse primer of reaction number two in order to generate a longer PCR product. Introduction of mismatches in the middle of the overlapping oligonucleotides generates point mutations in the sequence of choice. I will use the introduction of mutations in the Sp1 element as an example for all the mutations introduced in the *APP* promoter. 500 ng of EcoR I linearized pAPPCAT-3699, which contains the wild type *APP* promoter, was used as the template. The first reaction contained the forward primer APP-395FPst1 (this oligonucleotide contains one mismatch base pair in order to introduce a Pst I restriction site to facilitate the cloning of the final PCR products) and the reverse primer APPSP1-M1-2 generating of product of 355 bp. The second PCR reaction contained APPSP1-M1-1 as the forward primer along with APPRBamH1 as the reverse primer giving a fragment of 182 bp. The reaction products were ran on a 1.5% agarose gel and purified using the Qiaex gel extraction kit from Qiagen. In the second step of recombinant PCR, 100 ng of each PCR products were combined with forward primer APP-395FPst1 and reverse primer APPRBamH1 to generate a fragment of 514 pb. The final PCR product was gel purified and digested with Pst I and BamH I and introduced in the Pst I and BamH I sites of vector pBLCAT6 creating pAPPCAT-395mSp1. Mutations in the USF element were generated in a similar fashion using instead the overlapping primers APPUSF-M1-1 and APPUSF-M1-2 creating pAPPCAT-395mUSF. In order to create the double mutant pAPPCAT-395mSU, pAPPCAT-395mSp1 was linearized with the restriction enzyme EcoR I and used as template with the overlapping oligonucleotides APPUSF-M2-1 and APPUSF-M2-2. All mutations introduced in the *APP* promoter were verified by sequencing. The plasmids pAPPCAT-96mSp1, pAPPCAT-96mUSF, pAPPCAT-96mSU, pAPPCAT-77mSp1, pAPPCAT-77mUSF and pAPPCAT-77mSU were generated from pAPPCAT-395 derivatives as described previously for the wild type promoter.

Calcium phosphate transfections.

Derivatives of the plasmid pBLCAT6 were transfected using a modification of the standard calcium phosphate protocol (Chen and Okayama, 1987). Typical transfections used 10 ug of plasmid DNA for NG108-15 cells and 15 ug for HepG2 cells. Plasmid DNA was purified using the Qiagen Plasmid Maxi Kit following the manufacturer's specifications. Cells were incubated for 16 hours at 35°C in a 3% CO_2 incubator, rinsed with 5 ml of phosphate-buffered saline (PBS) and fresh medium was added. Cells were then transferred to a 5% CO_2 incubator for 24 to 48 hours. Transfection conditions were optimized for each cell line. Refer to the figure legends for the specific transfection conditions of each experiment. Each transfection included 1 ug of the reporter plasmid pUT535 which contains the ß-galactosidase gene under the control of the CMV enhancer/promoter region to standardize for transfection efficiencies.

Lipofectin™ transfections.

When mentioned, NG108-15 cells, 5 X 10^5 cells per 60 mm tissue culture dish, were transfected with lipofectin™ (Gibco-BRL). A total of 30 ug of DNA was used in combination with 30 ug of lipofectin™ per plate transfected. Transfections were performed according to the manufacturer's guidelines. CAT assays were performed as described previously except that the reactions were allowed to proceed for 16 hours in presence of 8 mM acetyl-CoA.

Transfection of *Drosophila* S2 cells.

Drosophila S2 cells were transfected using lipofectin™ . 1 X 10^6 cells per 2 ml were plated in a 6 well tissue culture plate (Falcon). Cells were allowed to attach for 3-4 hour. For each well to be transfected, 2 polystyrene tubes were prepared containing 500 ul of serum

free Shields and Sang M3 insect medium. One tube contained 10 µg of the DNA to be transfected whereas the other tube contained 10 µg of lipofectin™ (for detailed information on each transfection, see Figure legends). The DNA and lipofectin™ were gently combined and allowed to form a complex for 15 to 30 minutes at room temperature. The medium was removed from the wells and the cells were carefully rinsed with 2 ml of PBS, before the DNA-lipofectin™ mixture was gently added to the cells. Cells were incubated at room temperature on the bench for 6 hours before the DNA-lipofectin™ mixture was aspirated and replaced with 2 ml of Shields and Sang M3 medium supplemented with 10% FCS.

Reporter gene assays.

We have used the CAT reporter system to measure the activity of various constructs containing sequences from the APP promoter. Transfected cells were washed with ice cold PBS, removed from the culture dish mechanically, collected by centrifugation (1 000 RPM) and resuspended in 100 µl of TRIS-HCl 0.25 M pH 7.5. Cellular extracts from transfected cells were generated by three cycles of freezing and thawing. The cellular debris were removed by centrifugation at 13 000 RPM for 5 minutes. CAT and ß-galactosidase assays were performed as described in Sambrook et al., 1989. Briefly, 50 µl of cellular extracts were incubated with 50 µl of TRIS 1 M pH 8.0, 20 µl of acetyl co-enzyme A, 60 mM, and 1.0 µl of ^{14}C-chloramphenicol .0.1mCi/ml, for 30 minutes at 37°C . Acetylated and unacetylated ^{14}C-chloramphenicol was extracted with 1.0 ml of ethyl acetate. 900 µl of ethyl acetate (upper phase) was collected and evaporated under vacuum for 30 minutes. Acetylated and unacetylated ^{14}C-chloramphenicol was resuspended in 20 µl of ethyl acetate. Acetylated ^{14}C-chloramphenicol was separated from unacetylated ^{14}C-chloramphenicol by thin layer chromatography in 95:5 chloroform:methanol solvent. The 20 µl of ethyl acetate was spotted 3 µl at a time on a thin layer chromatography plate. The solvent front was allowed to migrate up the plate in a chromatography chamber for 20 to 30 minutes. Thin layer chromatography plates were exposed to a PhosphorImager screen overnight. The percentage of acetylation was determined using the ImageQuant software from Molecular

Dynamics. For ß-galactosidase assays, 30 µl of cellular extracts were incubated with 3 µl of 100X Mg solution (0.1 M $MgCl_2$, 4.5 M β-mercaptoethanol), 66 µl of *o*-nitrophenyl-β-D-galactopyranoside (ONPG), and 201 µl of 0.1 M sodium phosphate. The reaction were incubated for 10 to 30 minutes at 37°C. The reactions were stopped by adding 500 µl of 1.0 M Na_2CO_3. Reactions were quantitated by measuring the optical densities at 420 nM. The percentages of acetylation from the CAT assays were standardized to ß-galactosidase activity.

Preparation of nuclear extracts.

Crude nuclear extracts were prepared using a modification of Dignam et al., 1983. Cells were washed twice with cold PBS before they were harvested from the tissue culture dishes. Cells were then centrifuged at 1 000 RPM for 10 minutes, the pellet was resuspended in five packed cell pellet volume of buffer A (10 mM HEPES pH. 7.9 at 4°C, 1.5 mM $MgCl_2$, 10 mM KCl and 0.5 mM DTT) and incubated for 5-10 minutes on ice before NP-40 was added at a final concentration of 0.5% (v/v). Cells were lysed by pipeting 4-5 times in a 10 ml tissue culture pipette. Lysis was verified under the microscope. The lysate was centrifuged for 2 to 3 minutes at 3 000 rpm at 4°C, the supernatant discarded and the nuclei pellet rinsed with 5 packed volumes of buffer A without NP-40. The nuclei were centrifuged for 2-3 minutes at 3 000 RPM and resuspended in 1 packed volume of buffer B (20 mM HEPES pH 7.9, 25% glycerol, 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.5 mM phenylmethylsulfonyl fluoride (PMSF) and 0.5 mM DTT). Nuclei were lysed by passing 10-15 times in a Kontes all glass Dounce homogenizer with a B type pestle. Extent of the lysis was verified under the microscope. The nuclei lysate was gently agitated on ice for 30 minutes. The lysate was then centrifuged at 25 000 G for 20 minutes at 4°C. The supernatant was carefully removed and dialysed twice (2 hours and overnight) against 50 volumes of buffer C (20 mM HEPES pH 7.9, 20% glycerol, 0.1 M KCl, 0.2 mM EDTA, 0.5 mM PMSF and 0.5 mM DTT). The dialysate was centrifuged at 25 000 G for 20 minutes and the supernatant was quickly aliquoted and frozen at -80°C. All manipulations were performed on ice or at 4°C.

DTT and PMSF were added fresh. Protein concentrations were determined using the Pierce BCA Protein Assay Reagent kit (Pierce). HeLa nuclear extracts were purchased from Promega.

Electrophoretic mobility shift analysis.

For annealing, equimolar amounts of complementary single stranded oligonucleotides were heated at 95°C for 10 minutes and allowed to cool down at room temperature over a period of 2 hours in 10 mM TRIS-HCl pH 7.8, 1 mM EDTA and 100 mM NaCl. Double stranded oligonucleotides were end-labeled using γ-^{32}P-ATP and T4 polynucleotide kinase (Sambrook et al., 1989). When end-labelled restriction fragments were used, they were labeled according to Sambrook et al., (1989) using the Klenow fragment of DNA polymerase I, α-^{32}P-dCTP and/or α-^{32}P-dGTP and purified on a polyacrylamide gel (10%). Binding reactions were performed at room temperature in a final volume of 15 µl (4% glycerol, 1.0 mM $MgCl_2$, 0.5 mM dithiothreitol, 50 mM NaCl and 10 mM TRIS-HCl pH 7.5) in presence of 0.5-1.0 µg of poly (dI-dC) - poly (dI-dC) and 6 to 8 µg of nuclear proteins. Probes (0.1 ng: 3.5-5.0 x 10^5 cpm/ng) were added 10 minutes after the nuclear extracts and the binding reactions were allowed to proceed for 20-30 minutes at room temperature. The concentration of DTT was increased from 0.5 mM to 4.0 mM for the studies on the USF transcription factor. For binding reactions involving the AP-2 transcription factor, the following binding buffer was used: 25 mM TRIS-HCl pH 8.0, 6.25 mM $MgCl_2$, 0.5 mM EDTA, 0.5 DTT and 10% glycerol. When present, specific cold oligonucleotide competitor was added 5 minutes before the probe. For "supershifts", polyclonal antibodies (Santa Cruz Biotechnology Inc.) were added to the binding reaction after 20 minutes and incubated at room temperature for an additional 30 minutes. Samples were analyzed on a 5% polyacrylamide gel containing 2.5% glycerol. Electrophoresis was performed at 10 mA for 1.5-2.0 hours in 0.5X TRIS-borate-EDTA (TBE) (Sambrook et al., 1989) with circulating cold water. The gels were dried and exposed to Kodak XAR-5 film for 16 hours at -70°C or exposed to phosphor screens and analyzed on a Molecular Dynamic PhosphorImager using the ImageQuant software.

DNase I footprinting analysis.

DNase I footprinting analysis of the *APP* proximal promoter was performed on an end-labelled Xma III / BamH I (-203 to +100) fragment. In order to specifically label one strand, the plasmid pUC18BamHI3.8 was either digested with the restriction enzymes Xma III (for non-coding strand labelling) or BamH I (for coding strand labelling). The restriction fragments were end-labelled with α-^{32}P-dCTP and α-^{32}P-dGTP using Klenow polymerase (Sambrook et al., 1989). The end-labeled fragments were further digested in order to generate the 303 bp Xma III / BamH I fragment. The restriction fragments were gel purified before they were used in DNase I footprinting analysis. The binding of nuclear proteins to the end-labelled Xma III / BamH I fragments was performed under the same buffer conditions as those of the electrophoretic mobility shift analysis. The volume of the reaction was increased to 50 µl with 20 000 cpm of probe (3-10 X 10^4 cpm/ng) in presence of 5 µg of poly (dI-dC) - poly (dI-dC) and 60 µg of crude nuclear extract proteins. When purified Sp1 was used, the reaction included 0.05 µg poly (dI-dC) - poly (dI-dC) and 160 ng of Sp1. After 20 minutes of binding, 50 ul of solution A (5 mM $CaCl_2$, 10 mM $MgCl_2$) was added, DNase I digestion was performed at room temperature for 60 seconds and stopped with 100 µl of solution B (20mM EDTA, 1% SDS, 100 mg/ml tRNA). The products were phenol-chloroform extracted, ethanol precipitated and then resolved on a 6% denaturing polyacrylamide gel. The gels were dried and exposed to Kodak XAR-5 film for 16 hours at -70°C.

SDS-polyacrylamide gel electrophoresis.

Proteins (10 to 100 µg) were resuspended in sample buffer (final concentration: 50 mM TRIS-HCl pH 6.8, 2% SDS (w/v), 10% glycerol (v/v), 0.01% bromophenol blue (v/v) and 100 mM DTT (added fresh). The separating gel consisted of 10% acrylamide (29:1 acrylamide : BIS-acrylamide), 0.375 M TRIS-HCl pH 8.8 and 0.1% SDS (w/v). TEMED (0.05% v/v) and ammonium persulfate (0.05% w/v) were added to allow polymerization.

The stacking gel was composed of 4% acrylamide (29:1, acrylamide : BIS-acrylamide), 0.125 M TRIS-HCl pH 6.8 and 0.1% SDS (w/v). 0.1% (v/v) of TEMED and 0.05% (w/v) of ammonium persulfate were added for polymerization. Electrophoresis buffer consisted of 25mM TRIS, 250 mM glycine (pH 8.3) and 0.1% SDS. Proteins were electrophoresed at a 100 volts until bromophenol blue reached the bottom of the gel. Gels were dried and either exposed to Kodak XAR-5 film or to phosphor screen and analyzed on a Molecular Dynamic PhosphorImager with the ImageQuant software.

UV crosslinking.

UV crosslinking was performed once appropriate binding conditions were defined by EMSA. Reaction volumes were increased from 15 µl for EMSA to 50 µl for UV crosslinking which took place under the following conditions: 18 µg of crude nuclear extracts, 4% glycerol, 1.25 mM $MgCl_2$, 0.5 mM DTT, 50 mM NaCl and 10 mM TRIS-HCl pH 7.5, 10,000-20,000 cpm of end-labeled oligonucleotide probe (specific activity of 2-4 x 10^5 cpm/ng) and 3 µg of poly (dI-dC) - poly (dI-dC). The probe was added 10 minutes after the extracts and incubated another 20 minutes. When unlabelled specific oligonucleotide competitor was present, it was added 10 minutes before the labeled fragment. After the incubation period the reaction mixture was transferred from a 1.5 ml microcentrifuge tube to a 1.8 ml Nunc cryotube or to a Falcon (Lincoln Park, NJ) flat bottom polystyrene 96-well plate. The samples, on ice, were positioned 5 cm below the UV light source and exposed for various lengths of time (5 min - 60 min) (Bourbonnière et al., 1997). They were then resuspended in SDS sample buffer and electrophoresed on a 10% polyacrylamide gel (SDS-PAGE). To visualize the crosslinked proteins, gels were dried and exposed overnight at -70°C to Kodak (Rochester, NY) XAR-5 film or to phosphor screens and analyzed on a Molecular Dynamic PhosphorImager using the ImageQuant software.

In Vitro Transcription and Translation- TNT™ .

Coupled Reticulocyte Lysate System from Promega was used as recommended by the manufacturer to synthesize *in vitro* the 43 kDa USF-1 protein from 2 μg of plasmid pΔi2. Efficiency of USF-1 synthesis was verified by radioactively labelling an aliquot with ^{35}S-methionine and the molecular weight of the translation product was confirmed by SDS-PAGE.

In vitro Transcription.

Run-off *in vitro* transcription assays were performed using the HeLa Nuclear extract *in vitro* transcription system (Promega). Template pAPPCAT-96 was linearized by restriction enzyme digest with Msc I, resulting in a transcript of 638 nucleotide fragment after run-off transcription assays. The final reaction contained 1 μg of linearized templates incubated in 8 mM Hepes, 40 mM KCl, 0.08 mM EDTA, 1.25 mM DTT, 3 mM $MgCl_2$, 8% glycerol, 0.4 mM rATP, 0.4 mM rCTP, 0.4 mM rUTP, 0.016 mM rGTP and 1 ul of α-^{32}P-GTP (3000 Ci/mmol, 10 mCi/ml) in a volume of 25 ul. The amount of protein varied from 20 ug to 80 ug per 25 ul reactions (please refer to specific figure legends). The transcription reactions were incubated at 30°C for 60 minutes and stopped by adding 175 μl of 0.3 M TRIS-HCl (pH 7.4), 0.3 M sodium acetate, 0.5% SDS (w/v), 2 mM EDTA and 3 μg/ml tRNA. The resulting 200 μl were extracted with phenol:chloroform:isoamyl alcohol (25:24:1) and the aqueous phase was ethanol precipitated by adding 500 ul of ice cold ethanol. After 30 minutes at -80°C, the precipate was collected by centrifuging at 12 000 g for 20 minutes at 4°C. The supernatant was carefully decanted and the pellet was vacuum dried for 5-10 minutes. The pellet was resuspended in 10-20 ul of RNase free water. One volume of loading dye (98% formamide, 10 mM EDTA, 0.1% xylene cyanol and 0.1% bromophenol blue) was added to the samples before they were incubated at 95°C for 5 minutes and loaded onto a denaturing polyacrylamide gel (6% acrylamide (39:1 acrylamide:BIS), 7.0 M urea and 1X TBE (0.09 M TRIS-borate, 0.002 M EDTA)). The gel

was run in 1X TBE at 250 volts for 2.5 hours, dried and exposed to XAR-5 film or to phosphor screens and analyzed on a Molecular Dynamic PhosphorImager using the Image Quant software.

For run-on *in vitro* transcription assays, 2 µg of supercoiled pAPPCAT-96, pAPPCAT-96mSp1, pAPPCAT-96mUSF and pAPPCAT-96mSU were used as templates, radioactive nucleotides were omitted, consequently the final concentration of cold rGTP was increased to 0.4 mM, the amount of proteins was 160 µg in a final volume of 50 µl. After ethanol precipitation, the products of run-on *in vitro* transcription were subjected to primer extension assays in order to determine the transcriptional start sites of the *APP* promoter mutants.

Primer extension assays.

Primer extension assays were performed according to Sambrook et al., 1989. 1 x 10^5 cpm of the end-labeled primer ASAMY (Anti-Sens AMYloid), complementary to APP mRNA (+71 to +100), were added either to the products of run-on *in vitro* transcription assays or to 50 µg of total mRNA and ethanol precipitated by adding 0.1 volume of 3.0 M sodium acetate (pH 5.2) and 2.5 volume of ethanol. The mixture was stored at -20°C for 30 minutes. The RNA-primer precipitate was collected by centrifuging at 12 000 g for 20 minutes at 4°C. The pellet was rinsed with ice cold 70% ethanol, air dried and dissolved in 30 µl of hybridization buffer (40 mM PIPES (pH 6.4), 1 mM EDTA (pH 8.0), 0.4 NaCl and 80% formamide). heated at 85°C for 10 minutes and then quickly transfered to 30°C for 8 to 12 hours. After the hybridization was complete, 170 µl of RNase free water and 400 µl of ethanol were added and the mixture was stored at -20°C for one hour. The precipitate was collected as previously described and dissolved in 20 µl of reverse transcription buffer (50 mM TRIS-HCl (pH7.6), 60 mM KCl, 10 mM $MgCl_2$, 1 mM of each dNTP, 1 mM DTT, 1 unit/µl of placental RNase inhibitor (Gibco-BRL) and 50 µg/ml of actinomycin D). Murine reverse transcriptase, (50 units per reaction) was added

and polymerization was allowed to proceed for 2 hours at 37°C. To terminate the reaction, 1 µl of 0.5 M EDTA was added, followed by 1 µl of pancreactic DNase-free RNase (final concentration 5 µg/ml). The reaction was incubated for 30 minutes at 37°C before 150 µl of TNE (10 mM TRIS-HCl (pH 7.6), 0.1 M NaCl and 1 mM EDTA). The resulting 200 µl was extracted with phenol:chloroform (1:1) (200 µl). The aqueous phase was ethanol precipitated as previously described with 500 µl of ice cold ethanol. The pellet was dissolved in 4 µl of TE (pH 7.4). After completely resuspending the pellet, 6 µl of formamide loading buffer (80% formamide, 10 mM EDTA (pH 8.0), 1 mg/ml xylene cyanol and 1 mg/ml bromophenol blue) were added and the samples were heated at 95°C before being loaded onto at a denaturing polyacrylamide gel (6% acrylamide (39:1 acrylamide:BIS), 7.0 M urea and 1X TBE. Sequencing reactions (performed as recommended by the manufacturer, NEB) were present on the gels and were used to determine the size of the primer extension assay products.

Chapter 3

Results

Functional activity of proximal regulatory elements of the human ß-amyloid precursor protein promoter in NG108-15 cells.

Since overexpression of the *APP* gene, through its duplication, may be involved in the early formation of ß-amyloid deposits in DS, we initiated studies on the regulation of *APP* gene expression. We were particularly interested in cell type-specific regulation of *APP*, identification of functionally important *cis*-acting sequences and characterization of the *trans*-acting factors which bind them. In this chapter, results will be presented showing that sequences present in the *APP* promoter previously reported to be active in HeLa cells (Pollwein et al., 1992; Quitschke et al., 1992) are also functional in NG108-15 cells, a hybrid neuroblastoma X glioma cell line generated by fusion of mouse neuroblastoma N18TG2 and rat glioma C6-BU-1, which exhibits a cholinergic neuronal phenotype and can be induced to extend neurites when exposed to dibutyryl cAMP (Nelson et al., 1976). This cell line expresses *APP* constitutively (Shekarabi, 1996)

Salbaum et al., (1988), were the first to characterize the *APP* promoter. The *APP* promoter is located in a 3 800 bp BamH I genomic fragment on chromosome 21, has multiple trancriptional start sites and lacks a TATA box. To test for the presence of transcriptional activity in NG108-15 cells, we cloned 5' genomic DNA fragments of various lengths from the *APP* promoter upstream of the bacterial CAT gene present in pKK232-8 (Pharmacia Biotech Inc.). These constructs were transfected into NG108-15 using lipofectin™ reagent. The smallest construct, in which CAT expression was driven by 203 base pairs (bp) of *APP*

upstream sequence (Fig. 3.1 A) (shown previously to contain the minimal promoter required for expression in HeLa (Pollwein et al., 1992) and PC12 (Lahiri and Robakis, 1991) cells, had transcriptional activity in both NG108-15 cells and NG108-15 cells treated with dibutyryl cAMP (Fig. 3.1 B, lanes 2 and 4) since acetylated ^{14}C-chloramphenicol can be observed in lanes 2 and 4 (upper spots) where untranfected cells only show unacetylated ^{14}C-chloramphenicol (lanes 1 and 3). Thus these sequences are also sufficient for basal promoter function in the NG108-15 cell line and moreover, the transcriptional activity is not altered upon differentiation of the cells with dibutyryl cAMP (Fig. 3.1 B).

To identify possible sites for DNA binding proteins within this region, we performed an electrophoretic mobility shift analysis on restriction subfragments of the *APP* proximal promoter. Using this approach, the highest affinity binding was observed with a 48 bp Msp I fragment (-100 to -52) in the presence of nuclear extracts prepared from NG108-15 cells (Fig. 3.2). The specificity of this binding was verified by a competition assay in which the nuclear extracts were incubated with an excess of unlabelled 48 bp Msp I fragment prior to the addition of the labeled 48 bp fragment. A 10-fold excess of the unlabelled 48 bp fragment effectively diminished the observed shift(s) (Fig. 3.2, lane 5). However, no competition occurred in the presence of a 70-fold excess of an Sp1-specific double-stranded oligonucleotide (Fig. 3.2, lane 3), indicating that the observed shift was not due to recognition of the partial and putative Sp1 site present in this fragment. We also observed a weak mobility shift of the adjacent 25 bp MspI fragment (-123 to -98) which contains two GC boxes (data not shown).

The specific sequences bound by the nuclear proteins were identified by DNase I footprinting experiments on a Xma III / BamH I fragment, spanning -203 to +100, end-labeled on the non-coding strand. As shown in Figure 3.3 lane 2, the strongest DNase I footprinting activity detected in NG108-15 cells covered the -128 to -63 region of the promoter. In HeLa cells, sequences from -94 to -35 act as a transcriptional activator with three domains which interact with DNA-binding proteins as determined by DNase I footprinting analysis (A:-63 to -47; B:-82 to -75; and C:-97 to -87) (Pollwein et al. 1992). In

Fig. 3.1 Functional activity of the proximal promoter region of the human *APP* gene.

A) Schematic representation of the XmaIII / BamH I fragment (-203 to + 100) used in the transfection experiments. The open arrows indicate the MspI sites used to generate fragments for electrophoretic mobility shift analysis. The stippled boxes represent the 6 copies of the 9 bp GC-rich element, the solid box marks the putative AP-1 binding site. X: XmaIII; B: BamHI. The arrow marks the major transcriptional initiation site. Based on Salbaum et al. (1988). **B)** Functional activity of the proximal upstream region of the human *APP* gene in NG108 -15 cells. The CAT construct depicted in A) was transfected into NG108-15 cells (lane 2) and NG108-15 cells treated with dibutyryl cAMP (lane 4). NG108-15 cells were cultured for three days in the presence of 1mM dibutyryl cAMP prior to transfection and maintained in its presence during the transfection (24-48 hours). The corresponding mock transfections were carried out in parallel (lanes 1 and 3). Results of the CAT assays (TLC plate) were exposed overnight to a XAR-5 kodak film. Transfection efficiency was standardized through co-transfection with pUT535, a CMV-ß-galactosidase expression vector.

A

B

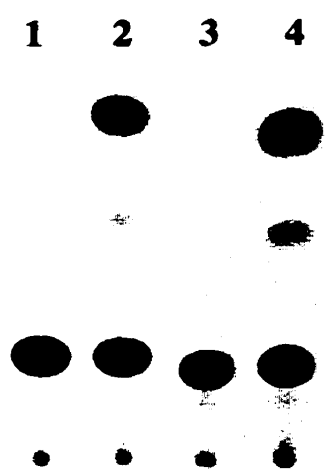


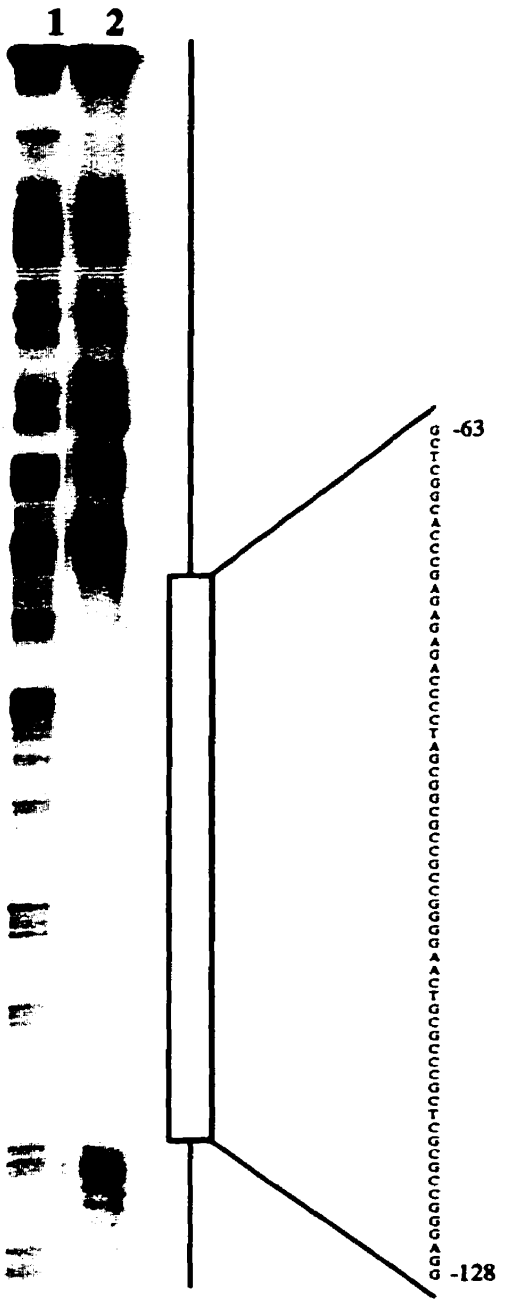
Fig. 3.2 Electrophoretic mobility shift analysis of the 48 bp MspI fragment (-100 to -52).

Competition of mobility shift (lane 2) by pre-incubation of nuclear extracts with an excess of 2.5-fold (lane 4), 10-fold (lane 5) and 50-fold (lane 6) of unlabelled 48 bp fragment. Note that no competition is observed in the presence of a 70-fold excess of unlabelled Sp1 double-stranded oligonucleotide (Promega) (lane 3). Free probe (in the absence of nuclear extracts) is shown in lane 1. F represents the free probe.


1 2 3 4 5 6
F

Fig. 3.3 DNAse I footprinting analysis of the restriction fragment Xma III / BamH I of the *APP* promoter.

DNAse I footprinting analysis in the absence (lane 1) and presence (lane 2) of nuclear extract from NG108-15 cells. Binding of nuclear extract proteins to the end-labeled XmaIII / BamHI fragment was performed under the same buffer conditions as those of the electrophoretic mobility shift analysis.


1
2
-63
-128

NG108-15 nuclear extracts, the footprinted region encompasses both the B element consisting of a pyrimidine tract, the C element characterized by a GC-palindrome as well as the GC-rich distal sequences (-133 to -122) described by Pollwein et al., (1992). The nuclear factors which bind element B and C have not been identified. We did not detect the protected area corresponding to region A when using nuclear extracts from NG108-15.

We focused our attention on the first 94 bp of upstream regulatory sequences since deletion analysis had shown that they were sufficient to confer full activity to the *APP* promoter. To further characterize the binding site(s) present within the 48 bp Msp I fragment, we used an oligonucleotide, APP-GC1 (-97 to -78), in EMSA with NG108-15 nuclear extracts. This oligonucleotide is part of the DNase I protected region when using NG108-15 nuclear extracts (-128 and -63) and contains the GC-palindrome (-97 to -87) region shown to be protected from DNase I digestion with HeLa nuclear extracts (Pollwein et al., 1992). It does not contains the full pyrimidine tract (-82 to -75) which was shown by site-directed mutagenesis not to affect *APP* regulation in transient transfection assays (Quitschke, 1994). Incubation of the double stranded APP-GC1 oligonucleotide with nuclear extracts from NG108-15 cells led to the formation of a specific complex (Fig. 3.4, lane 2). This complex could be competed with an 125 fold excess of unlabelled APP-GC1 (Fig. 3.4 lane 3) whereas the same molar excess of GC-rich oligonucleotides containing AP-2 (Fig. 3.4, lane 4) and Sp1 binding sites (Fig. 3.4 lane 5), had no effect.

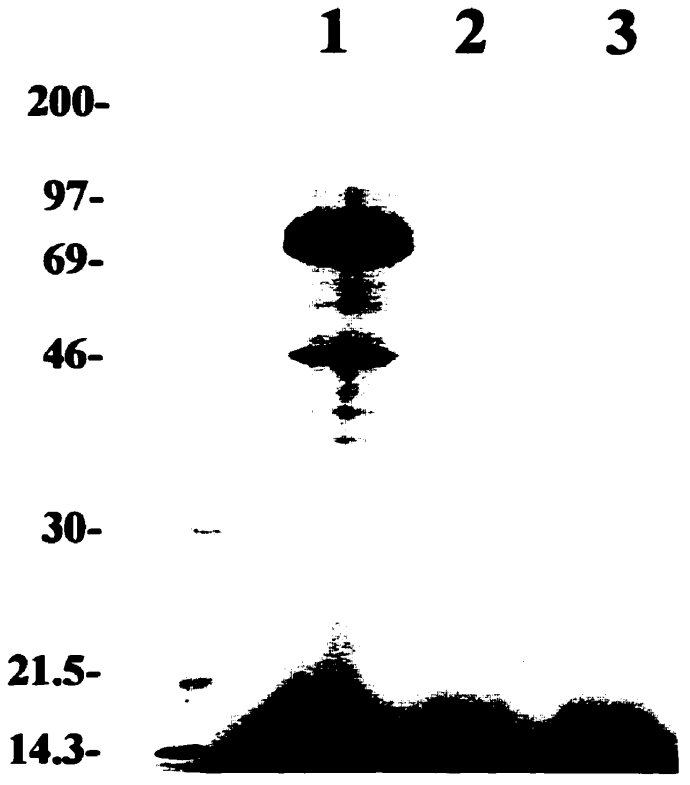
We used UV crosslinking of radio-labeled DNA to proteins in order to determine the molecular weight of the DNA binding protein(s)-complex recognizing the APP-GC1 oligonucleotide. Using conditions defined in EMSA, the labeled DNA-protein complex was UV crosslinked and electrophoresed on SDS-polyacylamide gels. Two major complexes were observed (Fig. 3.5 lane 2) between molecular weights of approximately 75 and 85 kDa. Another smaller complex could also be observed with a molecular weight of 48-50 kDa. Minor complexes were visible between 38 kDa and 100 kDa. Our attempts to identify, using a cDNA expression library, a gene encoding a protein capable of recognizing the oligonucleotide APP-GC1 did not yield positive results.

Fig. 3.4 Electrophoretic mobility shift analysis of oligonucleotide APP-GC1.
The major complex formed between APP-GC1 and proteins from NG108-15 nuclear extracts was efficiently competed by 125 fold molar excess of unlabeled APP-GC1 (lane 3). Oligonucleotides carrying the binding sites for transcription factors AP-2 and Sp1, lane 4 and 5 respectively, did not abolish the complex at similar molar excess of 125 fold. Lane 1 represents the migration of APP-GC1 in absence of crude nuclear extracts (F: free probe).

F

Fig. 3.5 UV-photocrosslinking of oligonucleotide APP-GC1.

The oligonucleotide APP-GC1 was UV crosslinked with proteins of nuclear extracts from NG108-15 cells. The binding conditions were the same one used in EMSA. 50 μg of NG108-15 crude nuclear extracts were used for lane 2 whereas 7 μg of purified transcription factor AP-2 were present for lane 3. Molecular weight markers (^{14}C- labeled) (BioRad) are shown in lane 1 and free probe in lane 4.


1
2
3
200-
97-
69-
46-
30-
21.5-
14.3-

In summary, these results show that the *APP* proximal promoter is functional in NG108-15 cells. DNase I footprinting analysis suggests that essentially the same proximal *cis*-elements are recognized by nuclear factors from both NG108-15 and HeLa cells. Furthermore, we demonstrate that an element located in the *APP* proximal promoter between positions -97 and -78, relative to the major transcriptional start site, is recognized specifically by multiple DNA binding proteins. Whether these proteins are expressed predominantly in neuronal tissue remains to be determined. More distal sequences also may be involved in the tissue-specific modulation of *APP* expression.

Chapter 4

Results

The helix-loop-helix transcription factor USF interacts with the basal promoter of human ß-amyloid precursor protein gene

The presence of a putative AP-1 element in the *APP* proximal promoter raised the issue of whether *APP* gene expression could be regulated by members of this family of immediate early genes, which includes *fos*, *jun* and *ATF* (reviewed in Karin et al., 1997). Characterization of the *APP* proximal promoter by DNase I footprinting analysis, methylation interference and EMSA revealed that the putative AP-1/AP-4 element, located between positions -50 and -39 relative to the major transcriptional start site, was recognized by DNA-binding proteins present in rat brain (Quitschke and Goldgaber, 1992) and HeLa cell (Pollwein, 1993) nuclear extracts. Neither an AP-1 consensus oligonucleotide nor an AP-4 consensus oligonucleotide could prevent nuclear proteins from binding to this region in EMSA (Quitschke and Goldgaber, 1992).

It is of interest that the core of the footprinted sequence of the AP-1/AP-4 element contains an E box (CANNTG) at position -49 to -44. DNA-binding proteins of the basic helix-loop-helix (bHLH) family have been shown to recognize the E box motif which was first characterized in the enhancer and promoter regions of the immunoglobulin genes (Ephrussi et al., 1985). The bHLH proteins contact DNA through their basic residues while the HLH domain, composed of two amphipathic α-helices separated by a loop, is responsible for dimerization (Kadesch, 1993). This family of proteins can be further subdivided according to their expression pattern (ubiquitous versus tissue-specific), their dimerization properties (homodimer versus heterodimer), and structural characteristics. In this respect, one class of

bHLH proteins contains a leucine zipper (LZ) located C-terminal to the HLH domain (Ferré D'amaré et al., 1993). The entire HLH-LZ domain is essential for proper dimerization but does not affect the activation function (Beckman and Kadesch, 1991). Members of this family include the Myc proteins as well as their partners Max, Mad (Ayer et al., 1993) and Mxi (Zervos et al., 1993); USF (Sawadogo and Roeder, 1985) (upstream stimulatory factor also known as major late transcription factor MLTF (Carthew et al., 1985)), first identified as an activator of the adenovirus late promoter; and TFE3 (Beckmann et al., 1990) which binds the immunoglobulin heavy chain enhancer.

To identify the nuclear factor which interacts with the *APP* E box, we performed UV crosslinking experiments between an end-labeled oligonucleotide from this region and nuclear extracts of HeLa cells. This preliminary analysis revealed that the nuclear proteins binding to this region were all below 50 kDa in molecular weight, ruling out Myc and E2A (67 kDa), and implicating USF (43-44 kDa) (data not shown). USF binding activity can be ascribed to homo- or heterodimers of the 43 kDa USF-1 protein or 44 kDa USF-2. The 43 kDa USF-1 can bind as a homodimer to the E box in the upstream region of several cellular genes and transactivate them (Carthew et al. 1987; Chodosh et al., 1987; Sato et al., 1989; Bresnick and Felsenfeld, 1993; Reisman and Rotter, 1993). USF-1 and USF-2 are expressed ubiquitously, interact physically to homo- or heterodimerize, recognize the same consensus sequence in DNA and transactivate genes carrying a USF binding site in co-transfection assays (Sirito et al., 1992; Sirito et al., 1994). The known characteristics of USF concorded well with the expression pattern of the *APP* gene: USF, a ubiquitous factor, has been shown to bind to promoters that are regulated in a developmental or tissue-specific manner (Carthew et al. 1987; Sato et al., 1989; Bresnick and Felsenfeld, 1993). In this chapter we show that USF binds the *APP* E box and activates transcription of a reporter gene linked to upstream sequences of the *APP* gene.

A USF-like factor present in nuclear extracts from HeLa cells binds to APP-E1.

To identify proteins which bind to the E box (APP-E1) closest to the transcription initiation site of the *APP* gene (Fig. 4.1), EMSA was performed with a 20 base pair end-labeled oligonucleotide (-56 to -37) incubated with nuclear extract from HeLa cells. Two major DNA-protein complexes were observed (Fig. 4.2A). The amount of complex formed varied in a dose-dependent manner on the amount of protein in the nuclear extract (data not shown). The binding specificity of the DNA-protein complex was verified by a competition assay in which the nuclear extracts were incubated with an excess of unlabelled APP-E1 oligonucleotide prior to addition of labeled oligonucleotide. A fifty-fold molar excess of unlabelled APP-E1 oligonucleotide effectively diminished the observed shift(s) (Fig. 4.2A, lane 4). The formation of the DNA-protein complex could also be abolished by competition with a 20 base pair oligonucleotide, Ad-USF, containing the E box sequence from the adenovirus major late promoter (-67 to -58) which is recognized by the transcription factor USF (Fig. 4.2A, lanes 5 to 7) (Sawadogo and Roeder, 1985). The competition by the Ad-USF oligonucleotide occurred at the same range of concentrations as that provided by APP-E1. The converse was also true in that the APP-E1 oligonucleotide interfered with the binding of nuclear protein(s), presumably USF, to Ad-USF (Fig. 4.2A, lanes 9 to 11). Although the APP-E1 oligonucleotide contains consensus recognition sites for AP-1, AP-4 and an incomplete Sp1 site, the formation of the complex between APP-E1 and HeLa cell nuclear proteins was not affected by the presence of a thousand-fold molar excess of unlabelled oligonucleotide containing the recognition sequence of either AP-1, Sp1 or the unrelated AP-2 (Fig. 4.2B). The results of the EMSA experiments suggest that APP-E1 and Ad-USF can be bound by nuclear proteins in a reciprocal fashion.

APP-E1 - protein complex is recognized by an antibody against USF-1 .

To further characterize the nuclear protein which binds to APP-E1 and to determine whether the complex contains either authentic USF or a USF-related protein, we performed EMSA in the presence of an antiserum against the 43 kDa USF protein. This rabbit polyclonal antibody reacts only very weakly with the 44 kDa USF-2 protein (Van Dyke te

Fig. 4.1 Structural features of the 5' upstream regulatory region of the human *APP* gene.

Illustration representing the putative binding sites of transcription factors present in the DNA sequences surrounding the transcription start site of the human *APP* promoter as first reported. GC boxes (Salbaum et al., 1988), GC-palindrome (Pollwein et al., 1992), Sp1 element (Pollwein, 1993) and AP-1/AP-4 (Salbaum et al., 1988). Numbers indicate the position relative to the major transcriptional star site (+1: arrow).

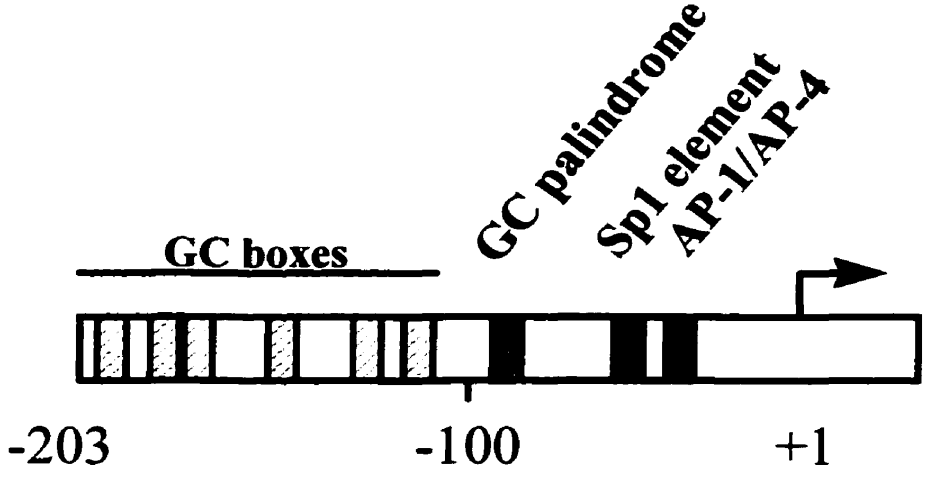
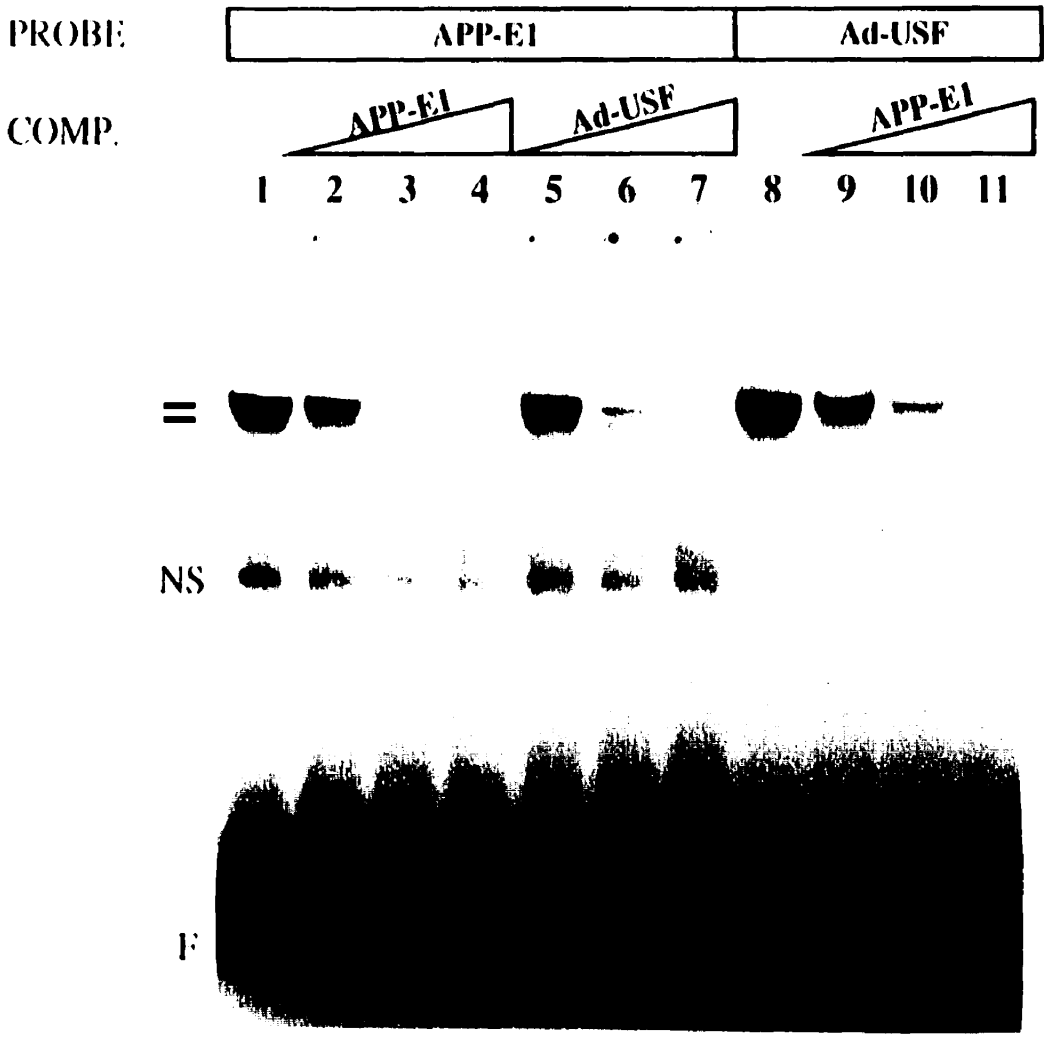

GC palindrome
Sp1 element
AP-1/AP-4
GC boxes
-203
-100
+1

Fig. 4.2 The APP-E1 and Ad-USF bind to a common factor(s) in HeLa cell nuclear extract.

EMSA was performed as described in Materials and Methods. **A)** End-labeled APP-E1 (lanes 1-7) or Ad-USF (lanes 8-11) oligonucleotides were incubated with nuclear extracts from HeLa cells in the absence (lanes 1 and 8) or presence of increasing amounts of unlabelled APP-E1 competitor (lanes 2 and 9, 10-fold; lanes 3 and 10, 25-fold; lanes 4 and 11, 50-fold molar excess) or Ad-USF competitor (lane 5, 10-fold; lane 6, 25-fold; lane 7, 50-fold molar excess). **B)** EMSA was carried out with an end-labeled APP-E1 oligonucleotide incubated with HeLa cell nuclear extracts in the absence (lane 1) or presence of a 1000-fold molar excess of unlabelled oligonucleotides containing the consensus recognition sequence of AP-1 (lane 2), AP-2 (lane 3) and Sp1 (lane 4). Specific DNA-protein complexes are labeled by a bar. Comp. indicates competitor oligonucleotides; NS, non-specific complexes; F, position of free probe.

A


PROBE
APP-E1
Ad-USF
COMP.
APP-E1
Ad-USF
APP-E1
1 2 3 4 5 6 7 8 9 10 11
=
NS
F

B

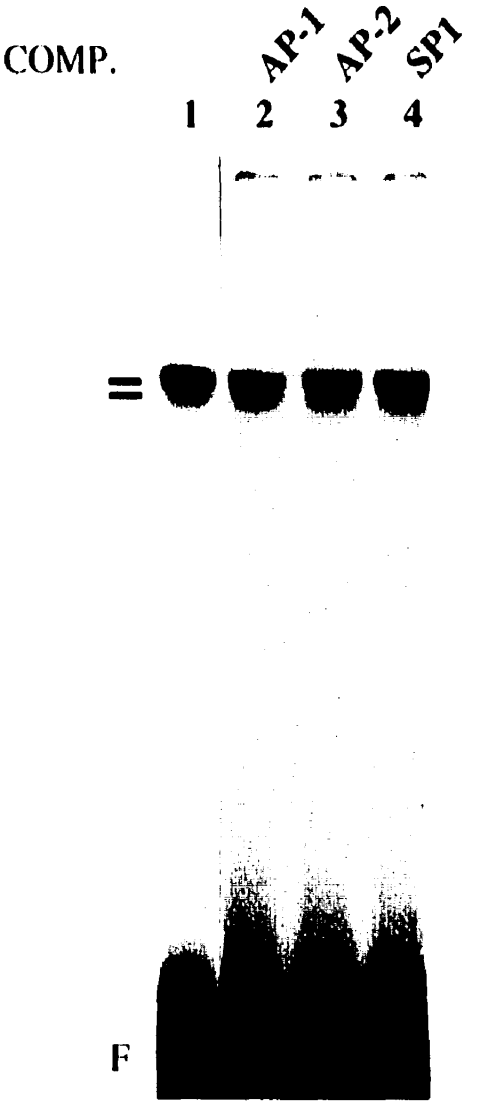
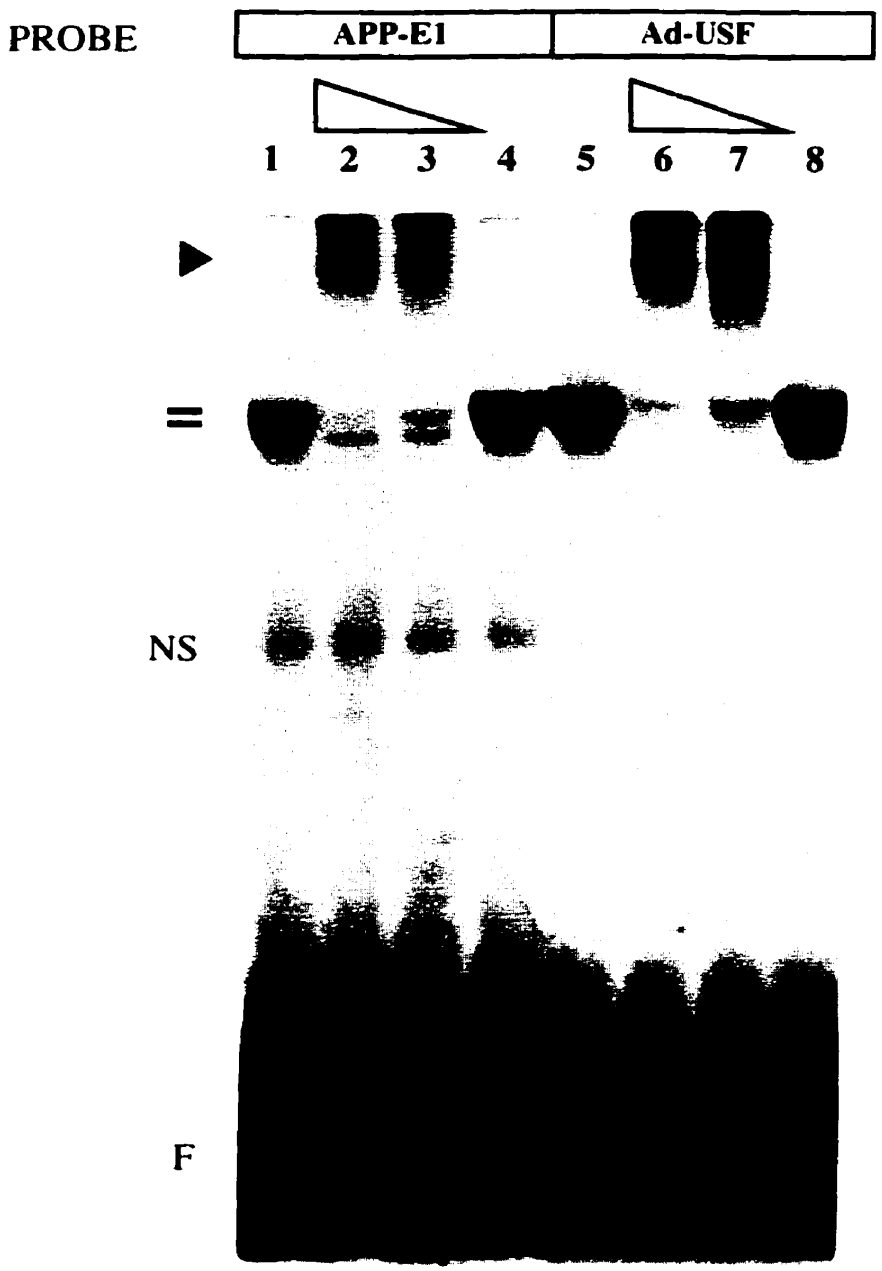

COMP.
AP-1
AP-2
SP1
1 2 3 4
=
F

Fig. 4.3 The APP-E1 - protein complex is recognized by an antibody against USF. End-labeled APP-E1 (lanes 1-4) or Ad-USF (lanes 5-8) oligonucleotides were incubated with nuclear extracts from HeLa cells and DNA-protein complexes were allowed to form prior to the addition of a rabbit antiserum directed against USF. Lanes 1 and 5, without antiserum; lanes 2 and 6, in the presence of a 1/500 dilution and lanes 3 and 7, a 1/1000 dilution of the anti-USF antiserum; lanes 4 and 7, in the presence of a 1/500 dilution of an unrelated rabbit antiserum. The arrowhead indicates the supershift which is observed in the presence of the anti-USF antiserum, specific DNA-protein complexes are labeled by a bar; NS, non-specific complexes; F, position of free probe.


PROBE
APP-E1
Ad-USF
1 2 3 4 5 6 7 8
NS
F

al., 1992). As shown in Fig. 4.3, the antibody binds to the complex formed between Ad-USF and the nuclear proteins which are present in HeLa cells, decreasing the electrophoretic migration of the complex ("supershift", Fig. 4.3, lanes 6 and 7). A similar change in electrophoretic pattern is observed when the APP-E1 - protein complex is incubated with anti-USF-1 antibody (Fig. 4.3, lanes 2 and 3). For both DNA-protein complexes, the reactivity which is obtained is dependent on the dilution of the antibody (Fig. 4.3). An irrelevant rabbit polyclonal antibody used at similar protein concentrations does not affect the migration of the complex (Fig. 4.3, lane 4). The complete shift in electrophoretic mobility observed in presence of the anti-USF antibody provides evidence that a protein which is antigenically related to USF is a major protein component of the DNA-protein complexes detected in EMSA.

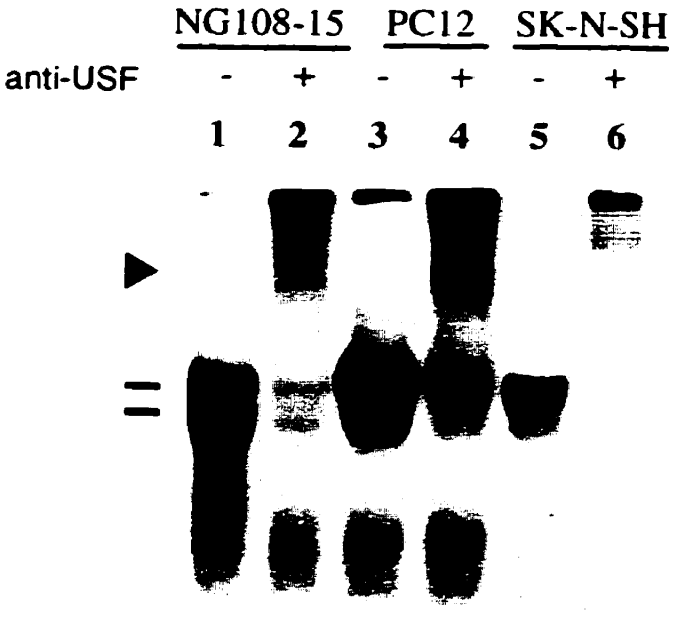
APP E1- binding protein is present in neuronal cell lines and is recognized by an anti-USF antibody.

The *APP* gene is highly expressed in the nervous system, mainly in neurons (Ohta et al., 1993; Salbaum and Ruddle, 1994). It was therefore of interest to verify whether nuclear extracts from neuronal cell lines had an APP-E1 binding activity. As shown in Fig. 4.4, EMSA performed with nuclear extracts from NG108-15 cells, PC12 cells (rat phaeochromocytoma) and SK-N-SH cells (human neuroblastoma) demonstrated that similar APP-E1 - protein complexes were formed in all three binding reactions. In each case, the complex was also recognized by the anti-USF antibody (Fig. 4.4, lanes 2, 4 and 6) indicating that the same family of protein binds APP-E1 in HeLa cells and neuronal cells.

USF-1 synthesized *in vitro* binds APP-E1

The results presented above suggested that the protein binding to APP-E1 recognized the consensus sequence of USF and shared immunoreactive epitopes with USF-1. To demonstrate that USF-1 itself was present in the APP-E1 - protein complex and that it could bind directly to APP-E1, we synthesized the USF-1 protein in a coupled *in vitro* transcription/translation system and performed EMSA with the expressed protein. Both APP-E1 and Ad-USF were bound by the *in vitro* translated USF-1 (Fig. 4.5A, lanes 2 and

Fig. 4.4 A USF-like APP-E1 binding protein is present in nuclear extracts of neuronal cell lines. EMSA was performed with an end-labeled APP-E1 oligonucleotide and nuclear extracts prepared from NG108-15 cells (lanes 1 and 2), PC12 cells (lanes 3 and 4), SK-N-SH cells (lanes 5 and 6) in the absence (lanes 1, 3 and 5) or in the presence (lanes 2, 4, and 6) of the anti-USF antiserum. The arrowhead indicates the supershift which is produced in the presence of the anti-USF antiserum. specific DNA-protein complexes are labeled by a bar; F, position of free probe.

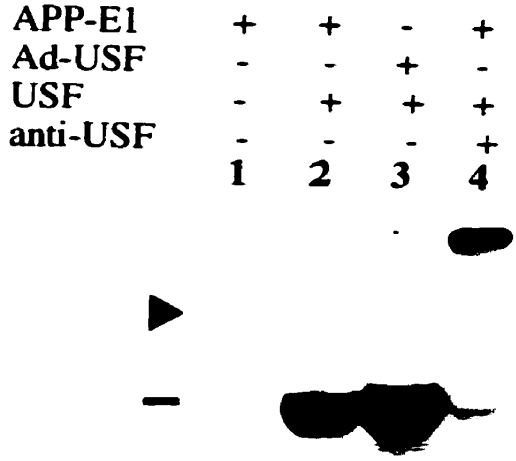

NG108-15
PC12
SK-N-SH
anti-USF
- + - + - +
1 2 3 4 5 6


F

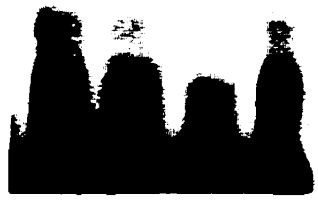
Fig. 4.5 USF can interact directly with APP-E1.

A) EMSA using *in vitro* synthesized USF-1 as a source of proteins was performed with labeled oligonucleotides APP-E1 (lane 2 and 4) and Ad-USF (lane 3). Each EMSA binding reaction contained 1 ul of a coupled *in vitro* transcription/translation reaction with or without USF-1. Lane 1 represents the coupled *in vitro* transcription/translation where T7 polymerase was omitted (absence of USF-1). No USF binding activity was detected. Mobility of both APP-E1 (lane 2) and Ad-USF (lane 3) is affected in presence of *in vitro* synthesized USF-1. The protein-DNA - complex formed by APP-E1 and USF-1 was recognized by a polyclonal antibody raised against the 43kDa USF. The arrowhead indicates the supershifted complex, the bar represents the protein- DNA - complex whereas F marks the free probe. **B)** UV crosslinking of Ad-USF (lane 2) and APP-E1 (lane 3) with HeLa nuclear extracts. Scaled up EMSA binding reactions (Materials and Methods) were exposed to UV light (254 nm) for 30 minutes and loaded onto a SDS- polyacrylamide gel. UV crosslinked protein-DNA - complexes between 50 to 60 kDa, indicated by arrowheads, could be observed with both oligonucleotides. APP-E1 also formed complexes with proteins of lower molecular weights. Lane 1 represents the molecular weight markers (^{14}C-labeled, BioRad).

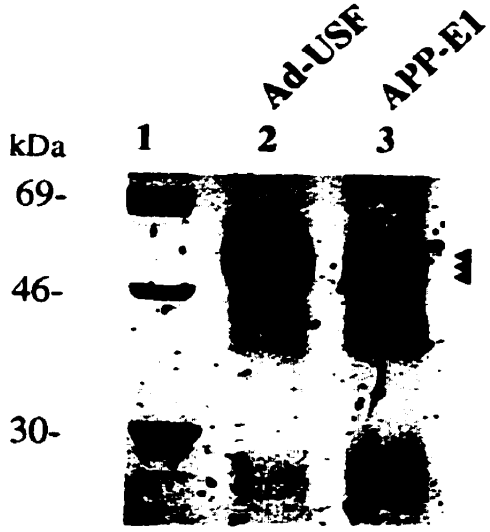
A



F



B



3). The APP-E1-USF complex was recognized by the anti-USF-1 antibody (Fig. 4.5A, lane 4). We also verified that nuclear factors of similar size classes were bound by APP-E1 and Ad-USF. The labeled oligonucleotide-protein complexes were UV crosslinked and analyzed by SDS-PAGE. Common polypeptides were labeled by APP-E1 and Ad-USF (Fig. 4.5B). The APP-E1 oligonucleotide was also crosslinked to additional nuclear proteins of lower molecular weight. These results suggest that USF which is present in nuclear extracts of HeLa cells (Sawadogo et al., 1988) interacts directly with APP-E1.

Co-expression of USF activates a reporter plasmid containing upstream sequences of the *APP* gene.

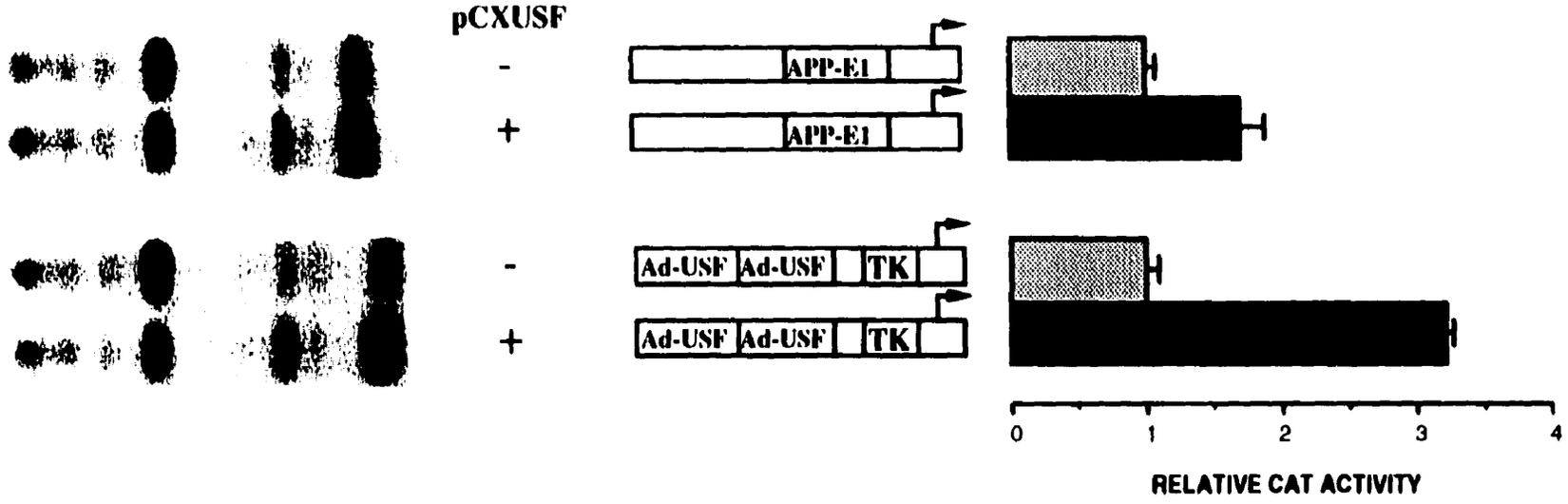
To demonstrate the biological significance of the binding of USF to APP-E1, co-transfection experiments were performed in NG108-15 cells using an *APP* promoter-CAT construct and a eukaryotic expression vector containing the USF-1 cDNA, pCXUSF, driven by the CMV promoter (Du et al., 1993). Co-expression of USF-1 resulted in a 1.7 fold increase of the CAT activity directed by the *APP* promoter (Fig.4.6). Similarly, in the presence of co-expressed USF, a 3.2 fold increase of CAT activity was observed for the plasmid p2XAdMLTF/TKCAT which contains two copies of the USF recognition element of the adenovirus major late promoter (-69 to -49) inserted upstream of the minimal promoter of the HSV TK gene (Shih and Towle, 1994) (Fig. 4.6). These results indicate that USF can interact *in vivo* with the *APP* basal promoter to modulate *APP* gene transcription.

Our results demonstrate that the transcription factor USF binds this E box we have labeled APP-E1. This conclusion is based on several observations. First, an oligonucleotide containing an authentic USF binding site from the adenovirus major late promoter competed very efficiently the binding of nuclear factor to labeled APP-E1 oligonucleotide. Second, an antiserum against USF-1 reacted with all APP-E1 - protein complexes which had formed. Third, USF synthesized *in vitro* from a human cDNA clone bound the APP-E1 oligonucleotide as well as its consensus recognition sequence, Ad-USF. These findings suggest that USF can interact with the *APP* promoter. Furthermore, an APP-E1 binding

activity related to USF is also present in neuronal cell lines of human and rodent origin. Our results also suggest that USF, upon binding to APP-E1, contributes to the expression of the *APP* gene since an *APP* promoter-CAT reporter construct was transactivated by co-transfection of a eukaryotic expression vector containing USF-1 cDNA into NG108-15 cells.

Fig. 4.6 Transactivation of human *APP* promoter by USF.

Co-transfection assays using an eukaryotic expression vector for the transcription factor USF (pCXUSF) in conjunction with a human *APP* promoter/CAT reporter gene construct (pKCXB) or the control plasmid p2XAdMLTF/TKCAT in the NG108-15 cell line. When pCXUSF was omitted, a carrier plasmid, pKK232-8 was used. The presence of pCXUSF caused a 1.7 fold increase in CAT activity of pKCXB whereas the CAT activity of p2XAdMLTF/TKCAT, which contains 2 USF binding sites, was increased 3.2 fold. Transfections were performed in triplicate using 2 independent plasmid preparations. Percentage of acetylation (between 10-40%) was normalized to protein concentrations. CAT activity is expressed relative to the control transfections. The error bars indicate the standard error of the mean (n=6).


pCXUSF
-
+
APP-E1
APP-E1
-
+
Ad-USF
Ad-USF
TK
Ad-USF
Ad-USF
TK
0
1
2
3
4
RELATIVE CAT ACTIVITY

Chapter 5

Results

Transcription factor Sp1 recognizes an essential element in the promoter of the human ß-amyloid precursor protein gene

The transcription factor Sp1 is one of the first proteins that was identified to play a role in transcriptional regulation (Kadonaga et al., 1987). Sp1 recognizes GC-rich DNA sequences, called GC-boxes, through three zinc fingers located at the C-terminal end of the protein (Kadonaga et al., 1987). In mice, it is ubiquitously expressed with highest levels observed in the developing hematopoietic system (Saffer et al., 1991). Sp1 is a member of a family of proteins that include the closely related Sp2, Sp3 and Sp4 (Hagen et al., 1992; Kingsley et al., 1992). All these proteins share overlapping patterns of expression and can recognize the same GC-rich and GT-rich sequences, suggesting a higher level of regulation (Hagen et al., 1992).

Using EMSA and HeLa nuclear extracts, Pollwein (1993) had previously reported that a complex formed with an oligonucleotide (-72 to -35) from the *APP* promoter, co-migrated with a complex formed between recombinant Sp1 and an oligonucleotide carrying a *bona fide* Sp1 element. This co-migrating complex formed with the *APP* promoter oligonucleotide could be partially competed with the Sp1 oligonucleotide. These results suggested that Sp1 could play a role in the transcriptional regulation of the human *APP* gene. It was also of interest that the oligonucleotide used by Pollwein contained the as yet to be identified USF element. We initiated studies to determine the relative contribution of each protein (USF and Sp1) to the transcriptional regulation of *APP*. In this chapter, we demonstrate that Sp1 recognizes *in vitro* the Sp1 element located in the *APP* minimal

promoter. However, exogenous Sp1 did not activate a construct bearing the *APP* promoter in co-transfection assays in *Drosophila melanogaster* S2 cells which are devoid of endogenous Sp1 activity.

Sp1 recognizes the consensus Sp1 element upstream of the USF binding site.

We investigated whether the transcription factor Sp1 could bind the consensus Sp1 sequence present in the *APP* minimal promoter. In order to determine the sequences recognized by the Sp1 protein in the *APP* promoter, we performed DNase I footprinting analysis in the presence of recombinant Sp1 protein. We used a DNA fragment end-labeled on the coding strand from the *APP* promoter spanning region -203 to +100 relative to the major transcriptional initiation site. Three regions of the *APP* promoter were protected from DNase I digestion (Fig. 5.1). Region -67 to -53 corresponds to the consensus Sp1 element of the minimal promoter. The second region, -137 to -122 (5'-gcgagggccctcccg-3'), also corresponds to a consensus Sp1 binding site as revealed by analysis with the TRANSFAC data base (Wingender et al., 1997). The third region could not be precisely determined due to its relative position in the gel. In the present study, the region upstream of the first -96 bp of 5' regulatory sequences and their role in *APP* basal expression were not further investigated. EMSA was used to confirm that the Sp1 transcription factor could bind the *APP* Sp1 consensus element. An oligonucleotide, spanning the region protected by Sp1 in DNase I footprinting analysis (APPSP1, -71 to -50) was synthesized and incubated in presence of recombinant Sp1 (Fig 5.2A). The APPSP1 oligonucleotide formed a complex with Sp1 (Fig. 5.2A. lane 2) that co-migrated with a the complex formed with Sp1 and an oligonucleotide carrying a SV40 Sp1 binding site (oligonucleotide SP1 from Promega). When using HeLa nuclear extracts, which contain Sp1 activity (Kadonaga et al., 1987), with the APPSP1 oligonucleotide, a specific complex was formed that could be efficiently competed with a 20 fold molar excess of the SP1 oligonucleotide. Furthermore, incubation of the EMSA reactions with an antibody directed against Sp1 further slowed down the migration of the APPSP1-Sp1 complex (supershift). Minor complexes could be observed that were not competed with SP1. These complexes were often observed with the APPSP1 oligonucleotide and could not be competed with unlabeled APPSP1 either,

Fig. 5.1 DNase I footprinting analysis of the *APP* promoter with recombinant Sp1 protein.

A Xma III / BamH I (-203 to + 100) restriction fragment end-labeled on the coding strand was incubated with either no protein (lane 1) or 160 ng of recombinant Sp1 (Promega) (lane 2) and subjected to DNase I digestion. The brackets illustrate the protected regions compared to control. The numbers represent the positions relative to the major trancriptional start site.

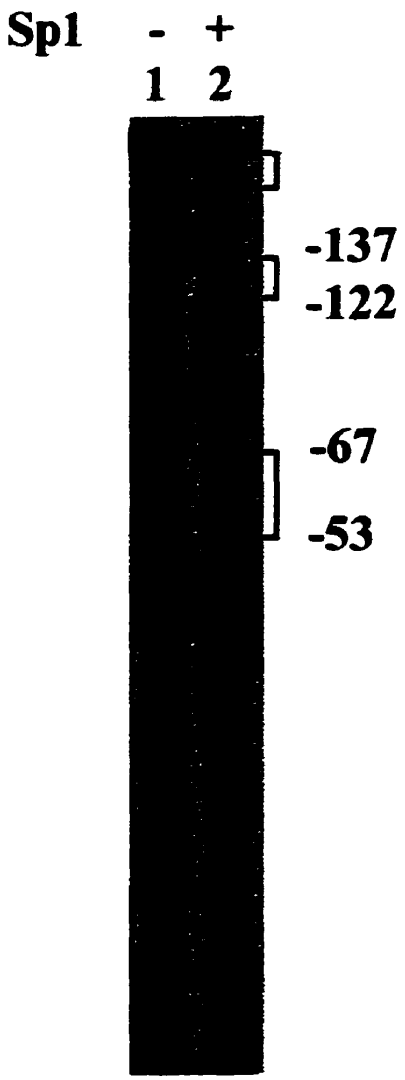
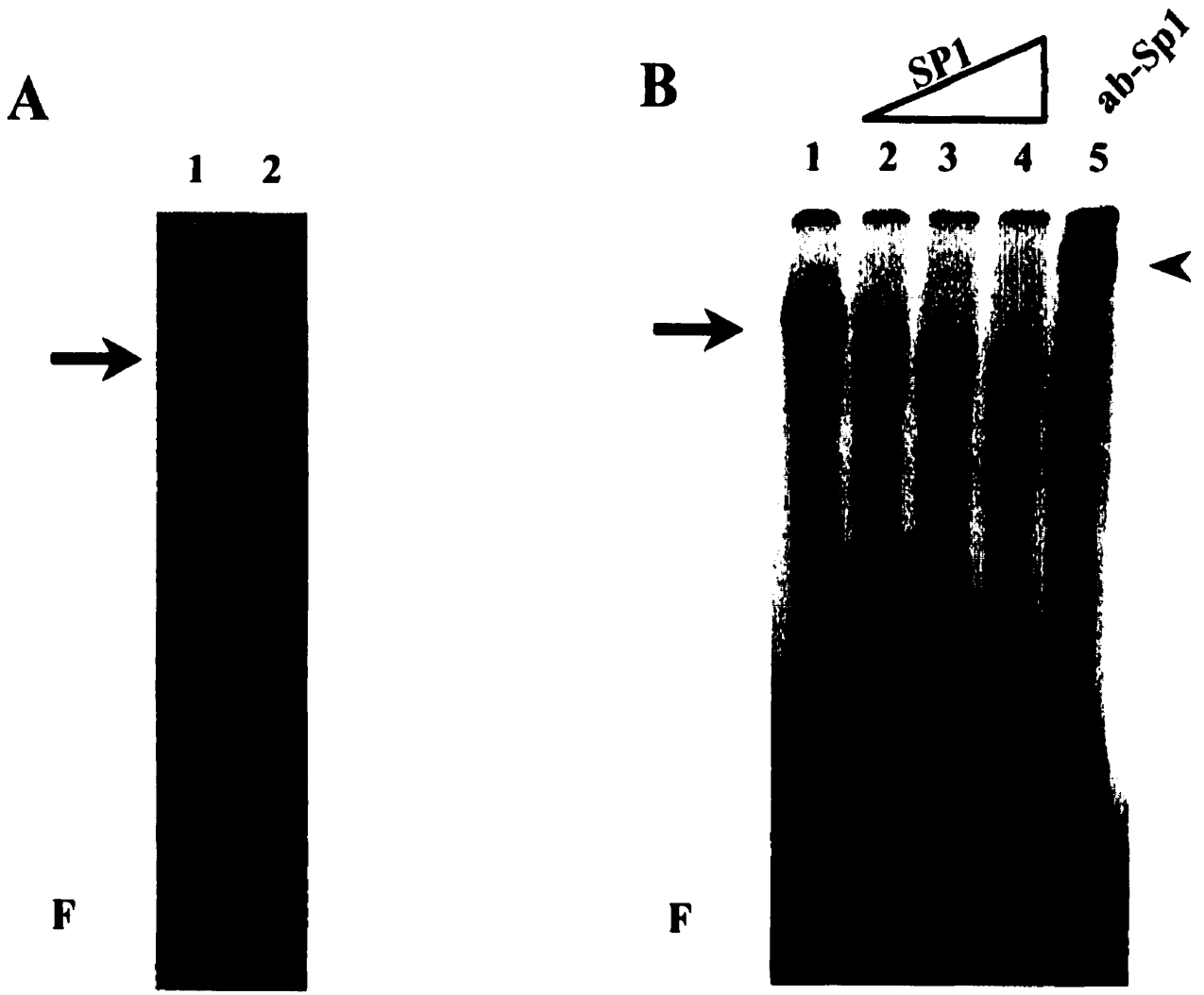

Sp1
-
+
1
2
-137
-122
-67
-53

Fig. 5.2 Sp1 binds the consensus Sp1 element in the *APP* promoter.

EMSA was performed with an oligonucleotide APPSP1, -71 to -50, containing the protected region observed by DNase I footprinting analysis. **A)** Recombinant Sp1 was incubated with oligonucleotide SP1 (lane 1) which contains a consensus Sp1 sequence from the SV40 promoter region and oligonucleotide APPSP1 (lane 2). The arrow indicates the complexes formed. F: free probe **B)** HeLa nuclear extracts were incubated with APPSP1 and formed multiple complexes when analyzed by EMSA. The slower migrating complex, indicated by an arrow, was competed with an excess SP1 oligonucleotide (lane 2, 20 fold molar excess; lane 3, 50 fold molar excess; lane 4, 100 fold molar excess). An antibody directed against Sp1 further slowed down this complex, indicated by an arrowhead (lane 5). F: free probe.


A
1 2
F
B
SP1
ab-Sp1
1 2 3 4 5
F

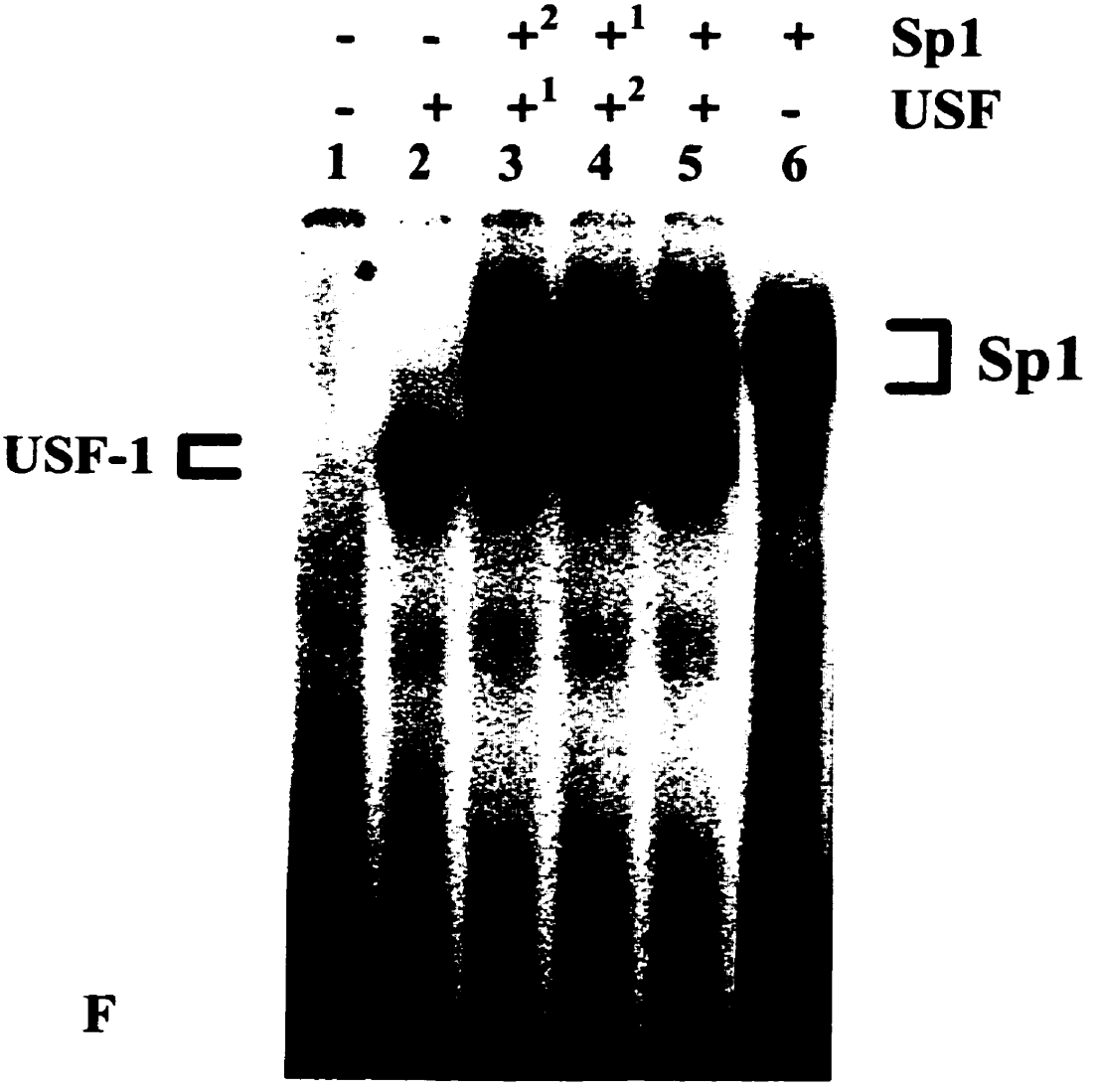
suggesting that they were non-specific in origin (data not shown and Fig. 6.3). These *in vitro* results demonstrate that the Sp1 transcription factor can bind directly the consensus Sp1 element present in the *APP* minimal promoter.

Similar results were obtained with an oligonucleotide containing both the Sp1 and USF elements (SP1-USF: -71 to -37). We first used recombinant Sp1 protein and *in vitro* synthesized USF-1 in EMSA with the SP1-USF oligonucleotide. Both Sp1 and *in vitro* synthesized USF-1 were able to recognize the SP1-USF oligonucleotide and form complexes (Fig 5.3, lanes 2 and 6). When both proteins were added at the same time to the binding reaction, the complexes were the sum of the individual complexes observed with Sp1 and *in vitro* synthesized USF-1 alone (Fig. 5.3 lane 5). The order in which Sp1 or *in vitro* synthesized USF-1 were added to the binding reaction did not affect the intensity of the complexes formed with the SP1-USF oligonucleotide (Fig. 5.3 lane 3 and 4). In the conditions used, we did not detect a slower complex containing both Sp1 and USF bound to the same DNA molecule.

To confirm the results with recombinant proteins, we used a similar approach using HeLa nuclear extract. Since both Sp1 and USF activity are present in HeLa nuclear extracts (Kadonaga et al., 1987; Sawadogo and Roeder, 1988), we used competition EMSA to visualize if removal of either Sp1 or USF had an effect on the recognition of the adjacent element (Fig 5.4). When the oligonucleotide SP1-USF was incubated in presence HeLa nuclear extracts, three major complexes were observed (Fig. 5.4, lane 1). Competition with the unlabeled SP1 oligonucleotide, which contains the Sp1 binding site from the SV40 promoter, abolished the slower migrating complex without significantly affecting the other complexes (Fig 5.4, lane 2-4). Furthermore, the complex which was competed by the SP1 oligonucleotide could be supershifted by an antibody directed against Sp1 (Fig. 5.4, lane 8) confirming the presence of Sp1 activity in the slower migrating complex. In the converse experiment, competition with an oligonucleotide (Ad-USF) containing the adenovirus major late promoter USF binding site abolished the two faster migrating

Fig. 5.3 Sp1 and USF-1 bind to an oligonucleotide from the *APP* promoter containing both elements.

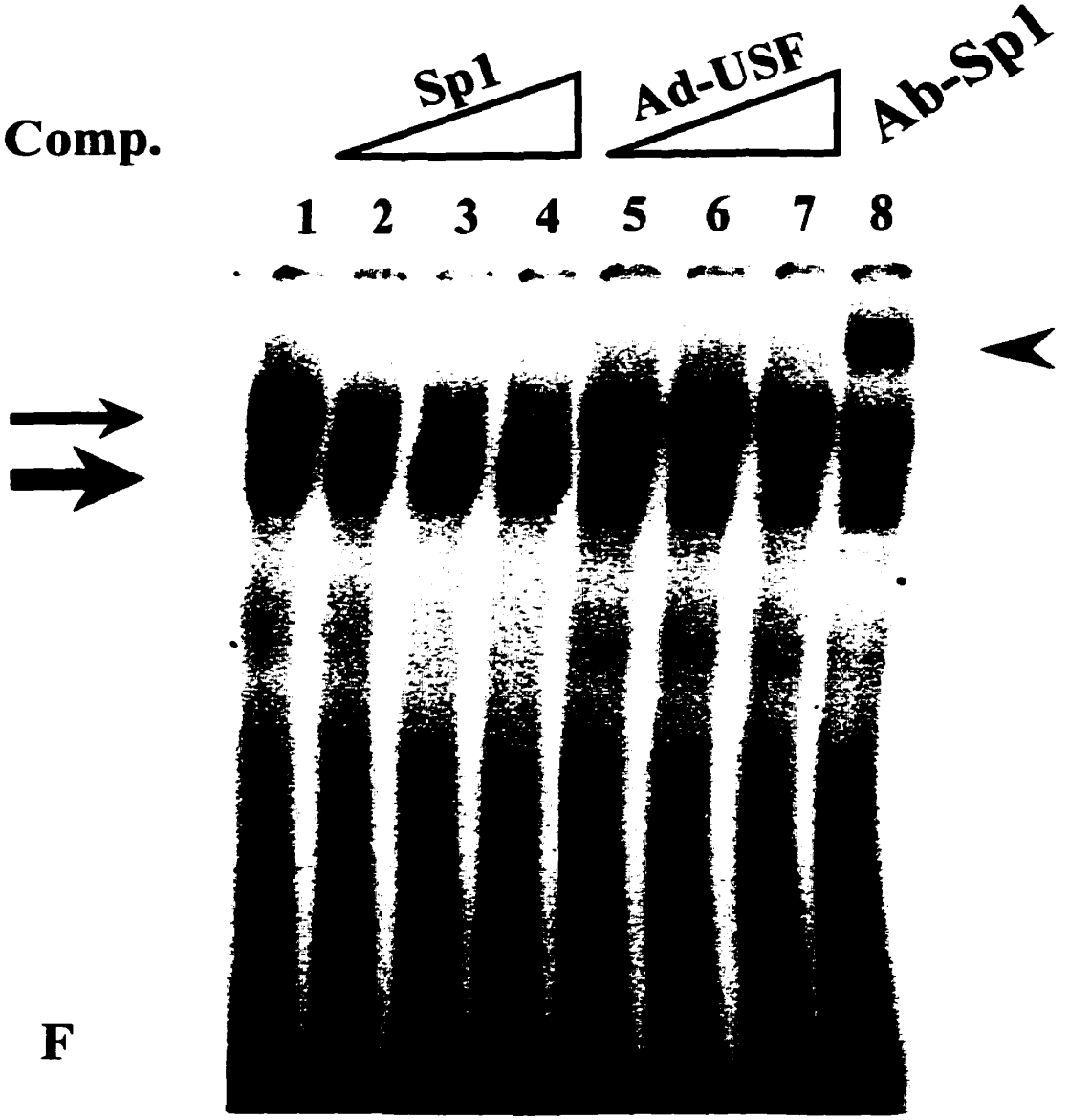
Recombinant Sp1 and *in vitro* synthesized USF-1 were used in EMSA with oligonucleotide SP1-USF spanning region -71 to - 37 of the *APP* promoter. Each binding reaction contained 1 μl of an *in vitro* coupled transcription/translation reaction. When recombinant Sp1 (100 ng) was present (lanes 3-6) it was added to *in vitro* coupled transcription/translation reactions where USF-1 was either present (lanes 3-5) or absent (lane 6). Lane 1 represents the probe SP1-USF incubated with 1 μl of *in vitro* coupled transcription/translation reaction where the T7 polymerase was omitted. The order in which Sp1 and USF-1 were added did not affect the formation of either complex (lanes 3 and 4).



F

Fig. 5.4 Oligonucleotide SP1-USF is recognized by proteins present in HeLa nuclear extracts.

HeLa nuclear extracts were used in EMSA with SP1-USF. Multiple complexes were formed (lane 1). The slower migrating complex was efficiently competed with 20, 50 and a 100 fold molar excess (lanes 2-4) of oligonucleotide SP1containing an Sp1 binding site from the SV40 early promoter (small arrow). When competitions assay were performed with oligonucleotide Ad-USF, the two faster migrating complexes disappeared (large arrow). An antibody directed against Sp1 supershifted the complex which could be competed with oligonucleotide SP1 (arrowhead).



F

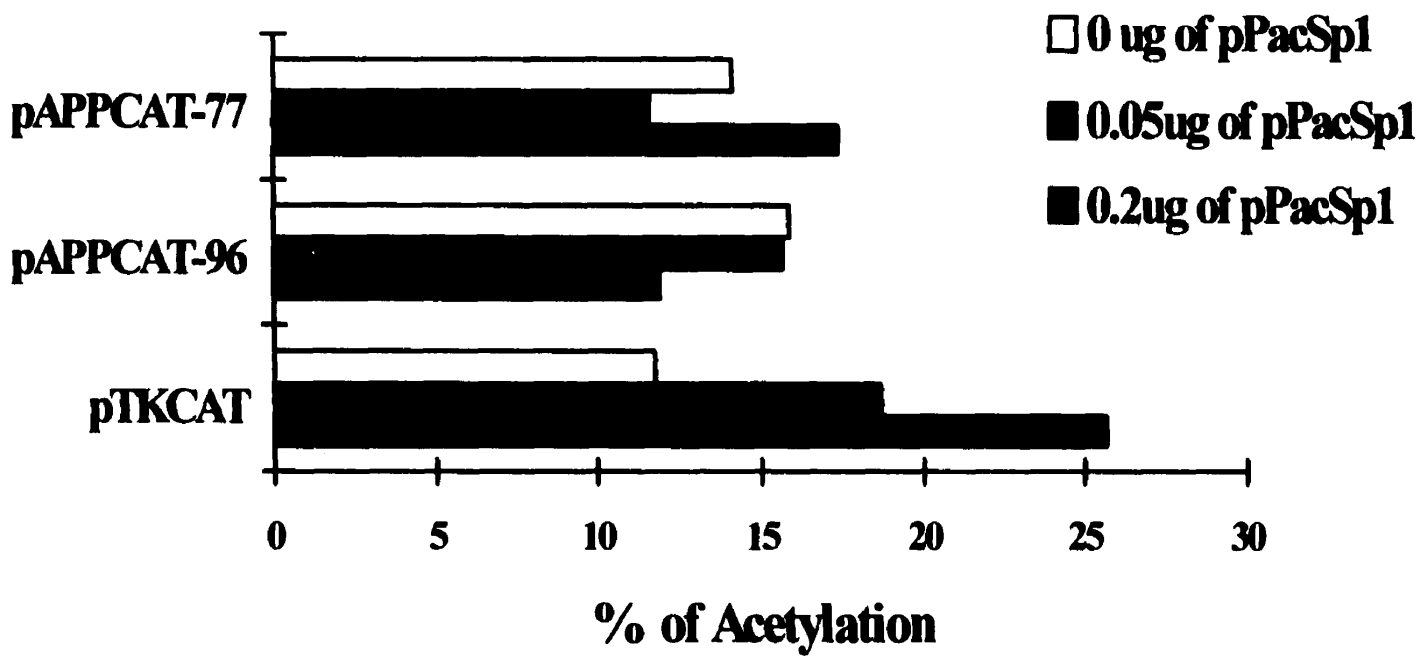
complexes without affecting the Sp1 complex (Fig. 5.4, lanes 5-7). The proteins present in HeLa nuclear extracts also formed minor complexes with the SP1-USF oligonucleotide. Taken together, these results suggest that Sp1 and USF recognize their respective adjacent elements in the *APP* promoter in an independent manner and that other proteins might also recognize these elements or other sequences within the SP1-USF oligonucleotide.

Sp1 fails to transactivate the *APP* promoter in *Drosophila* S2 cells

Co-transfection assays have been used regularly as a means of verifying the transactivation potential of specific transcription factors on a define promoter. We co-transfected the minimal *APP* promoter present in pAPPCAT-96, a CAT reporter plasmid under the control of 96 bp of upstream regulatory sequences from the *APP* promoter, along with an expression vector for Sp1 (pPacSp1) in *Drosophila melanogaster* S2 cells which are devoid of Sp1 activity (Courey and Tjian, 1988). As shown in Figure 5.5, Sp1 failed to transactivate the minimal *APP* promoter present in pAPPCAT-96 whereas similar conditions increased more than two fold the thymidine kinase promoter from the herpes simplex virus (HSV), which contains two Sp1 binding sites (Jones et al., 1985), present in pTKCAT. These results suggest that Sp1 can recognize the element located between position -67 and -53, but that it might have roles other than direct transactivation of *APP*.

Fig. 5.5 Co-transfection assays with Sp1 and the *APP* minimal promoter in *Drosophila* S2 cells.

The constructs pAPPCAT-77 and pAPPCAT-96 (1 μg) were co-transfected either with the *Drosophila* expression vector pPac0 or its derivative pPacSp1 which carries the cDNA for human Sp1 (pPacSp1). The amount of expression vector was kept constant at 0.2 μg: 0 μg of pPacSp1= 0.2 μg of pPac0; 0.05 μg of pPacSp1= 0.05 μg of pPacSp1 + 0.15 μg of pPac0). pPacSp1 had no effect on the *APP* promoter present in pAPPCAT-77 and pAPPCAT-96. The activity pTKCAT, which contains two Sp1 binding sites, was increased when 0.05 μg and 0.2 μg of pPacSp1 were present.


0 ug of pPacSp1
0.05ug of pPacSp1
0.2ug of pPacSp1
pAPPCAT-77
pAPPCAT-96
pTKCAT
0
5
10
15
20
25
30
% of Acetylation

Chapter 6

Results

The Sp1 and USF elements of the human ß-amyloid precursor protein gene are necessary for full promoter activity.

The general structure of the *APP* promoter is reminiscent of housekeeping genes. It possesses a high GC content, lacks a TATA-box and has multiple transcriptional start sites (Salbaum et al., 1988). Progressive deletions of the *APP* promoter revealed that the sequences located between -94 and +100 were sufficient to confer full activity to the promoter in transient transfection assays in a wide range of cell lines (Pollwein et al., 1992, Quitschke and Goldgaber, 1992). Two regions were identified to be responsible for the full activity of the promoter. One contained the GC-rich region located between -96 and -70 (Pollwein et al., 1992). The second one included a consensus sequence for the Sp1 transcription factor (-63 to -55) (Pollwein, 1993) and the USF element (-50 to -43) (Kovacs et al., 1995; Vostrov et al., 1995; Bourbonnière et al., 1996). Deletion of the GC-element reduced the activity of the *APP* promoter to 10-30% whereas further deletion of the Sp1 and USF elements abolished the remaining activity (Pollwein et al., 1992; Quitschke, 1994). Furthermore, the region located between -94 and -40 had been shown to activate an heterologous promoter when introduced upstream of its TATA box but had no activity when positioned upstream of its full promoter (Pollwein et al., 1992). Again, deletion of the upstream GC-element reduced the activity of the heterologous TATA-box promoter, suggesting that the proteins recognizing the region located between -94 and -40 function as a unit to regulate transcription and that this region's proximity to the transcriptional machinery is important (Pollwein et al., 1992). Results presented in Chapter 6 will attempt to clarify the roles of the closely positioned Sp1 and USF elements in the transcriptional

regulation of the *APP* gene by using transfection assays in combination with site directed mutagenesis of both the Sp1 and USF elements in presence or absence of the GC-element. In these studies, in addition to the NG108-15 cell line, we used human HepG2 cells. In our experience, human HepG2 cells, which express *APP* at high levels (data not shown), gave superior results in primer extension assays on total RNA and in transient transfection and co-transfection assays when compared to HeLa cells.

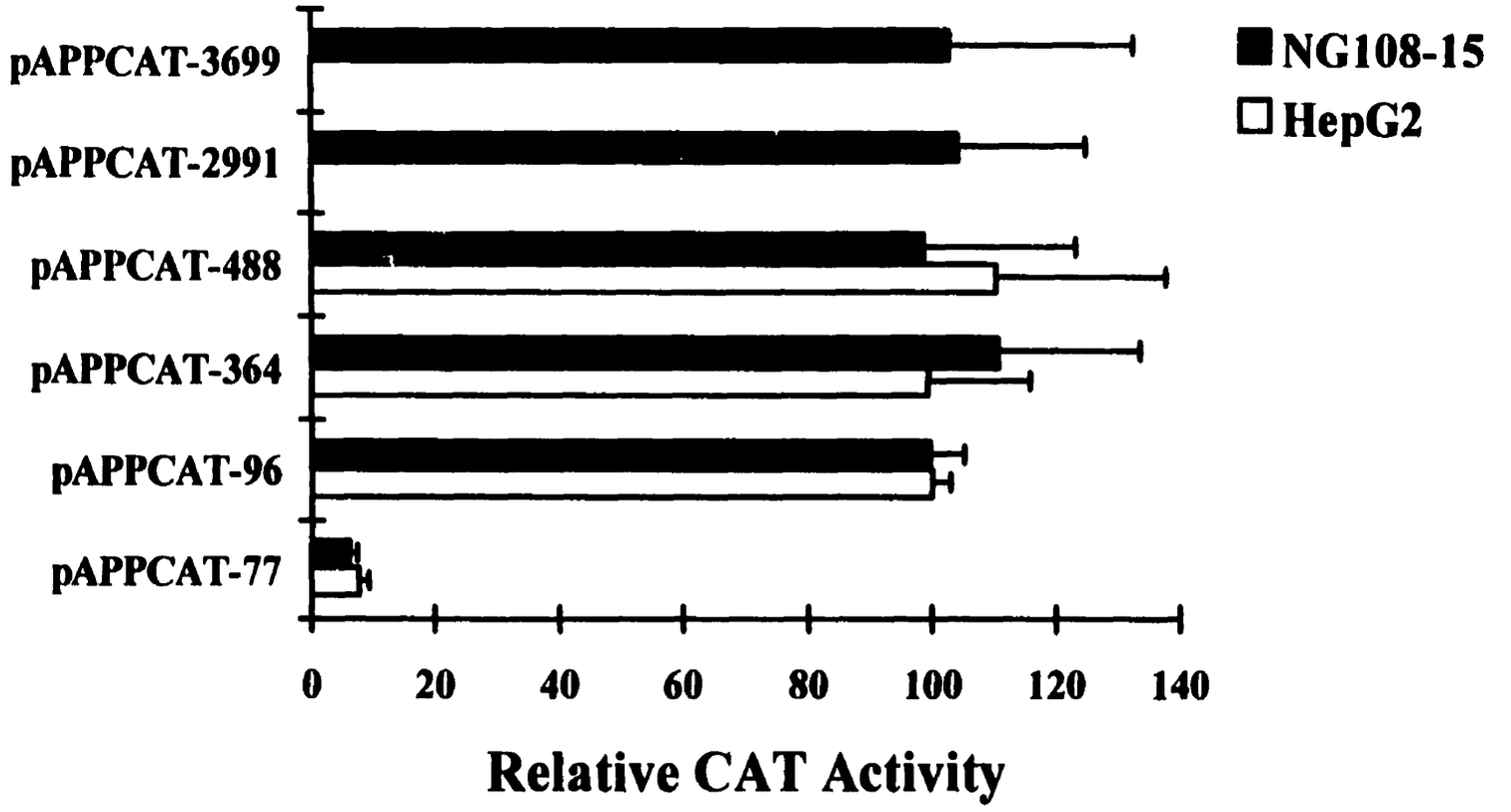
Transcriptional activity of deletions of the *APP* promoter in NG108-15 and HepG2 cells.

As a first step, a genomic fragment containing the 3699 bp of 5' regulatory sequences was introduced upstream of the reporter gene CAT present in the pBLCAT6. This particular vector was chosen over the previously used pKK232-8 (see Chapter 3) for its large number of unique restriction sites, its high copy origin of replication and its low background in transient transfection assays (Boshart, 1992). As illustrated in Figure 6.1, results of transient transfection assays in both NG108-15 and HepG2 cells show that sequences present upstream of the first -96 bp do not significantly affect the activity of the promoter. Deletion of the sequences present between -96 and -77 reduced the activity of the *APP* promoter to approximately 10% in both cell lines (pAPPCAT-77). These results are in agreement with what was previously reported with other cell lines (Pollwein et al., 1992; Quitschke and Golgaber, 1992)

Introduction of mutations in the Sp1 and USF elements

Previous mutational analysis of the human *APP* promoter resulted in the proposal of contradicting roles for the Sp1 and USF elements. In one case, mutations introduced in the Sp1 element had no effect on *APP* activity whereas mutation in the USF element reduced the activity by only 20% (Pollwein et al., 1993). An *APP* construct carrying both mutations lost 90% of activity compared to the wild type promoter suggesting a role for both elements. Similar results were also observed with the rat *APP* promoter which is conserved up to 85% with human *APP* (Hoffman and Chernak, 1995). However, Quitschke (1996) observed that

Fig. 6.1 Transient transfection of the *APP* promoter in NG108-15 and HepG2 cells. NG108-15 and HepG2 cell lines were transiently transfected using a modification of the calcium phosphate protocol. For NG108-15, each transfection was performed with a total of 10 μg of DNA composed of 5 μg of pAPPCAT reporter plasmids, 4 μl of carrier DNA (pBLCAT6) and 1 μg of pUT535, a ß-galactosidase reporter plasmid. HegG2 cells were transfected with a total of 15 μg of pAPPCAT reporter plasmids and 1 μg of pUT535. The CAT activity of each construct is expressed as a percentage of the minimal promoter contained in pAPPCAT-96. The error bars represent the standard error of the mean (NG108-15, n=10-15; HepG2, n=5-9). Results were obtained with at least two independent preparations of plasmid DNA for each construct. Transfections was performed in triplicate.


pAPPCAT-3699
pAPPCAT-2991
pAPPCAT-488
pAPPCAT-364
pAPPCAT-96
pAPPCAT-77
NG108-15
HepG2
0
20
40
60
80
100
120
140
Relative CAT Activity

mutations in both the Sp1 and USF elements (i.e double mutant) had no effect on activity of the *APP* promoter in this system.

In our efforts to characterize the individual function of the elements present in the minimal *APP* promoter we introduced mutations into the *APP* promoter (see Fig. 6.2) to study their effects in the context of various promoter sequences. As a first step, we used EMSA to confirm that the mutations to be introduced in the Sp1 and USF elements would not be recognized by their respective transcriptional activator. Oligonucleotides containing mutations in Sp1 and USF elements were used in competition EMSA. The mutant oligonucleotide APPSPm failed to compete (Fig. 6.3, lanes 2-4) with the end-labeled oligonucleotide APPSP1 whereas, in similar conditions, the oligonucleotide APPSP1 abolished the complex corresponding to Sp1 (Fig. 6.3, lanes 5-7). A second faster migrating complex (Fig. 6.3, arrowhead) was also disrupted in a concentration dependent manner. Other protein-DNA complexes were observed with the APPSP1 probe. These complexes could not be competed in a concentration dependent manner with the unlabeled APPSP1 oligonucleotide, suggesting non-specific interactions of the probe APPSP1 with nuclear proteins. Competition experiments with oligonucleotides containing the USF wild type and mutant elements yielded similar results. The mutant oligonucleotide APPUSF-M1 was unable to interfere with the USF complexes (Fig. 6.4, lanes 2-4) formed with the APP-E1 end-labeled oligonucleotide, which contains the E-box from the *APP* promoter recognized by USF (Chapter 4). When similar conditions were used with the oligonucleotide APP-E1 as a source of competitor, it successfully abolished the USF complexes. We concluded from these competition studies that these mutations, when introduced into the *APP* promoter, would prevent the binding of Sp1 and USF to their respective elements.

Mutations in Sp1 and USF elements do not affect the selection of multiple transcriptional start sites.

The mutations present in the APPSP1-M1 and APPUSF-M1 oligonucleotides were introduced separately and in combination into the *APP* promoter by recombinant PCR (see

Fig. 6.2 Schematic representation of the *APP* minimal promoter.

Elements present in the *APP* promoter are indicated above the sequence. The sequence represents the coding strand of the *APP* gene located between position -100 and -30 relative to the major transcriptional start site. The core binding sequences of each element are indicated in capital. The mutation introduced in the Sp1 and USF elements are indicated below the sequence in ***bold italics***.

-100 -30

cggcggcgCCGCTAGGGGtctctctcgggtgccgagcGGGGTGGGCcggaTCAGCTGActcgcctggctct

tgccgagcGG*AACA*GGCcggat

gccggaTC*GATT*GActcgcc

Fig. 6.3 EMSA of the mutations introduced into the Sp1 element.

Oligonucleotide APPSP1 was end-labeled and incubated with HeLa nuclear extracts (lane 1). Competition EMSA with oligonucleotide APPSP1m had no effect on the formation of the complexes observed in lane 1: lane 2, 20 fold molar excess; lane 3, 50 fold molar excess; lane 4, 100 fold molar excess. Competition with the oligonucleotide SP1, removed the slower migrating complex indicated by an arrow (lane 5-7, 20, 50 and 100 fold molar excess respectively). Competition under similar conditions with APPSP1 also removed the slower migrating complex (lane 8-10, 20, 50 and 100 fold molar excess respectively). Comp., competition; F, free probe.Materials and Methods).

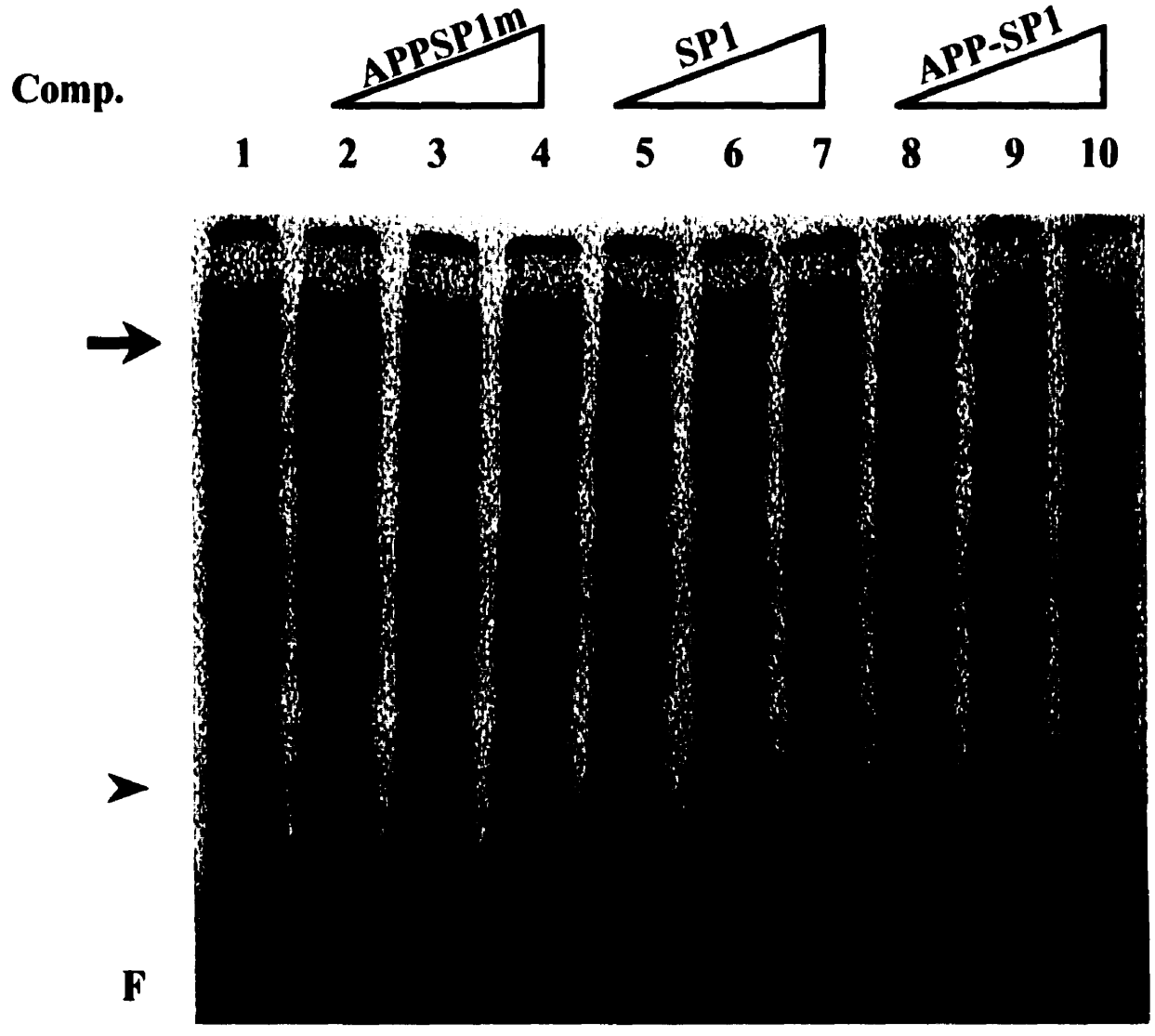
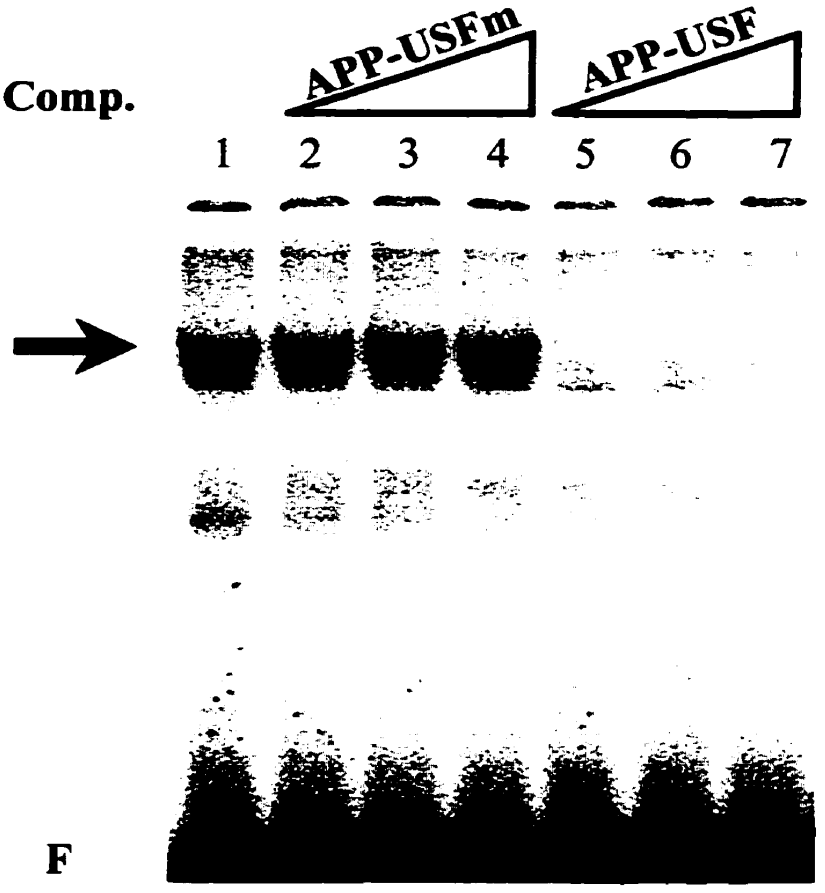

Comp.
APPSP1m
SP1
APP-SP1
1 2 3 4 5 6 7 8 9 10
F

Fig. 6.4 EMSA of the mutations introduced into the USF element.

HeLa nuclear extracts were used in competition EMSA with end-labeled oligonucleotide APP-E1 which contains the USF element from the *APP* promoter. APP-E1 formed two major complexes with proteins from HeLa nuclear extract (large arrow). These complexes were not affected by the increasing amount of unlabelled competitor APPUSFm (lane 2, 20 fold ; lane 3, 50 excess; lane 4, 100 fold molar excess), whereas competition with APP-E1 totally removed the complexes (lane 5, 20 fold; lane 6, 50 fold ; lane 7, 100 fold molar excess). Comp., competition; F, free probe.


Comp.
APP-USFm
APP-USF
1
2
3
4
5
6
7
F

We first verified that the mutations introduced in the SP1 and USF elements did not affect the utilization of the major transcriptional start sites of the *APP* promoter. In order to detect the transcriptional start sites of the various constructs of the minimal *APP* promoter, we performed run-on *in vitro* transcription assays, using HeLa nuclear extracts, followed by primer extension assays (Sambrook et al., 1989). As seen in Figure 6.5, mutations in the Sp1 and USF elements (lanes 2-4) of the *APP* promoter did not affect the utilization of transcriptional start sites when compared to the wild type *APP* promoter (lane 1). The transcriptional start sites used *in vitro* by the *APP* promoter with nuclear extracts from HeLa cells are identical to the ones used *in vivo* by HepG2 cells (Fig. 6.5, lane 5). When *in vitro* transcription templates were used for primer extension assays, we observed 2 close bands corresponding to the major transcriptional start site (+1). Determination of the ratio (average of three independent experiments) between the two transcriptional start sites (+1/-4) used by the *APP* promoter revealed a slight reduction in the constructs carrying a mutation in the USF element. Furthermore, we constantly observed a decrease in the efficiency of *in vitro* transcription run-on and run-off assays with constructs carrying mutations (Fig. 6.5 and data not shown) reflecting an effect on transcriptional activity. These results suggest that Sp1 and USF are not involved in start site selection but rather in transcriptional activity of the *APP* promoter.

USF-1 fails to activate an *APP* promoter bearing a mutated USF element.

We had previously demonstrated that USF-1 could transactivate the *APP* promoter in co-transfection assays in NG108-15 cells (Chapter 4, Fig. 4.6). Here we show that USF-1 can transactivate the minimal *APP* promoter in a concentration dependent manner in HepG2 cells and that this activation is dependent on the presence of a functional USF element. In co-transfection assays, increasing amounts of the pCMVUSF-1 expression vector activated pAPPCAT-96 up to two-fold (5 μg of pCMVUSF-1) (Fig. 6.6). We confirmed the role of USF in transcriptional activation of the *APP* promoter by co-transfecting 5 μg of pCMVUSF-1 expression vector in combination with the reporter plasmids pAPPCAT-96 and pAPPCAT-96mUSF. As shown in Figure 6.7, mutations introduced in the USF element reduced the activity of the promoter to 40% of the wild type promoter. When 5 μg

Fig. 6.5 Analysis of the transcriptional start sites of mutants of the *APP* promoter. Run-on *in vitro* transcription assays were performed with 80 μg of HeLa nuclear extracts on 2 μg of supercoiled plasmids pAPPCAT-96, pAPPCAT-96mSP1, pAPPCAT-96mUSF and pAPPCAT-96mSU. Transcriptional start sites were determined by primer extension assays with the antisense oligonucleotide ASAMY (+71 to +100) on the products of run-on *in vitro* transcription assays (lane 1-4) or using 50 μg of total RNA from HepG2 cells (lane 5). The products were separated on a 6% denaturing polyacrymide gel and their size was assessed by comparing with a sequencing reaction used as a ladder. The major transcriptional start site is indicated by +1. The ratio +1/-4 is indicated below.

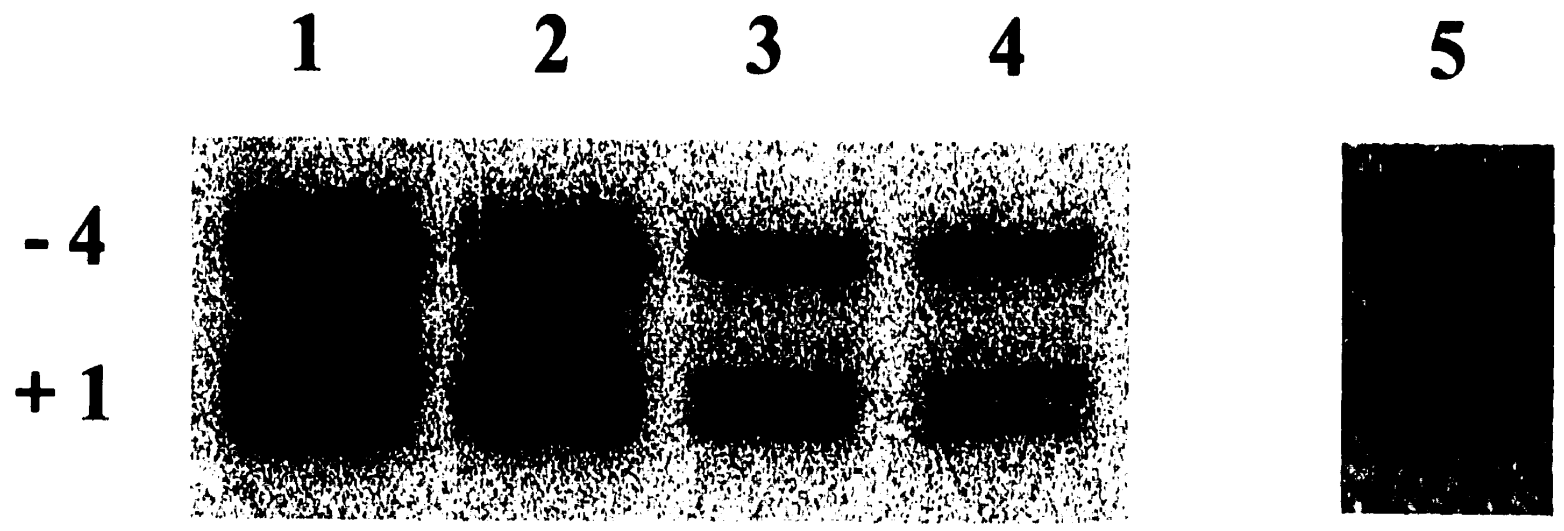

1
2
3
4
5
- 4
+ 1
+1/-4
1.33
1.40
1.12
1.19
1.72
WT
mSp1
mUSF
mSU
HepG2

Fig. 6.6 Co-transfection assays of USF-1 and the *APP* promoter in HepG2 cells.
HepG2 cells were co-transfected with a combination pcDNAI/amp (5, 4, 3 or 0 μg) or pCMVUSF-1 (0, 1, 2 or 5 μg), keeping the amount of plasmids bearing the CMV promoter constant at 5 μg; 5 μg of the reporter plasmid pAPPCAT-96; 4 μg of pBLCAT6 (carrier) and 1 μg of pUT535 (ß-galactosidase) for transfection efficiency, for a total of 15 μg. Fold increases are expressed in function of untreated pAPPCAT-96. The error bars indicate the standard error of the mean (SEM), n=3.

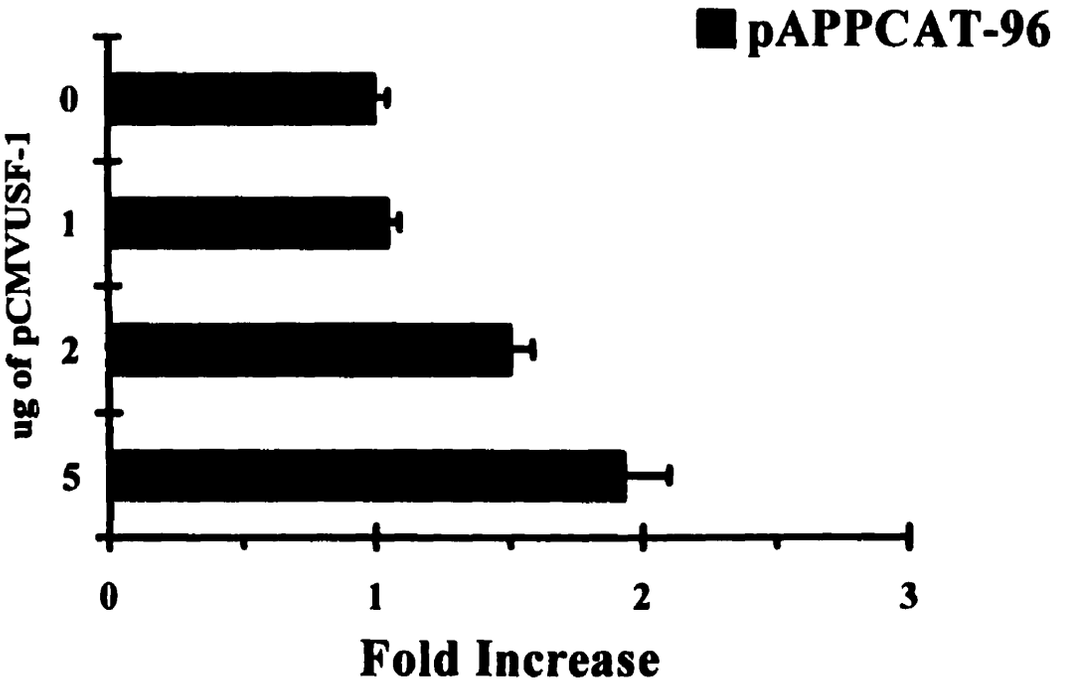
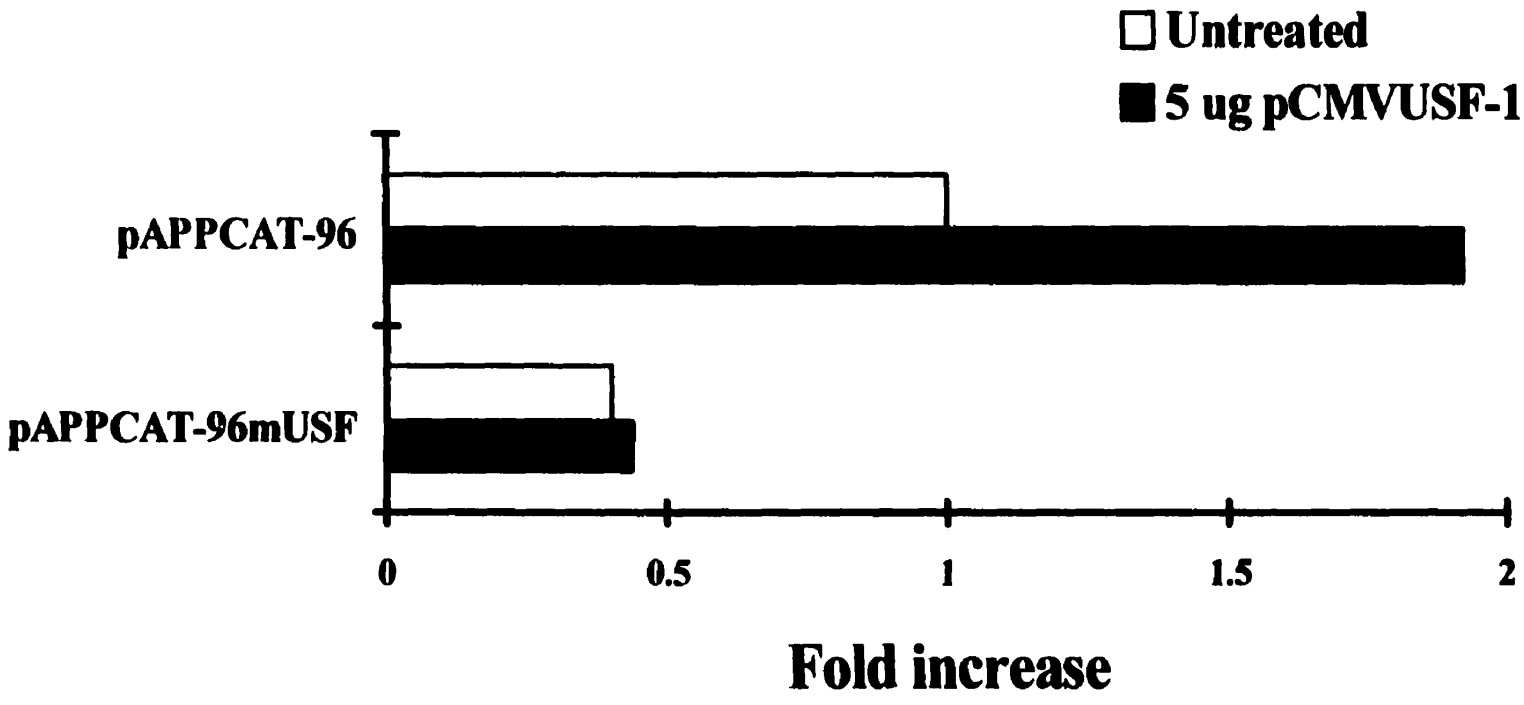

pAPPCAT-96
ug of pCMVUSF-1
0
1
2
5
0
1
2
3
Fold Increase

Fig. 6.7 Co-transfection assays in HepG2 cells of USF-1 and an *APP* promoter carrying a mutated USF element.

HepG2 cells were co-transfected with a total of 15 μg composed of either 5 μg of pAPPCAT-96 or 5 μg of pAPPCAT-96mUSF, 5 μg of pCMVUSF-1, 4 μg of pBLCAT6 (carrier) and 1 μg of pUT535 to standardize for transfection efficiency. Fold increases are expressed in function of the untreated pAPPCAT-96. The bar graph represents the average of 2 independent transfections.


Untreated
5 ug pCMVUSF-1
pAPPCAT-96
pAPPCAT-96mUSF
0
0.5
1
1.5
2
Fold increase

of pCMVUSF-1 were present, pAPPCAT-96 was activated 1.9 fold whereas pAPPCAT-96mUSF remained unaffected. These co-transfection studies underline the importance of USF in *APP* expression.

The Sp1 and USF elements are necessary for full promoter activity.

In order to investigate the effects of the mutations introduced in the Sp1 and USF elements, we transfected NG108-15 and human HepG2 cells with constructs containing the mutations described previously. We first analyzed the effects of mutating the Sp1 and USF elements separately or in combination in context of the full *APP* promoter present in the first 96 bp of upstream regulatory sequences (Figs. 6.8, 6.10). As shown in Figure 6.8, pAPPCAT-96mSp1 activity was reduced to 67% whereas pAPPCAT-96mUSF had a more important effect reducing activity to 42% when compared to pAPPCAT-96 in NG-108-15 cells. In HepG2 cells, similar results were obtained: pAPPCAT-96mSp1 had a reduced activity of 59% and pAPPCAT-96mUSF, 40%. The double mutant pAPPCAT-96mSU was further affected by the mutations of both Sp1 and USF elements to 22.3% in NG108-15 cells and to 14.3% in HepG2 cells. pAPPCAT-488 had a level of activity similar to pAPPCAT-96. The relative activity of pAPPCAT-395mSU in NG108-15 was34% and in HepG2 32%. These differences between pAPPCAT-96mSU and pAPPCAT-395mSU suggest that sequences located upstream of -96 could participate in *APP* gene regulation in certain circumstances.

To further characterize the role of Sp1 and USF elements, we studied the functional activity of the mutants in the absence of the GC-element of the *APP* promoter in NG108-15 (Figs. 6.9, 6.10). The wild type promoter present in pAPPCAT-77 had a relative CAT activity of 7.4% when compared to pAPPCAT-96. When the Sp1 element was disrupted, in pAPPCAT-77mSp1, we observed an increase in relative CAT activity to 10.7 %. Absence of the USF element (pAPPCAT-77mUSF) led to a reduction of relative CAT activity to 4.8 %. The activity of the double mutant pAPPCAT-77mSU, 4.8%, was similar to pAPPCAT-77mUSF, suggesting that the proteins recognizing the Sp1 element need the presence of the GC-element binding proteins to properly activate the *APP* promoter. These results

illustrate well that both the Sp1 and USF elements are essential and that they cooperate with the GC-element, located between -96 and -77, to confer full activity on the *APP* promoter. Furthermore, the data obtained with the double mutation present in pAPPCAT-96mSU and pAPPCAT-395mSU clearly show that the proteins binding to the GC-element cannot be held responsible for the entire activity of the *APP* promoter as previously proposed (Quitschke et al., 1996).

Fig. 6.8 Transient transfections of mutants of the *APP* promoter in NG108-15 and HepG2 cells.

NG108-15 cells were transfected with 5 μg of pAPPCAT derivatives, 5 μg of pBLCAT6 (carrier) and 1 μg of pUT535 (ß-galactosidase) to standardize for transfection efficiency. Transient transfection assays with HepG2 cells used 15 μg of pAPPCAT derivatives and 1 μg of pUT535. Transfection were performed in triplicate with 2 independent preparation of plasmid DNA for each construct (n=4). The presence or absence of the GC-element, Sp1 and USF elements are indicated by boxes in the schematic representation of the *APP* promoter. Relative CAT activity is expressed as a function of pAPPCAT-96.

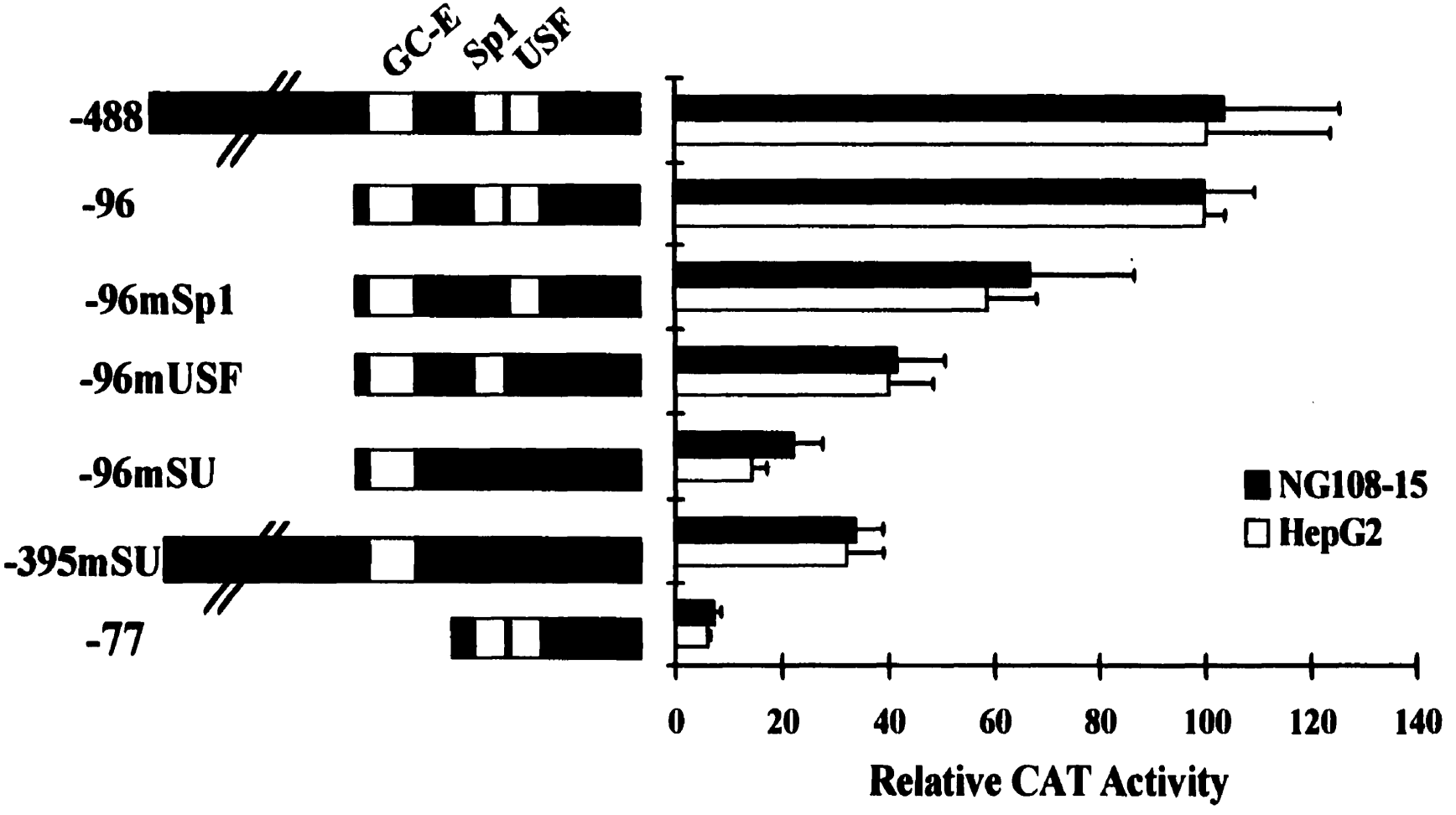

GC-E
Sp1
USF
-488
-96
-96mSp1
-96mUSF
-96mSU
-395mSU
-77
NG108-15
HepG2
0
20
40
60
80
100
120
140
Relative CAT Activity

Fig. 6.9 Transient transfections of truncated mutants of the *APP* promoter in NG108-15.

NG108-15 cells were transfected with 10 μg of pAPPCAT-77 derivatives and 1 μg of pUT535 for ß-galactosidase standardization of transfection efficiency. Presence or absence of the Sp1 and USF elements is indicated by the boxes in the schematic drawing of the truncated *APP* promoter. The relative CAT activity is expressed as a function of pAPPCAT-96 (not shown). Transfections were performed in triplicate with at least two independent preparations of plasmid DNA. The error bars indicated the standard error of the mean (SEM), n=3.

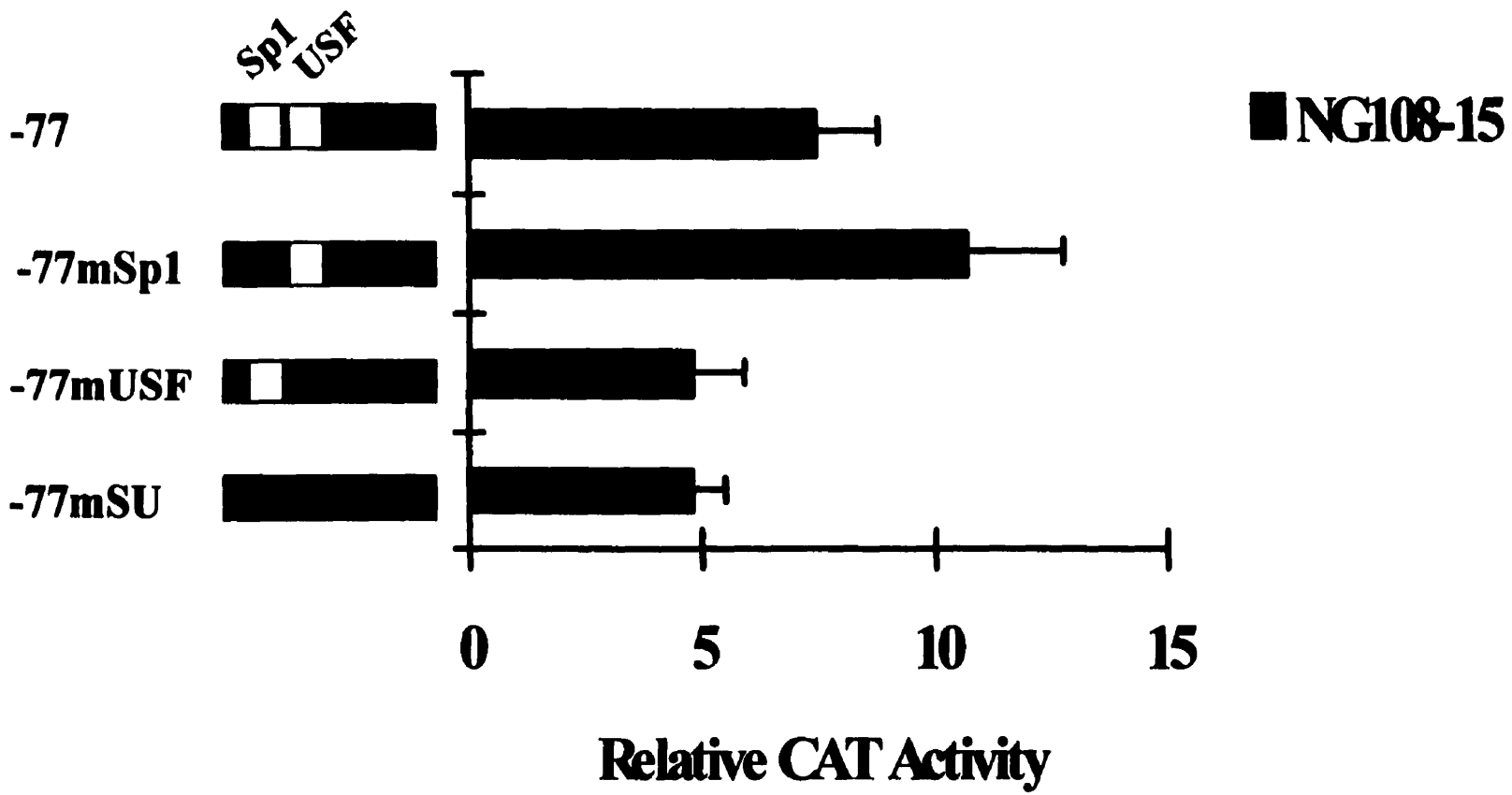
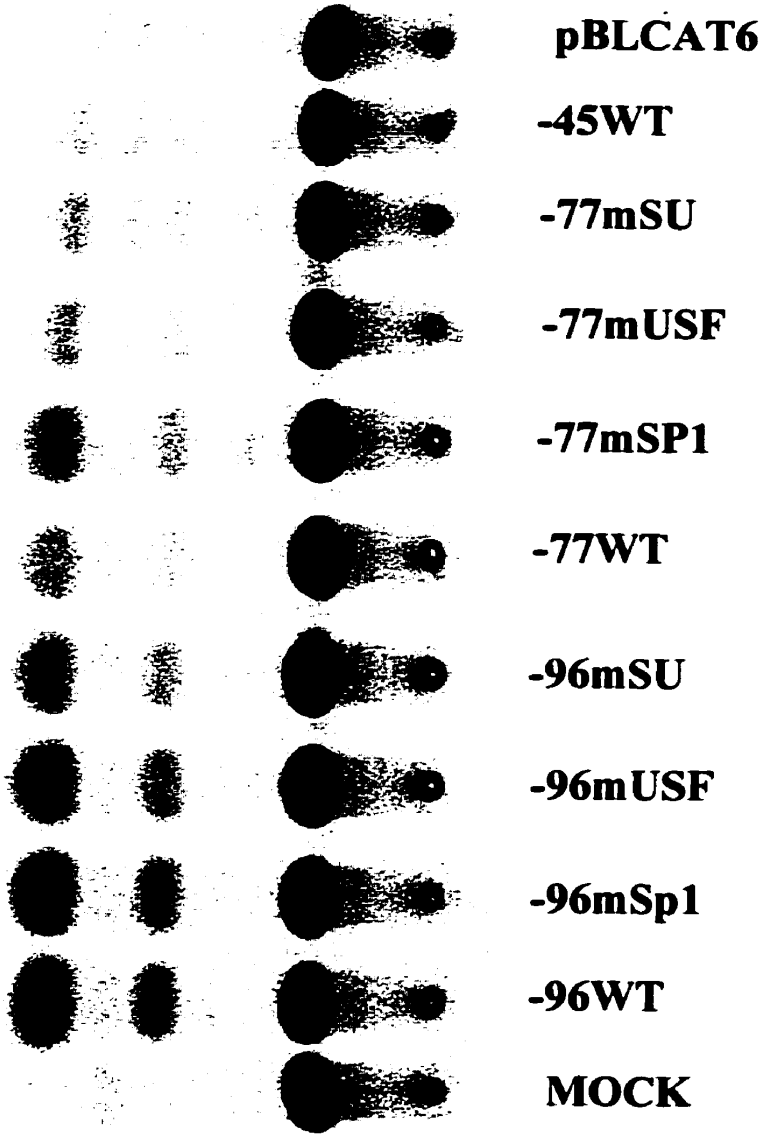

Sp1
USF
-77
-77mSp1
-77mUSF
-77mSU
NG108-15
0
5
10
15
Relative CAT Activity

Fig. 6.10 Representative CAT assays of mutants of the *APP* promoter.

NG108-15 cells were transfected with 10 μg of pAPPCAT derivatives and 1 μg of pUT535. For the purpose of this Figure, cellular extracts were standardized to ß-galactosidase activity before CAT assays were performed. Mock transfection contained no DNA.


pBLCAT6
-45WT
-77mSU
-77mUSF
-77mSP1
-77WT
-96mSU
-96mUSF
-96mSp1
-96WT
MOCK

Chapter 7

Results

Enhanced expression of the ß-amyloid precursor protein gene in response to dibutyryl cAMP is not mediated by the transcription factor AP-2

Although the structure of the *APP* promoter is typical of housekeeping genes, its pattern of expression implies that it is regulated in a developmental and tissue specific manner (Salbaum and Ruddle 1994). *APP* expression can be modulated *in vitro* by treatment of endothelial cells and cultured astrocytes with interleukin-1ß (IL-1ß) (Goldgaber et al., 1989; Gray et al., 1993a), transforming growth factor ß (TGFß) (Gray et al., 1993a), or basic fibroblast growth factor (bFGF) (Gray et al., 1993b). In neuronal cell lines, treatment with growth factors leads to upregulation of *APP* mRNA, but this seems to be a consequence of the differentiation state of the cells. For example, treatment of PC-12 cells with NGF, bFGF, phorbol ester and retinoic acid will enhance the expression of *APP* mRNAs through stimulation of the *APP* promoter (Lahiri and Nall 1995). However, in comparison to the untreated cells these authors did not observe any increase in basal level promoter activity when the factors were added to the cells after transfection (post-treatment only), suggesting that the stimulatory effect on promoter activity was dependent on the state of differentiation of the PC-12 cells by the factors.

The expression of *APP* can be regulated by different signal transduction pathways. For example, the increase in the level of *APP* mRNAs induced by IL-1 is blocked by H-7, an inhibitor of protein kinase C (Goldgaber et al. 1989). Recently, Trejo et al. (1994) have

found that activation of protein kinase C upregulates *APP* gene expression through the function of the transcription factor Jun/AP-1. They demonstrated that phorbol 12-myristate 13-acetate (PMA) induces nuclear proteins that bind specifically to an AP-1 recognition sequence in the *APP* promoter at -346 and that the binding is effectively competed by a consensus AP-1 oligonucleotide.

Cyclic AMP is a key intracellular second messenger in nervous tissue. Our laboratory has shown that during treatment of NG108-15 cells with dibutyryl cAMP, an inhibitor of cAMP phosphodiesterase which results in an increase in the cellular pool of cAMP, expression of the *APP* gene is upregulated three fold after 5-6 days, and that this increase in expression occurs through transcriptional mechanisms (Shekarabi et al., 1997). *APP* does not contain a canonical cyclic AMP response element (CRE) (Salbaum et al., 1988), but of the putative *cis*-elements which are found in the proximal upstream region, the AP-2 site centered at position -205 can be considered a good candidate since AP-2 is known to mediate the effects of cAMP. Results will be presented in Chapter 7 that demonstrate by co-expression studies and EMSA that this putative AP-2 site is not functional.

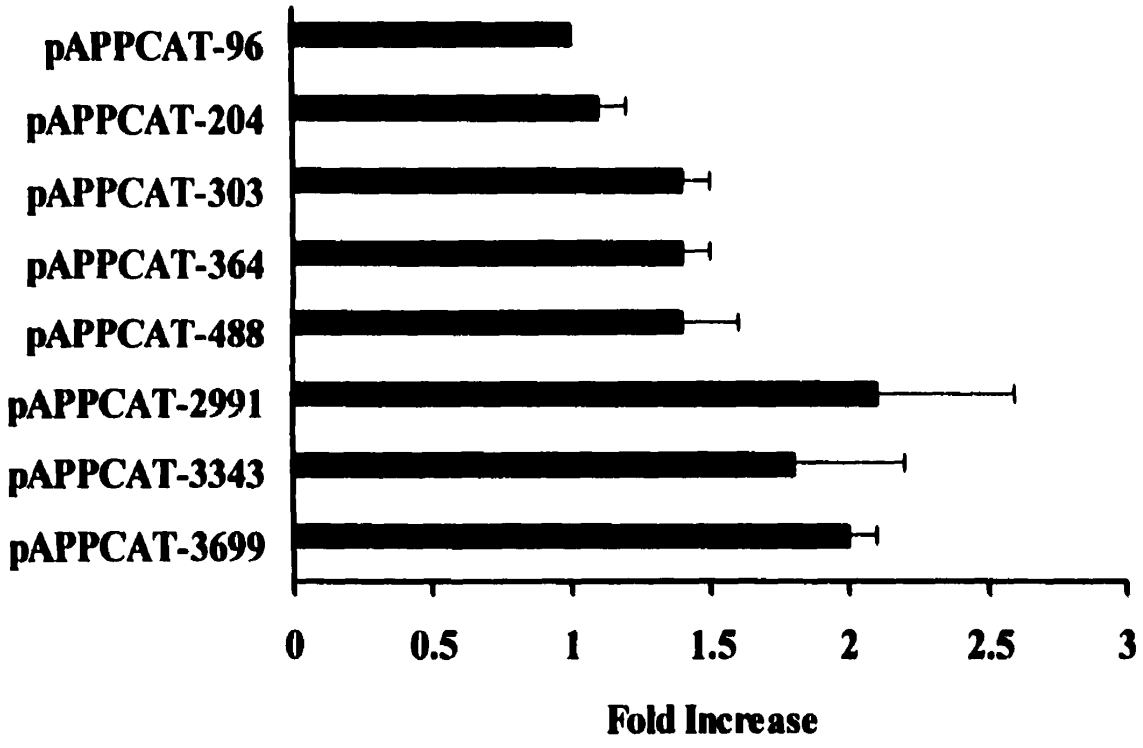
Cyclic AMP increases *APP* transcription

NG108-15 cells were treated with 1 mM dbcAMP, and steady-state level of *APP* mRNA was quantitated by northern blot analysis. A short-term effect of dbcAMP was observed leading to a 1.8-fold increase in *APP* expression after 12 hours of treatment (Shekarabi, 1996). Similarly, in the presence of forskolin, which is an activator of adenylate cyclase and increases intracellular cAMP levels (Seamon and Daly, 1986), a 1.7-fold increase was observed after about 7 hours of treatment of NG108-15 cells (data not shown).

To determine the *cis*-element responsible for the upregulation of *APP* levels, *APP* promoter activity was assayed by transiently transfecting NG108-15 cells with different promoter-reporter constructs containing progressively longer 5' upstream regulatory sequences of the *APP* gene. After sixteen hours of transfection, the cells were divided into two groups, one being treated with 1 mM dbcAMP and the other left untreated. As illustrated in Figure 7.1,

Fig. 7.1 Transcriptional activation in NG108-15 cells of the *APP* promoter by dbcAMP.

NG108-15 cells were transiently transfected with various portions of the *APP* promoter introduced upstream of the bacterial *CAT* gene. Each transfection contained 5 μg of pAPPCAT derivatives, 4 μg of pUC18 used as carrier and 1 μg of pUT535. After 16 hrs, the transfected cultures were divided in two and one culture was treated with 1 mM dbcAMP. The calculated fold increase represents the overall effect of dbcAMP on the *APP* promoter when normalized to the basal promoter present in pAPPCAT-96. Exact fold increases are indicated on the right of the graph. The error bars represent standard error of the mean (SEM), n=3-4 with at least two different preparations of plasmid DNA.


pAPPCAT-96
pAPPCAT-204
pAPPCAT-303
pAPPCAT-364
pAPPCAT-488
pAPPCAT-2991
pAPPCAT-3343
pAPPCAT-3699
0
0.5
1
1.5
2
2.5
3
Fold Increase

the different *APP* promoter constructs responded differently to the presence of dbcAMP. A 1.4-fold increase in transcriptional activity was regulated by sequences from -303 to -204 and was maintained in all *APP* promoter-reporter constructs up to the sequence -488. A further 50 % increase in transcriptional activity in response to dbcAMP was directed by sequences from -2991 to -488.

Co-expression of AP-2 activates *APP* promoter

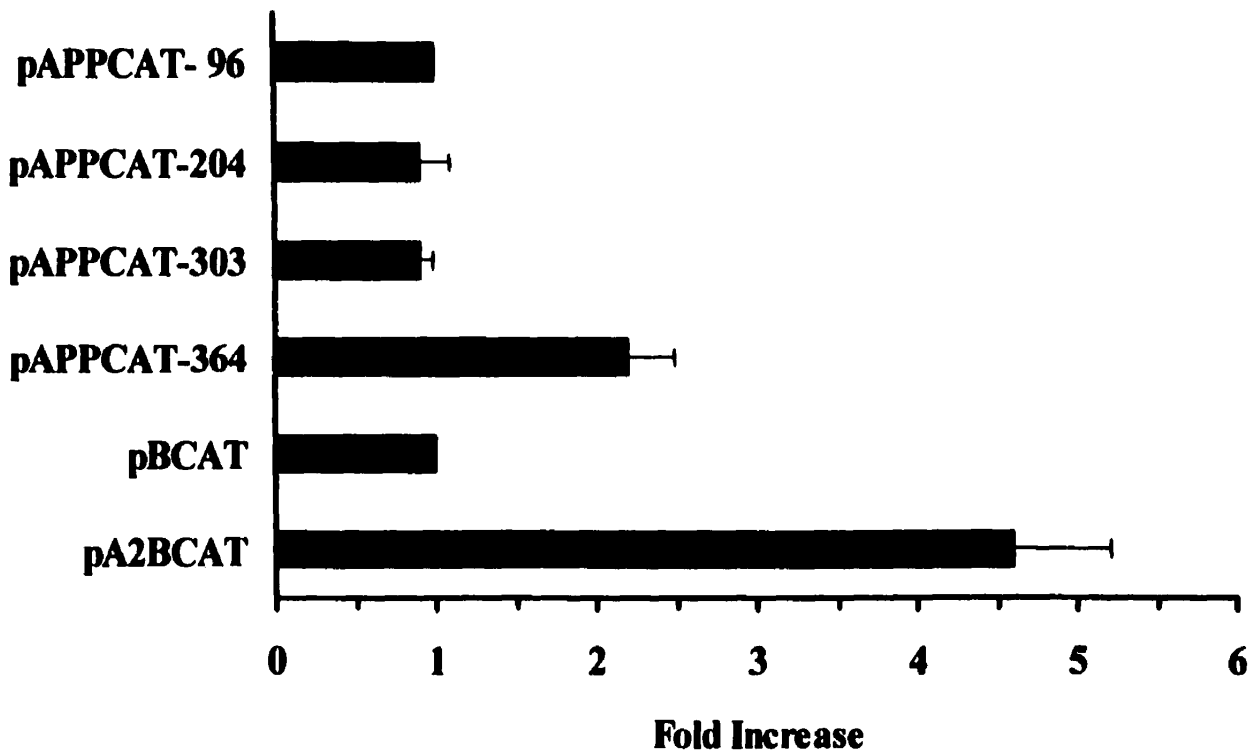
The above results suggest that regulatory sequences between -303 and -204 may mediate some of the increase detected in steady-state levels of *APP* mRNA in the presence of dbcAMP. Although there is no canonical CRE in this region (Salbaum et al., 1988), there is a binding site centered at -205 for the transcription factor AP-2 which is also inducible by cAMP (Imagawa et al., 1987). To verify whether AP-2 plays a role in this response of the *APP* gene, the different *APP* promoter-reporter constructs were co-transfected into HepG2 cells with an expression vector in which the full-length AP-2 cDNA is driven by the RSV LTR (Williams et al., 1991a). The HepG2 cell line lacks endogenous AP-2 activity and hence provides an efficient assay system for testing the effect of transfected AP-2 (Williams et al., 1991a). As shown in Figure 7.2, co-expression of AP-2 in HepG2 cells with a plasmid containing two AP-2 recognition sites (pA2BCAT) increases reporter gene levels 4.6-fold compared to a plasmid devoid of AP-2 elements (pBCAT). Similarly, exogenous AP-2 leads to activation of the *APP* promoter, with a 2-fold increase in CAT activity (Fig. 7.2). However, the response to AP-2 occurs not in the -303 fragment which contains the AP-2 element, but in the -364 fragment which has functional binding sites for AP-1 (Trejo et al., 1994) and a heat shock factor (Dewji et al., 1995), in addition to the AP-2 recognition sequence. These results indicate that the effect of AP-2 may not be through a direct transactivation of the promoter but may occur through action of AP-2 on the expression of other genes.

***APP* promoter does not contain a functional AP-2 site**

As shown above, co-expression of AP-2 and an *APP* promoter-reporter construct which contains a putative AP-2 site did not result in enhancement of transcriptional activity as

Fig. 7.2 Transcriptional activation in HepG2 cells of the *APP* promoter by the transcription factor AP-2.

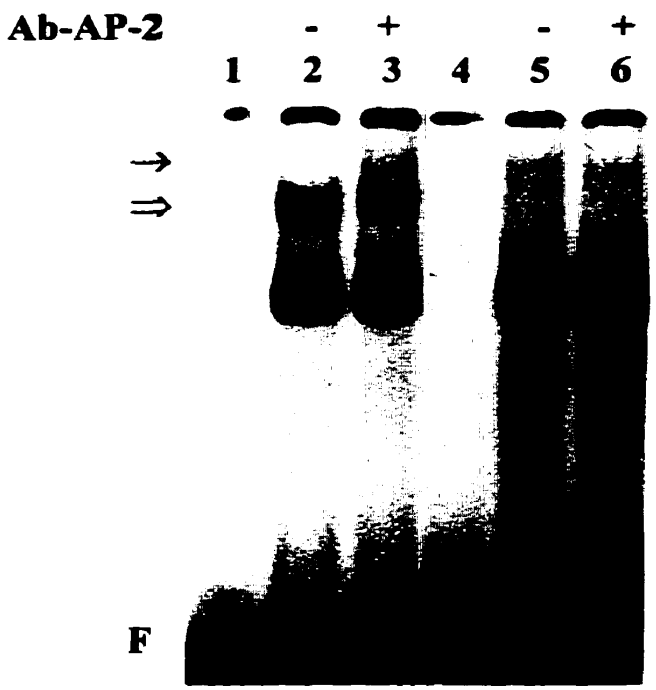
HepG2 cells were transiently co-transfected with *APP* promoter-reporter plasmids (pAPPCAT) and eukaryotic expression vectors containing AP-2 cDNA (pRSVAP2) or no insert (pRSV). Each 60 mm dish contained 2 μg of CAT reporter plasmids, 0.5 μg of pRSV or pRSVAP2, 1 μg of pUT535 and 11.5 μg of pBLCAT6, used as carrier, for a total of 15 μg. The positive control experiment for the effect of AP-2 consists of the pair pBCAT and pA2BCAT (two AP-2 elements). Fold increases represent the specific effect of the transcription factor AP-2 from cells co-transfected with pRSVAP2 compared to cells co-transfected with pRSV (control plasmid). Results were normalized to the basal promoters pAPPCAT-96 or pBCAT. The error bars represent the standard error of the mean, n=3-4 with two separate preparations of plasmid DNA.


pAPPCAT- 96
pAPPCAT-204
pAPPCAT-303
pAPPCAT-364
pBCAT
pA2BCAT
0
1
2
3
4
5
6
Fold Increase

would have been expected with a functional AP-2 site. Since the AP-2 protein was initially cloned from a HeLa cell cDNA library and to determine whether the AP-2 site at -205 can bind AP-2, we performed EMSA in the presence of HeLa cell nuclear extracts with a double-stranded oligonucleotide spanning from -222 to -195 (APP-AP2). No binding was observed in HeLa nuclear extracts (Figure 7.3, lanes 5 and 6) although these contain *bona fide* AP-2 activity as shown with the AP-2 consensus oligonucleotide which forms a specific complex (Figure 4, lane 2) that can be recognized and "supershifted" with an antibody against AP-2 (Figure 7.3, lane 3). Taken together, these results suggest that the AP-2 recognition site at -205 cannot be bound by AP-2 nor can it be used by exogenous AP-2 to transactivate the human *APP* promoter.

Fig. 7.3 The transcription factor AP-2 fails to recognize the putative AP-2 element located in the *APP* promoter.

EMSA of oligonucleotides containing a *bona fide* AP-2 binding site (lanes 1-3) and a putative AP-2 element present in the *APP* promoter (lanes 4-6) (APP-AP-2: -222 to -195). Oligonucleotides were incubated in absence (lanes 1 and 4) or presence (lanes 2-3 and 5-6) of HeLa nuclear extracts. The large arrow indicates the complexes specific to the AP-2 oligonucleotide (lanes 2 and 3). The small arrow shows the supershift induced by the AP-2 antibody in lane 3. (Note the absence of the AP-2 complexes in lanes 5 and 6 which contain the oligonucleotide APP-AP-2).

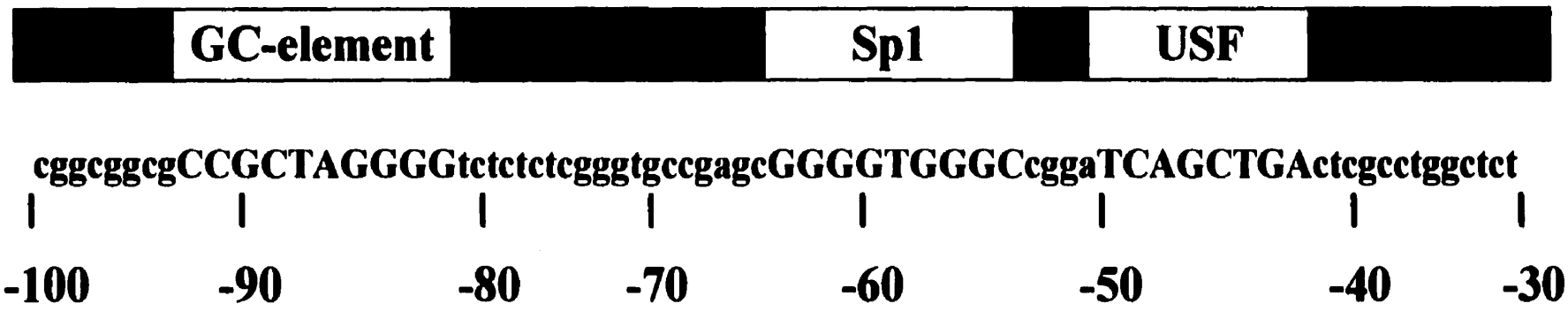

Ab-AP-2
-
+
-
+
1
2
3
4
5
6
F

Chapter 8

Discussion

The APP gene is expressed ubiquitously. The sequences surrounding the major transcriptional start site have high a percentage of GC (85%) and do not contain the consensus sequence referred to as TATA-box which is recognized by the TATA binding protein (TBP), a component of the transcription factor II D (TFIID) subunit of the RNA polymerase II. We have shown using transient transfection assays that the *APP* promoter was functional in NG108-15 and dibutyryl cAMP treated NG108-15 cells. DNA sequences of the proximal *APP* promoter recognized by proteins present in NG108-15 nuclear extracts were essentially the same as those previously reported in HeLa cells (Pollwein et al., 1992; Bourbonnière and Nalbantoglu, 1993). We also showed that an oligonucleotide (APP-GC1) carrying sequences located between positions -97 and -78 relative to the major transcriptional start site was recognized in a specific manner by proteins present in nuclear extracts from NG108-15 cells. Sequences responsible for the transcriptional activity of the *APP* promoter have been narrowed down to 96 bp upstream of the major transcriptional start site (Pollwein et al., 1992; Quitschke and Goldgaber, 1992). We investigated the role of three elements located between positions -96 and -30 in *APP* regulation (see Fig 8.1). Presence of the GC-element is essential for *APP* expression. The protein(s) recognizing the GC-element have a molecular weight ranging from 45 kDa to 85 kDa. We demonstrated that the other two elements were recognized by the Sp1 (-63 to -55) and USF (-50 to -43) transcription factors and that of the two only USF was capable of transactivating the *APP* promoter in transient co-transfection assays. Our *in vitro* results coupled with deletion and mutational analyses of the *APP* promoter showed that these three elements located within a 66 bp region, between -96 and -30, of the *APP* promoter are necessary for full activity in two different cells lines, the NG108-15 and the HepG2 cell lines.

Fig. 8.1 Schematic representation of regulatory elements of the *APP* promoter.
Elements present in the *APP* promoter are indicated above the sequence. The numbers indicate the position relative to the major transcription start site of *APP*. The core binding site of each element is indicated in capitals letter.


GC-element
Sp1
USF
cggcggcgCCGCTAGGGGtctctctcgggtgccgagcGGGGTGGGCcggaTCAGCTGActcgcctggctct
-100
-90
-80
-70
-60
-50
-40
-30

Pattern of binding of nuclear proteins to proximal sequences of the *APP* promoter.

Our studies of the *APP* promoter using DNase I footprinting analysis revealed a protected region located between -123 and -68 of the *APP* promoter. This footprint spans a region that includes two of the putative GC-boxes (-123 to -114 and -113 to -104) described by Salbaum et al. (1988), a GC-palindrome (-97 to -87) and a fourth element consisting of a pyrimidine tract (-82 to -75) (Pollwein et al., 1992). The occupancy of these sequences by many different proteins may explain the large protected region observed in the DNase I footprint in Fig. 3.2.

Using NG108-15 nuclear extracts we did not observe the presence of a protected area corresponding to region -65 to -47 as described by Pollwein and co-workers (1992). This could be due to the absence of the protein recognizing this portion of the promoter from NG108-15 nuclear extract. This region corresponds to the element later identified as binding Sp1 (-63 to -55). In EMSA with the APPSP1 oligonucleotide (-71 to -50) and nuclear extracts from HeLa cells, we observed the formation of a specific complex containing Sp1 activity (Chapter 5). However, this complex was absent when NG108-15 nuclear extracts were used in EMSA (data not shown).

In the footprinting analysis of the *APP* promoter performed by Pollwein and colleagues (1992), a protected area was not observed which corresponded to the putative AP-1/AP-4 element, later characterized as being recognized by the USF transcription factor (USF element). Our own DNase I footprinting analysis of the *APP* promoter also failed to detect the USF element. The element corresponding to the USF binding site was first reported to be protected from DNase I digestion by Quitschke and Goldgaber (1992). In this DNase I footprinting analysis of the *APP* promoter, the experimental procedure was specifically optimized to visualize the USF element. These workers used a shorter probe (-96 to +53) combined with longer exposure times to visualize the results. These differences in experimental procedures could explain why we and Pollwein (1992) did not detect the USF element.

Characteristics of the GC-element.

Deletions of the *APP* promoter have shown that sequences upstream of the first 94 base pairs are not essential for the activity of the promoter (Pollwein et al., 1992; Quitschke and Goldgaber, 1992). Similar results were obtained with the mouse and rat *APP* promoters which show a high level of conservation between position -100 and + 1 (86% with the mouse promoter and 85% with the rat promoter) (Izumi et al., 1992; Hoffman and Chernak, 1994). These results suggest that the footprint observed by Pollwein et al. (1992) that contains the GC palindrome (-97 to -87, 5'-GCGGCGCCGC-3') does not contain the core binding site of the factor(s) involved in *APP* promoter activity. A similar footprint (-98 to -77) was also observed by Quitschke (1996) that encompasses the GC palindrome (-97 to -87) and the pyrimidine tract (-82 to -75) identified by Pollwein et al. (1992). Mutational analysis of the sequences located between -99 and -70 revealed that region -93 to -82 is critical for *APP* promoter activity, lowering its activity by 60% to 90% (Quitschke, 1994). Mutations introduced in the pyrimidine tract did not affect the activity of the *APP* promoter. These results suggest that the sequence 5'-GCGCCTAGGGGT-3' (-92 to -82) is the core binding site of the factor(s) recognizing this region of the *APP* promoter. This sequence is conserved between rodents and humans except for the G residue located at position -92 which is replaced by a T residue in rodents.

Our results have shown that the APP-GC1 oligonucleotide (-97 to -78), which contains the region of the promoter we refer to as the GC-element, was recognized in a specific manner by proteins present in NG108-15 nuclear extracts. Using UV crosslinking of nucleic acids to proteins present in NG108-15 nuclear extracts, we observed that the APP-GC1 oligonucleotide was recognized by numerous proteins. The presence of multiple complexes suggests a family of DNA-binding proteins recognizing the GC-element to regulate expression of the *APP* promoter. Our attempts to identify a gene encoding for a protein recognizing this element by screening a HeLa cDNA expression library failed. This might have been caused by the nature of the GC-element. The GC-

element contains a palindrome of 10 bp (5'-CCGCTAGGGG-3') with a single mismatch (underlined). Palindromes are usually recognized by homo- or heterodimer proteins. It is conceivable that the transcription factor(s) recognizing the GC-element is a dimer and part of a larger family of proteins that either homo- or heterodimerize to recognize their target sequence. This interpretation of our results would agree with our UV crosslinking data that show two major complexes (Fig. 3.5, 75 to 85 kDa) of about the same intensity suggesting equimolecular amounts of proteins having a similar molecular weight, as often observed with this type of transcription factors (for example USF-1, 43 kDa and USF-2, 44 kDa). Due to these characteristics, purification of the binding activity from nuclear extracts might be more suitable for identifying the gene(s) encoding the protein(s) recognizing the GC-element.

Characteristics of the E box - USF element.

The E box found in the proximal promoter region of the APP gene at position -49 to -44 has previously been shown to be important for expression of the human and rodent genes (Izumi et al., 1992; Pollwein et al., 1992; Quitschke and Goldgaber, 1992; Pollwein, 1993; Hoffman and Chernak, 1994; Quitschke, 1994). EMSA studies (Quitschke and Golgaber, 1992; Pollwein, 1993; Hoffman and Chernak, 1994; Quitschke, 1994), DNase I footprinting (Izumi et al., 1992; Pollwein et al., 1992; Quitschke and Goldgaber, 1992; Hoffman and Chernak, 1994) and methylation interference (Quitschke and Goldgaber, 1992) had revealed that a sequence of 12 base pairs, 5'-GGATCAGCTGAC-3' (-53 to -42), was essential for nuclear factor binding (Quitschke and Goldgaber, 1992; Quitschke, 1994). This motif which is conserved 100% between human, mouse and rat genes is also footprinted by nuclear proteins in rodents. While this sequence overlaps with the consensus recognition sequences of transcription factors AP-1 (TGACTCG, -45 to -39) and AP-4 (TCAGCTGA, -50 to -43) neither factor appears to bind to it (Quitschke and Goldgaber, 1992).

The competition experiments in EMSA have shown that USF binding to oligonucleotide APP-E1 occurs with approximately the same affinity as its binding to oligonucleotide Ad-USF. This is somewhat surprising in view of the difference of the *APP* E box (CAGCTG) from the USF consensus sequence of CACGTG (2 out of 6 nucleotides) (Bendall and Molly, 1994). However, USF binding to non-consensus sequences has been documented (Chodosh et al., 1987; Carthew et al., 1987). Furthermore, it has recently been demonstrated that $MgCl_2$ has a significant influence on the DNA binding specificity of USF: in the absence of $MgCl_2$, specificity is relaxed at positions ±1 and ±4 of the consensus half-site (Bendall and Molly, 1994). Since protein contacts to positions 1 and 4 of the half-site are not as critical in the absence of Mg^{++} ions, the substitution of CG by GC in oligonucleotide APP-E1 does not appear to affect USF binding under our assay conditions (1 mM $MgCl_2$). Although increasing Mg^{++} ion concentrations do diminish the affinity of binding, APP-E1 - protein complexes are easily detectable at 5mM $MgCl_2$ (data not shown).

Our results also suggest that USF, upon binding to oligonucleotide APP-E1, contributes to the expression of the *APP* gene since an *APP* promoter-CAT reporter construct was transactivated by co-transfection of a eukaryotic expression vector containing USF-1 cDNA into NG108-15 cells and HepG2 cells. Furthermore, the introduction of mutations in the *APP* E box prevented this activation of the *APP* promoter by USF-1 in HepG2 cells (Fig. 6.7). Our results are confirmed by other reports which have shown comparable data concerning the role of USF and the E box in both rat and human *APP* promoters (Hoffman and Chernak; 1995; Kovacs et al., 1995; Vostrov et al., 1995).

Besides activating the adenovirus major late promoter by binding to an E box (Sawadogo and Roeder, 1985), USF has been shown to regulate a variety of cellular genes through interactions with upstream E boxes (Carthew et al., 1987; Chodosh et al., 1987; Sato et al., 1989; Bresnik and Felsenfeld, 1993; Reisman and Rotter, 1993). The stimulatory effect of USF may be mediated through direct protein-protein interaction between USF and the transcriptional apparatus (Roy et al., 1991). It has also been proposed that binding of USF to its recognition sequences may lead to formation of a nucleosome-free region over the

promoter, increasing the accessibility of the region surrounding its binding site to basal initiation factors (Workman et al., 1990). Further support for the involvement of USF in chromatin dynamics is provided by the report that USF binds with high affinity to an E box in the DNase I-hypersensitive site HS2 in the ß-globin locus control region (Bresnick and Felsenfeld, 1993). This might also be the functional mechanism in regulating basal expression of the APP gene since a DNase I hypersensitive site has been demonstrated in the proximity of the USF binding site (Lukiw et al., 1994).

Characteristics of the Sp1 element.

DNase I footprinting analysis and methylation interference of the proximal *APP* promoter using HeLa nuclear extracts identified a region containing a putative Sp1 binding site (-65 to -47) (Pollwein et al., 1992; Pollwein 1993). Similar results were observed with the mouse *APP* promoter although the protected area (-67 to -41) included as well the entire E box corresponding to the USF element (Izumi et al., 1992). In competition EMSA with HeLa nuclear extracts, Pollwein (1993) observed that an oligonucleotide containing a previously described Sp1 binding site could partly remove one of the complexes formed with an oligonucleotide representing the *APP* promoter spanning region -72 to -35, suggesting that the Sp1 transcription factor could recognize sequences present between -72 and -35. Also, in EMSA studies using an oligonucleotide containing both the Sp1 and USF elements from the rat *APP* promoter, Hoffman and Chernak (1995) reported that one of the complexes formed could be recognized by an antibody directed against Sp1. The Sp1 element (-63 to -55: 5'-GGGGTGGGC-3') is conserved between rodents and humans except for the G residue at position -63 which is replaced by a C residue in rodents.

Our results using recombinant Sp1 in DNase I footprinting analysis and EMSA with oligonucleotide APPSP1 (-71 to -50) are in complete agreement with what was proposed by Pollwein (1993) and by Hoffman and Chernak (1995) for the rat *APP* promoter.

Furthermore, the use of an antibody directed against Sp1 clearly demonstrated that Sp1 (Fig. 5.2B) was part of a complex formed between proteins present in HeLa nuclear extracts and oligonucleotide APPSP1. These *in vitro* results suggested that Sp1 participated in the regulation of the *APP* promoter. However, we failed to observe a transactivation of the *APP* promoter in *Drosophila melanogaster* S2 cells, a system from which Sp1 activity has been shown to be lacking (Courey and Tjian, 1988). One of the reasons for this lack of activation could be that the affinity of Sp1 for the *APP* Sp1 element. Although we did not perform affinity studies, two observations suggest the Sp1 does not have the same affinity for the *APP* Sp1 element when compared with the SV40 Sp1 element. First, intensity of the complex formed between recombinant Sp1 and the SP1 oligonucleotide is stronger then the one observed between recombinant Sp1 and oligonucleotide APPSP1 (Fig. 5.2A). Second, in competition EMSA with HeLa nuclear extracts and oligonucleotide APPSP1, the SP1 oligonucleotide totally removed the complex containing Sp1 activity with a 20 fold molar excess whereas for oligonucleotide APPSP1 the complex disappeared with a 50 molar excess (Fig 6.3, compare lane 5 to lane 8 and 9). Another factor that could have been influential in our transactivation study is the number of Sp1 binding sites present in the *APP* promoter. Our positive control (pTKCAT) which was activated more than two fold contained two Sp1 binding sites (Jones et al. 1985). The activation of pTKCAT by Sp1 in our transactivation assays is well within what was previously reported under similar conditions (2.8 fold) (Courey and Tjian, 1988). The presence of only one low affinity binding site for Sp1 in the *APP* promoter could explain why we did not detect any increase in activity during co-transfection studies in *Drosophila* cells. It remains to be seen if transactivation would have been observed within the context of a longer portion of the *APP* promoter, in which there is an additional Sp1-binding site in close proximity (-137 to -122) as shown in Figure 5.1.

The activity of both pAPPCAT-77 and pAPPCAT-96 (Fig. 5.5) is very similar when transfected in *Drosophila* S2 cells whereas in mammalian cell lines we observed a decrease of 90% in activity (Fig. 6.1). This suggests that the sequences located between -

96 and -77 (GC-element) are not active in *Drosophila* cells. Although the transcriptional machinery is mostly conserved in eukaryotic cells, some components are species specific. One example is the human TBP associated factor (hTAF) 55 (55 kDa) of TFIID which is absent from *Drosophila* cells and shown to physically interact with Sp1 (Chiang and Roeder, 1995; reviewed in Burley and Roeder, 1996). Whether this particularity affected the potential activation of the *APP* promoter by Sp1 is not known.

The role of Sp1 in *APP* regulation might not only be to directly activate transcription of the *APP* promoter. Binding sites for Sp1 in promoters of housekeeping genes, usually located in CpG islands in the chromatin, are protected from *de novo* methylation which turns off gene transcription (Brandeis et al., 1994). Another possible function of Sp1 would be to create an environment free of nucleosomes and facilitate the assembly of a transcriptionally active RNA polymerase II complex (Li et al., 1994). Sp1 has been shown to directly interact with two subunits of TFIID, hTAF 135 and hTAF 55 and possibly help in the recruitment of TFIID to TATA-less promoters (reviewed in Burley and Roeder, 1996).

Role of the GC-, Sp1 and USF elements in transcription of *APP*.

We studied the roles of the Sp1 and USF elements in presence (pAPPCAT-96 derivatives) and in absence (pAPPCAT -77 derivatives) of the GC-element in order to clarify their participation in the transcriptional regulation of the *APP* promoter. The proximity of an Sp1 binding site in the TATA-less human Ha-ras promoter influences the selection of transcriptional start sites (Lu et al., 1994). In the *APP* promoter, neither the Sp1 nor the USF binding sites affected the choice of transcriptional start sites although we observed a decrease in overall activity of the mutants reminiscent of the results obtained in transient transfection assays. We determined in these assays that both elements were necessary for full activity of the *APP* promoter (Fig. 6.8, Fig. 6.9 and

Table 1 Mutational analysis of the *APP* promoter in NG108-15 and HepG2 cells.
The active elements present in each construct are indicated. The relative CAT activity is expressed in function of the wild type promoter present in pAPPCAT-96.

Plasmids	Elements	Relative CAT Activity	Relative CAT Activity
		NG108-15	HepG2
pAPPCAT-96	GC - Sp1 - USF	100.0%	100.0%
pAPPCAT-96mSp1	GC - USF	66.8%	58.9%
pAPPCAT-96mUSF	GC - Sp1	41.5%	40.1%
pAPPCAT-96mSU	GC	22.3%	14.3%
pAPPCAT-77WT	Sp1 - USF	7.4%	6.1%
pAPPCAT-77mSp1	USF	10.7%	-
pAPPCAT-77mUSF	Sp1	4.8%	-
pAPPCAT-77mSU	-	4.8%	-

summarized in Table 1). Our approach is the first one that looks at the effects of mutations in the Sp1 and USF elements, separately or in combination, in presence or absence of the GC-element (for a comparison with previous studies see Table 2).

The roles of the Sp1 and USF elements in regulation of the *APP* promoter have been difficult to define. Pollwein (1993) observed that mutations introduced into the Sp1 element (which only differs from our mutation by one nucleotide, see Table 2) had no effect on the activity of the *APP* promoter in HeLa cells. Mutations in the USF element (not known at the time to be recognized by USF) reduced the activity to 78% of the wild type promoter. When both mutations were present, the activity of the *APP* promoter fell to 13% (a level which is similar to what we observed in human HepG2 cells, 14.3%). Pollwein proposed that the close proximity of the Sp1 and USF elements led to competition of the factors in their recognition and regulation of the *APP* promoter and that the factors recognizing these elements could compensate for the absence of each other. When both factors were prevented from interacting with their respective element, the activity of the promoter was compromised. In the rat, opposite results were observed (Hoffman and Chernak, 1995). Mutations in the Sp1 element reduced the activity to 70% whereas mutation in the USF element had no effect. Hoffman and Chernak (1995) demonstrated the role of USF by mutating the *APP* USF element into a previously described consensus sequence for USF (Sawadogo and Roeder, 1985) and obtaining higher levels of activation. This study did not look at the consequences on rat *APP* promoter activity of the effect of mutations in both elements. Our results show that mutations introduced into both the Sp1 and USF element, alone or in combination, affect the *APP* promoter. These differences could be due to the specific changes introduced in the *APP* promoter. For example, to inactivate the Sp1 element, we changed four bases (GGTG to AACA) in order to disrupt the binding of 2 out of the 3 zinc fingers of Sp1. Pollwein (1993) reported only partial competition in EMSA with an oligonucleotide containing the mutated Sp1 element (GTG to ACA, one zinc finger) whereas in our case the oligonucleotide APPSP1m did not interfere with formation of the complex between APPSP1 and Sp1 under similar conditions. Since mutations in both elements have an

Table 2 Comparison of mutational analysis of the *APP* promoter.

Summary of mutations introduced in the *APP* promoter. The promoter context in which the mutational analysis took place is indicated in parenthesis by the relative position to the major transcriptional start site. The wild type core binding site of each element is underlined. Mutations are indicated in ***bold italics***. Differences between human and rat sequences are underlined. The % of activity is expressed in function of the wild type promoter used in each separate study.

	Sp1 element	USF element	% of activity
(-96)	AGC GGG GTG GGC CGG	GAT CAG CTG ACT	100
Bourbonnière			
(-395)	AGC GG*A ACA* GGC CGG	GAT C*GA T*TG ACT	32
(-96)	AGC GG*A ACA* GGC CGG		67
		GAT C*GA T*TG ACT	42
	AGC GG*A ACA* GGC CGG	GAT C*GA T*TG ACT	22
(-77)	AGC GGG GTG GGC CGG	GAT CAG CTG ACT	8
	AGC GG*A ACA* GGC CGG		11
		GAT C*GA T*TG ACT	5
	AGC GG*A ACA* GGC CGG	GAT C*GA T*TG ACT	5
Pollwein (1993)			
(-94)	AGC GGG *ACA* GGC CGG		100
		GAT CAG C*CA* ATC	78
	AGC GGG *ACA* GGC CGG	GAT CAG C*CA* ATC	13
Quitschke (1994)			
(-488)		GA*G AC*G CTG ACT	100
		GAT CA*T AG*G ACT	100
		GAT CAG CT*T CA* T	100
(-77)		GAT CAG CTG ACT	30
		GA*G AC*G CTG ACT	5
		GAT CA*T AG*G ACT	5
		GAT CAG CT*T CA* T	5
Hoffman and Chernak (1995)			
(-219) (rat)	AGC CGG GT*A TAT* GGG		70
		GAT *GTT AGA* ACT	100
		GAT CA*C G*TG ACT	180
		G*G*T CA*C A*TG ACT	160
Quitschke et aL, (1996)			
(-488)	AGC GGG GT*T TT*C CGG	GAT CA*T AG*G ACT	100

effect, our results do not support the compensatory mechanism between Sp1 and USF proposed by Pollwein (1993), but rather point to an additive mechanism where both the Sp1 and USF elements contribute to *APP* promoter activity. However, our data do not rule out the possible competition between Sp1 and USF for the regulation of *APP* promoter. Whether Sp1 and USF are simultaneously present on the same DNA molecule when they participate in *APP* transcription cannot be determined by transient transfection assays or EMSA studies which use an excess of DNA molecules. Moreover, *in vitro* studies so far reported, including ours, do not take into consideration DNA topology, the presence of histones and the nearby RNA polymerase II holoenzyme. An *in vitro* analysis of the complete transcriptionally active complex formed on the *APP* promoter could determine whether Sp1 and/or USF are part of this complex. Taken together, the previously described mutational analyses provide evidence for the involvement of the Sp1 and USF elements in *APP* transcriptional regulation.

In contrast to the studies described above, in experiments performed by Quitschke and colleagues (1996), mutations introduced into the Sp1 and USF elements had no effect on *APP* promoter activity in presence of 488 base pairs of upstream regulatory sequences. In a previous report by the same author (Quitschke, 1994), mutations introduced in the USF element affected the *APP* promoter only in absence of the GC-element (77 bp of upstream regulatory sequences), but not in its presence (488 bp of upstream regulatory sequences), both in transient transfection and *in vitro* transcription assays. These results led to the proposal that the Sp1 and USF elements had no role to play in *APP* transcriptional regulation in presence of the proteins recognizing the GC-element (Quitschke et al., 1996). These results contrast with our own and those reported by Pollwein (1993). We observed a strong effect on *APP* activity when mutating both elements in presence of both 96 and 395 bp of upstream regulatory sequences. The high level of conservation of the *APP* promoter between species and the observation of similar regions protected by DNase I digestion argue against the fact that the Sp1 and USF elements are not essential. Again, the specific mutations introduced in the Sp1 and USF elements could in part explain these discrepancies. However, in their studies of the *APP* promoter, Quitschke and co-workers

used a reporter plasmid carrying two reporter genes under the control of two different promoters located on the same DNA strand. One is under the control of the *APP* promoter and controls the CAT reporter gene whereas a ß-galactosidase gene is controlled by the chicken ß-actin gene to standardize for transfection efficiency. Previously, presence of the SV40 promoter/enhancer sequences downstream of two separate promoters had been shown to activate transcription in a non-specific manner (Schatt et al., 1990). When only the TATA box of these promoters was present, the SV40 promoter/enhancer had no effect suggesting that the binding sites for proteins located upstream of the TATA box are essential for this sort of activation. Similarly, presence of the ß-actin promoter in *cis* could affect the *APP* promoter in an unidentified manner and mask the effects of the mutations. In absence of the GC-element, however, mutations in the USF element affected *APP* activity suggesting that this *cis*-activation needed the presence of the protein(s) recognizing the GC-element (Quitschke, 1994).

Our results have revealed that in the presence of the GC-element, the Sp1 and USF elements regulate the *APP* promoter in additive manner (Figs. 6.8, 6.10 and Table 1). However, in the absence of the GC-element, there seems to be competition between the proteins recognizing the Sp1 and USF elements since mutating the Sp1 element increased the activity of the *APP* promoter (Figs. 6.9, 6.10 and Table 1), although the converse was not true in that mutating the USF element actually reduced the activity of the promoter to levels similar to the double mutant (Fig. 6.9 and Table 1, compare pAPPCAT-77mUSF and pAPPCAT-77mSU). These results suggest that Sp1 needs the presence of the protein(s) binding the GC-element to properly activate the *APP* promoter whereas USF does not. Possible roles for Sp1 and USF in *APP* regulation could be in the recruitment of TFIID to the *APP* promoter since both Sp1 and USF have both been reported to physically contact hTAF 55, a subunit of TFIID. Recruitment of TFIID to the promoter regions of genes has been shown to be a limiting event in transcriptional initiation. TFIID recognizes the TATA box presents in promoters through the its TBP subunit. Although the *APP* promoter does not possess a consensus TATA box, TFIID has been shown to be present in TATA-less promoters in a region located approximately 30 bp upstream of the

transcriptional start site and its recruitment seems to involve proteins located upstream of the -30 region which could implicate Sp1 and USF (Wiley et al., 1992).

Our mutational analysis shows that Sp1 and USF synergize with the proteins recognizing the GC-element. This conclusion comes from the observation that deletion of the GC-element reduces the activity of the *APP* promoter in NG108-15 and HepG2 cells to 7.4 % and 6.1% respectively. However, in presence of an intact GC-element, inactivation of both the Sp1 and USF elements reduced the activity of the promoter to 22.3 and 14.3 % in NG108-15 and HepG2 cells (Table 1, pAPPCAT-96mSU) instead of the predicted 92-94%. This suggests that the proteins binding to the Sp1 and USF elements are essential for providing full activity to the *APP* promoter.

Regulation of APP transcription by cAMP.

The results presented in Chapter 7 show that agents which elevate intracellular levels of cAMP increase the steady state levels of *APP* transcripts through transcriptional activation of the *APP* gene. Thus, expression of the *APP* is responsive to signaling through the protein kinase A pathway in addition to protein kinase C (Trejo et al., 1994). Hormones and neurotransmitters acting through G-protein coupled seven-transmembrane domain receptors are well-documented regulators of cAMP levels through either activation or inhibition of adenylate cyclase (reviewed by Krupinski, 1991). Although this is an important way of altering the relative abundance of cAMP in a given cell type, cAMP levels can also be increased, for example, by activation of NMDA receptors and the subsequent influx of Ca^{++} into neurons (Chetkovich et al., 1991). Therefore many different types of stimuli, by affecting cAMP levels, could ultimately lead to changes in *APP* gene expression.

Much information has accumulated about the mechanism underlying transcriptional stimulation by cAMP of genes that contain CREs in their promoter. Cyclic AMP binds to the regulatory subunit of protein kinase A, induces dissociation of the catalytic subunit that is then

translocated to the cell nucleus (reviewed by McKnight, 1991). The catalytic subunit phosphorylates the CRE-binding transcription factor CREB at serine residue 133 (Gonzalez et al., 1989). A co-activator called CBP (CREB binding protein) can specifically interact with phosphorylated CREB to mediate transcriptional activation (Chrivia et al., 1993; Kwok et al., 1994; Arias et al., 1994) by acting as an adaptor between CREB and the basic transcriptional apparatus. The CRE consists of the core octamer sequence TGACGTCA, a sequence which is not present within the 3.7 kb region upstream of the transcription initiation site of the human *APP* gene (Salbaum et al., 1988). Thus, the activation of pAPPCAT-303 by dbcAMP (Fig. 7.1) does not occur via CREB binding to a CRE site.

Alternatively, cAMP can act through the AP-1 transcription factor which can recognize CREs or AP-1 sites (MacGregor et al., 1990; Sassone-Corsi et al., 1990). In this context, it is significant that induction of AP-1 activity by cAMP is a cell-type specific response (Bravo et al., 1987; deGroot et al., 1992). AP-1 sites are recognized by proteins of the *fos* and *jun* gene families which can function either as homodimers (in the case of Jun) or heterodimers (in the case of Fos and Jun). The induction of AP-1 activity by cAMP can occur through several mechanisms: by increasing c-*fos* transcription (Schilling et al., 1991), and/or by protein kinase A activation of c-Jun (deGroot et al., 1992). Depending on the cell type, these modifications will lead to greater AP-1 binding and enhanced transactivation.

The promoter of the human *APP* gene contains a functional AP-1 site, centered at position -346, which is responsive to PMA treatment of astroglial or HeLa cell lines (Trejo et al., 1994). Furthermore, constructs of *APP* promoter containing this site (-488 to +100) can be activated by expression of exogenous c-*jun* (Trejo et al., 1994). However, transfection experiments in which NG108-15 cells were treated with dbcAMP revealed that the presence of a functional AP-1 site in the APP promoter does not result in a difference in the level of CAT activity between constructs pAPPCAT-364 and pAPPCAT-303 (Fig. 7.1), i.e. dbcAMP treatment of pAPPCAT-364 did not lead to potentiation of the effect observed with pAPPCAT-303 (which is devoid of the AP-1 site). Thus, our results suggest that the AP-1 site at -364 is not involved in the upregulation of the APP promoter by cAMP in NG108-15 cells.

Another transcription factor which is inducible by cAMP is AP-2 (Imagawa et al., 1987), a 52 kDa protein isolated from HeLa cells first identified through its interactions with the enhancer region of SV40 and human metallothionein IIA (Mitchell et al., 1987). In addition to cAMP, its activity has been shown to be regulated by protein kinase C (Imagawa et al., 1987) and retinoic acid (Lüscher et al., 1989). During development, AP-2 is expressed in the neural crest cell lineages (Mitchell et al., 1991) and mice lacking the *AP-2* gene die before birth of severe defects in neural tube formation, craniofacial structures, and skeletal abnormalities (Schorle et al., 1996; Zhang et al., 1996). The AP-2 transcription factor recognizes the consensus sequence GCCNNNGCC (Williams et al., 1991a; 1991b) as a homodimer through which it regulates both basal (Greco et al., 1995; Medcalf et al., 1990) and inducible gene expression (Park et al., 1993; Descheemaeker et al., 1992).

We have shown by electrophoretic mobility shift assay and co-expression studies that the putative AP-2 site at -205 is not functional. Hence this element can be classified in the same category as the putative AP-1/AP-4 element at -49 of the human APP promoter. This sequence is not bound by authentic AP-1 factor since double-stranded oligonucleotides containing AP-1 consensus sequence cannot compete binding of nuclear proteins (Bourbonnière et al., 1996). However, unlike the AP-2 sequence at -205, the putative AP-1/AP-4 site is highly conserved between rodents and human (Salbaum et al., 1988; Izumi et al., 1992; Chernak, 1993), and it is occupied by nuclear proteins (Quitschke et al., 1992), specifically the transcription factor USF which recognizes a core E-box within this sequence. Furthermore, expression of exogenous USF can transactivate the basal promoter of the *APP* gene. In contrast to USF, transactivation of the human *APP* promoter by exogenous AP-2 occurs not through the binding of an authentic AP-2 site, but through an indirect effect. In this regard, it is of interest that the promoter of c-*fos* contains AP-2 binding sites (Lavrovsky et al., 1994), and the activation of pAPPCAT-364 by exogenous AP-2 may be mediated by the AP-1 site at -346.

For many genes, the effect of cAMP can be due to an interplay between several *trans*-acting factors binding to different *cis*-elements to govern the overall increase in gene expression

(Medcalf et al., 1990; Descheemaeker et al., 1992; Berkowitz et al., 1989). It remains to be seen whether this mechanism might also apply to the upregulation of the *APP* gene by cAMP.

Conclusion

Characterization of the *APP* proximal promoter has identified three elements necessary for its activity. Two of these elements can be recognized by ubiquitously expressed transcription factors whereas the third one still eludes us. The differences in tissue expression of the *APP* gene could be caused by the varying proportions of the factors as well as the combination of their family members. So far there is no indication that the proximal promoter of the *APP* gene and the proteins that recognize it could play a role in increasing *APP* expression. Although neither Sp1 nor USF seem to be under specific regulation, the activity of the GC-binding protein may be under the control of different regulatory mechanisms that might ultimately result in upregulation of *APP*. However, other sequences outside of the proximal region of the *APP* promoter could be involved not only in tissue-specific expression but also cause an alteration of *APP* expression under certain conditions, as mentioned in the introduction.

The advent of high throughput screening methods of an almost limitless number of bio-molecules could allow the identification of compounds able to recognize specific DNA sequences. In conditions where overexpression of the *APP* gene could lead to symptoms related to AD, for example DS, interfering with the basal activity of the *APP* promoter, alone or in combination with other therapies, could slow down the development of the disease, even if the source of the signal leading to altered expression is unknown.

Chapter 9

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