The EMBO Journal vol.7 no.9 pp.2807 – 2813, 1988

The promoter of Alzheimer's disease amyloid A4 precursor gene

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Communicated by K.Beyreuther

The promoter of the gene for the human precursor of Alzheimer's disease A4 amyloid protein (*PAD* gene) resembles promoters of housekeeping genes. It lacks a typical TATA box and shows a high GC content of 72% in a DNA region that confers promoter activity to a reporter gene in an *in vivo* assay. Transcription initiates at multiple sites. Sequences homologous to the consensus binding sites of transcription factor AP-1 and the heat shock control element binding protein were found upstream of the RNA start sites. Six copies of a 9-bp-long GC-rich element are located between positions −200 and −100. A protein–DNA interaction could be mapped to this element. The 3.8 kb of the 5′ region of the *PAD* gene include two Alu-type repetitive sequences. These findings suggest that four mechanisms may participate in the regulation of the *PAD* gene and could be of relevance for the progression of amyloid deposition in Alzheimer's disease.

Key words: housekeeping gene/heat shock element/GC content/mRNA start/transcription factors

Introduction

The pathological hallmark of Alzheimer's disease (AD) is the deposition of fibrillar aggregates of the 42/43-residue amyloid A4 protein (also termed β-protein) (Glenner and Wong, 1984a; Masters *et al.*, 1985a,b). Protein sequencing of amyloid isolated from brains of patients with AD and aged invididuals with Down's syndrome (DS) revealed the presence of the A4 protein in both conditions (Glenner and Wong, 1984b; Masters *et al.*, 1985a,b; Beyreuther *et al.*, 1986).

Recently, molecular cloning based on the sequence of the A4 protein indicated that it is encoded as part of a larger precursor (PreA4) that maps to chromosome 21 (Kang *et al.*, 1987; Goldgaber *et al.*, 1987; Tanzi *et al.*, 1987). Two mRNA-bands (Kang *et al.*, 1987) have now been accounted for by the demonstration of three alternative splicing products of the amyloid gene (Ponte *et al.*, 1988; Tanzi *et al.*, 1988; Kitaguchi *et al.*, 1988). The smallest of these products, the 695-residue precursor protein ($PreA4_{695}$), has been synthesized *in vitro* and shown to be a *N*-glycan membrane protein that spans the lipid bilayer once (Dyrks *et al.*, 1988). At least two other forms of PreA4 exist ($PreA4_{751}$ and $PreA4_{770}$), both containing a 56-residue insert which has a protease-inhibitory function (Kitaguchi *et al.*, 1988). The amyloidogenic A4 protein is derived in part from the transmembrane domain and from part of the adjacent extracellular *N*-glycan domain (Kang *et al.*, 1987). A precursor–product relationship has been demonstrated (Dyrks *et al.*, 1988). This suggests that membrane damage and proteolytic cleavage could be important events which precede the release of the A4 protein.

The A4 gene is expressed in brain and peripheral tissues, such as muscle and epithelial cells (Bahmanyar *et al.*, 1987; Goedert, 1987; Shivers *et al.*, 1988; Tanzi *et al.*, 1988; Zimmermann *et al.*, 1988), yet for reasons still unknown the amyloid deposits in AD are confined to the brain.

Recently, *in situ* hybridization analyses were published that indicate an alteration of the amount of PreA4 mRNA in brains of AD patients when compared to normal individuals (Cohen *et al.*, 1988; Higgins *et al.*, 1988; Lewis *et al.*, 1988). These results implicate a role for gene regulation in AD. To address questions on the transcriptional control of the amyloid A4 precursor gene, which range from the identification of cellular transcription factors to the biochemical mechanisms of the regulatory processes that are relevant for AD pathogenesis, it is first necessary to isolate the promoter of the amyloid A4 precursor gene.

In this paper we describe the cloning and characterization of the promoter of the amyloid A4 protein precursor gene, which hereafter we refer to as *PAD* (for Precursor of Alzheimer's Disease A4 amyloid protein gene) gene, also termed AD-AP gene (Goldgaber *et al.*, 1987), amyloid β-protein gene (Tanzi *et al.*, 1987), AAP gene (Robakis *et al.*, 1987), A4 amyloid gene (Van Broeckhoven *et al.*, 1987), APP gene (Tanzi *et al.*, 1988) or CVAP gene (Bendotti *et al.*, 1988) on the basis of cDNA cloning. This report, however, deals with the genomic locus. The *PAD* promoter strongly resembles the promoters of 'housekeeping' genes. The *in vivo* activity of the promoter and putative transcription factor binding sites are also demonstrated.

Results

Cloning of the PAD gene promoter

We isolated genomic clones from the 5′ end of the *PAD* gene. A *Bam*HI–*Acc*I fragment from position −47 to +227 of A4 amyloid precursor cDNA (Kang *et al.*, 1987), which encodes the shortest 695-residue product ($PreA4_{695}$) was used to screen a library of flow-sorted human chromosome 21. Clone H1.30 was found to contain a 2.8-kb *Hin*dIII fragment which was subcloned into pUC19. The fragment contained a single *Bam*HI site. The 593-bp *Hin*dIII–*Bam*HI fragment of clone H1.30 was cloned into M13 vectors and the DNA sequence of both strands was determined. We

Fig. 1. DNA sequence of the 5′ end region of the *PAD* gene. Base numbers are relative to the strongest signal of the 5′ end mapping experiments (see text and Figure 3). The unique *Hin*dIII site is indicated. *Msp*I sites are given between the *Hin*dIII site and the *Bam*HI site at the 3′ end of the sequence. Six copies of a 9-bp-long GC-rich element are underlined. Symbols: #, mapped 5′ termini of PreA4 mRNA; ~ ~, homology to AP-1 binding site consensus; **, homology to the heat shock consensus sequence.

found this DNA fragment to contain 99 bp upstream of the *Bam*HI site which were identical to the reported 5′ untranslated cDNA sequence of $PreA4_{695}$, except for one additional G in the genomic DNA at cDNA position −96 to −99. The genomic DNA showed a stretch of five consecutive Gs versus four in the cDNA. The 488 bp upstream of the cDNA sequence were expected to be the promoter region of the *PAD* gene. In order to obtain a clone which contains more upstream DNA, we used the 593-bp *Hin*dIII−*Bam*HI fragment as a probe to screen a human genomic library. Clone PN.1 was isolated and found to have 3.8-kb *Bam*HI fragment which hybridized to the probe.

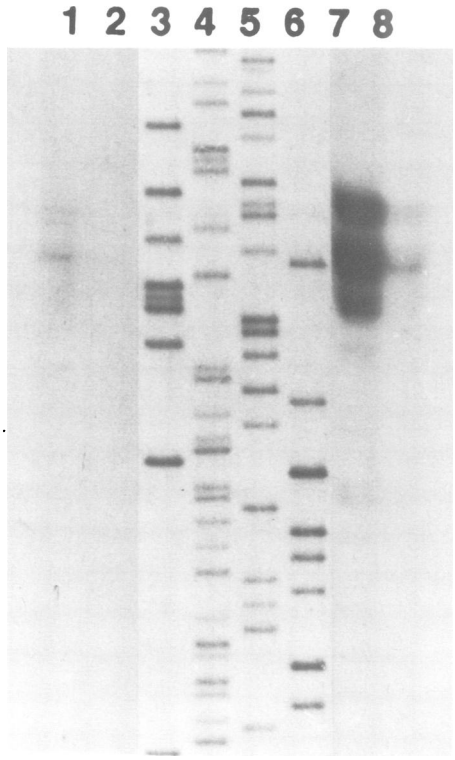
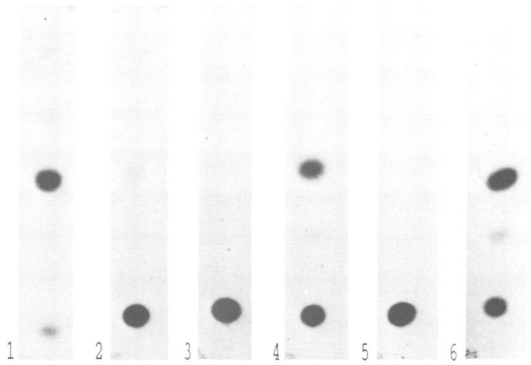


Fig. 2. Mapping of the 5′ termini of PreA4 mRNA. Primer extension analysis of 20 μg (**lane 1**) and 2 μg (**lane 2**) of total fetal brain RNA. **Lanes 3–6**, reactions A, C, G and T of DNA sequence as size marker. S1 nuclease protection analysis of 10 μg total fetal brain (**lane 7**) and 20 μg total RNA from the SY5Y neuroblastoma cell line (**lane 8**). Primer extension, DNA sequencing and S1 nuclease protection analysis were performed with the oligonucleotide 5′-GCCGCGTCCTTGCTCTGC-3′ as primer. The primer is complementary to positions +82 to +99 of the sequence.



LANE	SAMPLE	CONVERSION
1	pSV2CAT	>100%
2	pBLCAT3	5%
3	A4 5'HB rev./pBLCAT3	2%
4	A4 5'HB/pBLCAT3	42%
5	hMTII/pBLCAT3	1.5%
6	hMTII/pBLCAT3, induced	70%

Fig. 3. CAT assays to show promoter activity *in vivo*. Transfected plasmids are: **lane 1**, pSV2CAT containing the SV40 promoter; **lane 2**, pBLCAT3, the vector used for all further constructs; **lane 3**, A4 5′HB rev./pBLCAT3, a construct with the 593-bp *Hin*dIII–*Bam*HI promoter fragment in reverse orientation with respect to the CAT gene; **lane 4**, A4 5′HB/pBLCAT3 with the *PAD* promoter in proper orientation to the CAT gene; **lane 5**, hMTII/pBLCAT3 contains the promoter of the human metallothioneine gene in front of the CAT gene; **lane 6**, hMTII/pBLCAT3 with induction of the hMTII promoter with 2 mM Zn^{2+}. Labelled acetylated chloramphenicol is separated by TLC from [^{14}C]acetylCoA, which stays at the origin. Conversion of radioactivity is indicated for each construct.

This fragment was subcloned and its DNA sequence was determined (Figure 1). The DNA contains two copies of an Alu-type repetitive sequence in the same orientation at positions −2436 to −2179 and −2020 to −1764.

Multiple transcription start sites

To determine the 5′ end of the PreA4$_{695}$ mRNA we performed an S1 nuclease protection experiment (Figure 2, lanes 7,8). A uniformly ^{32}P-labelled single-stranded DNA probe was prepared by annealing the oligonucleotide primer 5′-GCCGCGTCCTTGCTCTGC-3′ to mha6 template DNA, a M13mp19 clone of the 593-bp *Hin*dIII–*Bam*HI 5′ end fragment. After extension with Klenow polymerase and digestion with *Hin*dIII a 582-bp-long probe was generated. Hybridization of excess probe to 10 μg of total fetal brain RNA and subsequent digestion with S1 nuclease results in a heterogeneous population of protected fragments. Sizes of 104, 103, 99, 96 and 95 bp were observed. The same pattern was obtained with RNA from the SY5Y neuroblastoma cell line (Biedler *et al.*, 1973). A DNA sequence ladder was primed with the same oligonucleotide and served as size marker. Thus we were able to correlate the protected fragments to bases in the DNA sequence. The signal at 99 bp, which is the strongest we observed, exactly corresponds to the first nucleotide of the PreA4$_{695}$ cDNA. To confirm the results of the S1 nuclease protection, a primer extension analysis was carried out. Whereas S1 identifies not only 5′ ends of the mRNA but also mismatches between the mRNA and the probe, primer extension with AMV reverse transcriptase maps 5′ termini and stop sites for reverse transcriptase (RT). A ^{32}P-labelled 29-bp primer with the same 5′ end as the S1 probe was annealed to 20 μg of total fetal brain RNA and extended with RT. Bands of 103, 102, 99 and 95 bp were observed (Figure 2, lanes 1,2). The differences between S1 and RT might be due to preferential termination by RT or to unequal sensitivity of certain bases to S1 digestion. However, the similarity of the band patterns generated by the two independent 5′ end mapping techniques indicates that the true 5′ termini of the PreA4$_{695}$ mRNA were mapped. From these experiments we conclude that more than one transcription initiation site is used at the *PAD* gene promoter.

PAD promoter elements

The DNA sequence upstream of the multiple RNA 5′ termini (Figure 1) does not contain a typical TATA box and has a high GC content. Between positions −1 and −400 the DNA is comprised of 72% GC. The ratio of the dinucleotide CpG, the target site for DNA methylation, versus GpC is ~1:1 around the RNA start site. At position −45 as well as −350, relative to the strongest 5′ end signal, we find heptamer sequences which are in good agreement to the binding consensus sequence of the transcription factor AP-1 (TGACTCA; Lee *et al.*, 1987). The sequence starting

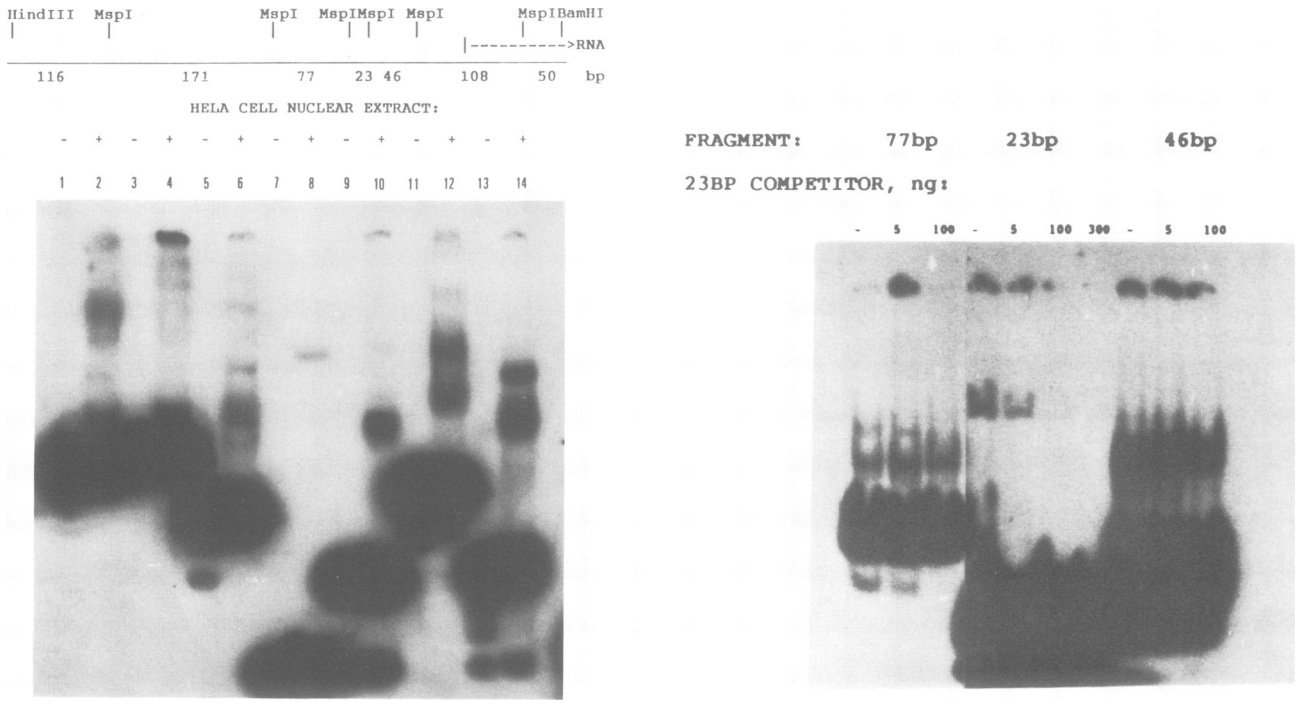


Fig. 4. Gel-retardation assays. (**A**) *Msp*I fragments of the *PAD* promoter were used to probe for sequence-specific protein binding. **Lanes 1** and **2**, the *Hin*dIII−*Msp*I fragment of 116 bp; **lanes 3** and **4**, the 171-bp *Msp*I fragment; **lanes 5** and **6**, the 77-bp *Msp*I fragment; **lanes 7** and **8**, the 23-bp *Msp*I fragment; **lanes 9** and **10**, the 46-bp *Msp*I fragment; **lanes 11** and **12**, the 108-bp *Msp*I fragment; and **lanes 13** and **14**, the 50-bp *Msp*I−*Bam*HI fragment. Absence (−) or presence (+) of a nuclear extract prepared from HeLa cells is indicated. Presence of the nuclear extract results in bands of lower electrophoretic mobility (+ lanes compared to the corresponding − lanes). (**B**) The *Msp*I fragments of 77, 23 and 46 bp were incubated with the HeLa cell nuclear extract and increasing amounts of synthetic DNA with the sequence of the 23-bp *Msp*I fragment as sequence-specific competitor. The amount of competitor DNA is indicated above each lane. Shift bands of the three probe fragments show a different sensitivity to the presence of the competitor DNA.

at position −317 corresponds very well to the heat shock control element (HSE), whose consensus sequence is CT-GAA--TTC-AG (Wu *et al.*, 1987). Furthermore, six copies of a GC-rich element following a consensus of GGGCGC$^{G}_{A}$GG can be located between positions −198 and −105. The features of multiple RNA start sites, the absence of a typical TATA box, the high GC content of the DNA upstream of the RNA starts and the presence of a GC-rich box places the promoter of the *PAD* gene in the class of promoters of 'housekeeping' genes, like the adenosine deaminase gene (Valerio *et al.*, 1985) or the gene for dihydrofolate reductase (Crouse *et al.*, 1982). The promoter of the hamster PrP gene (Basler *et al.*, 1986), the product of which gives rise to brain amyloid deposits in scrapie-infected animals, is organized in a similar fashion: it lacks a TATA box, shows multiple 5′ termini of the mRNA, contains an AP-1 binding consensus sequence (positions 232−238) and is GC rich. In addition, there are three potential binding sites for the transcription factor SP-1 (Dynan and Tjian, 1983).

Promoter activity in vivo

The 593-bp *Hin*dIII−*Bam*HI fragment was tested for its ability to show promoter activity *in vivo*. It was cloned into pBLCAT3 (Luckow and Schütz, 1987) in correct as well as in reverse orientation to the chloramphenicol acetyltransferase (CAT) gene. These constructs were transfected into HeLa cells and CAT activity was measured (Figure 3). The SV40 promoter of pSV2CAT served as high control (lane 1), resulting in a complete conversion of chloramphenicol into its acetylated derivatives. Transfection with the *PAD* promoter in reverse orientation yielded 2% conversion (lane 3), comparable to the CAT vector without an inserted promoter (5%, lane 2) or the non-induced human metallothionine II (hmtII) promoter (1.5%, lane 5). When induced with 2 mM Zn^{2+}, the hmtII promoter produced 70% conversion in our assay conditions. The *PAD* promoter fragment in correct orientation gave 42% conversion (Figure 3, lane 4). This result clearly shows that the 593-bp genomic fragment can function as a promoter in an *in vivo* assay. Together with the results of the 5′ end mapping of the PreA4$_{695}$ mRNA we conclude that we have indeed isolated the promoter of the *PAD* gene from our genomic clones.

Identification of a transcription factor binding site

For a further characterization of the *PAD* gene promoter we performed gel-retardation assays (Figure 4). As we could show that the *PAD* promoter is active when introduced into HeLa cells, we concluded that possible transcription factors interacting with this promoter would be present in nuclear extracts prepared from HeLa cells. The 593-bp *Hin*dIII−*Bam*HI fragment was digested with *Msp*I. Seven DNA fragments with sizes of 116, 171, 77, 23, 46, 108 and 50 bp (fragments ordered from *Hin*dIII to *Bam*HI) were obtained and used to probe for sequence-specific formation of protein−DNA complexes. Probe DNAs were incubated with nuclear extract from HeLa cells in the presence of the non-specific competitor poly[d(I-C)] to allow complex formation to occur. Protein−DNA complexes were separated from free DNA on polyacrylamide gels and

compared to probe DNAs which were incubated with BSA instead of nuclear extract (Figure 4A). A variety of shift bands was observed. We directed our attention to the 23-bp fragment, which contains two copies of the GC-rich element. When this fragment is incubated with the nuclear extract, a strong shift band occurs (lane 8 versus 7 in Figure 4A). A band of similar mobility but different intensity appears with other probe fragments, notably with the 77-bp fragment, which also carries copies of the GC-rich element. Also the 46-bp as well as the 108-bp fragments, which have no copies of the GC-rich element, show shift bands in this range. We therefore synthesized complementary oligonucleotides covering the 23-bp *Msp*I fragment. These oligonucleotides were annealed to give double-stranded DNA and used as a specific competitor in the binding assay (Figure 4B). Three consecutive *Msp*I fragments with 77, 23 and 46 bp were incubated with nuclear extract in the absence or presence of the synthetic 23-bp competitor DNA. The reactions were analysed on a 5% polyacrylamide gel. The shift band of the 23-bp fragment disappeared with increasing amounts of specific competitor (Figure 4B, lanes 4–7). The 77-bp fragment showed two shift bands. In contrast to the strong lower band, the upper band could be competed with synthetic 23-bp DNA (Figure 4B, lanes 1–3). The 46-bp fragment showed a strong shift band which was not sensitive to competition (Figure 4B, lanes 8–10). From this experiment we conclude that a sequence-specific protein–DNA interaction occurs between positions −124 and −105 of the *PAD* gene promoter. As two copies of the GC-rich element are located in this DNA region we suggest that the GC-rich element plays a role in regulating transcription of the *PAD* gene.

Discussion

We cloned human genomic DNA fragments which hybridize to a 5′ end restriction fragment of the $PreA4_{695}$ cDNA (Kang *et al.*, 1987) in order to isolate the promoter of the *PAD* gene. Since the gene is located on chromosome 21, a library of flow-sorted human chromosome 21 was used initially. The first clone we obtained had 488 bp of 5′ overlapping sequence relative to the first nucleotide of the cDNA clone. We found the genomic clone to contain an additional nucleotide when compared to the published cDNA sequences (Kang *et al.*, 1987; Ponte *et al.*, 1988). However, a genomic probe was protected from nuclease S1 digestion at this site when hybridized to PreA4 mRNA, suggesting no mismatch between the genomic sequence and the mRNA. When tested in an *in vivo* promoter assay, the DNA fragment carrying the junction of genomic and cDNA sequence is clearly able to drive the transcription of a reporter gene. This result provided evidence that we have isolated the promoter and not an intron–exon border.

The promoter of the human *PAD* gene has features similar to the promoters of so-called 'housekeeping' genes (Basler *et al.*, 1986). We mapped the 5′ termini of the PreA4 mRNA by two independent techniques in order to exclude artefacts associated with the particular enzyme reactions. Although the results were not fully identical with each other, at least three different 5′ ends could be mapped by S1 nuclease as well as by primer extension. This result strongly suggests the existence of multiple RNA start sites at the *PAD* gene promoter. The most prominent start site corresponds exactly to the first nucleotide of the $PreA4_{695}$ cDNA clone 9-110 (Kang *et al.*, 1987), which we could show in this work to be a full-length clone. The DNA sequence upstream of the transcription initiation sites is characterized by an unusually high GC content, which reaches 72% within the region from −1 to −400. It also does not contain a TATA box. These features are typical for promoters of housekeeping genes. The observation that promoters lacking a TATA box have multiple mRNA starts has been described for a variety of mRNAs, including those of adenosine deaminase (Valerio *et al.*, 1985), Thy-1 (Giguère *et al.*, 1985) and hamster PrP (Basler *et al.*, 1986). The almost ubiquitous expression of transcripts derived from the *PAD* gene (Tanzi *et al.*, 1987, 1988) is also consistent with the concept of a housekeeping gene. This term, however, does not imply that the gene is expressed constitutively.

We have observed a considerable high frequency of CpG versus GpC dinucleotides in the promoter sequence, being 1:1 instead of 1:5 as seen in normal eukaryotic DNA (Razin and Riggs, 1980). The dinucleotide CpG is the target for DNA methylation, a mechanism known to control gene expression (Doerfler, 1983).

We found two heptamer sequences which resemble the consensus binding site TGACTCA of the transcription factor AP-1, a phorbol-ester-inducible enhancer binding protein (Lee *et al.*, 1987), one at position −45 with a G in position 7 instead of the A and the other at position −350 with a T substituting the central C. With the products of the oncogenes v-*jun* and c-*fos* having the ability to bind to the same consensus sequence (Bohmann *et al.*, 1987; Rauscher *et al.*, 1988), it remains to be established whether these sites in the *PAD* promoter are used and which genetic control circuit acts on the *PAD* gene.

The sequence at position −317 is in good agreement with the consensus sequence CT-GAA--TTC-AG of the heat shock control element (HSE, Wu *et al.*, 1987) with the *PAD* promoter sequence having a C at position 6 instead of the A. The term 'heat shock' includes exposure to stress conditions which are not limited to heat but may include hypoxia and exposure to a variety of toxic organic as well as inorganic chemicals such as ethanol or cadmium chloride (Lindquvist *et al.*, 1986). Another amyloidogenic protein is related to stress conditions. The serum amyloid A protein (SAA) is upregulated in response to a variety of inflammatory or toxic conditions (Benditt and Eriksen, 1971). Whether the SAA gene promoter also contains HSE control elements remains to be shown. The degenerative pathology of the AD brain could easily be seen in the context of neurons undergoing stress reactions. DNA footprint analyses are presently being undertaken to prove whether these elements in the *PAD* promoter are of importance.

A putative protein-binding region was identified in the sequence between positions −200 and −100, where six copies of a 9-bp-long GC-rich element with a consensus of GGGCGC$^{G}_{A}$GG are located. Gel-retardation assays together with sequence-specific as well as non-specific competitor DNAs provide evidence that binding occurs in a region of the DNA with two copies of the GC-rich element. This binding could be competed with double-stranded oligonucleotide DNA having the sequence from position −123 to −101. Other binding reactions were not influenced by the addition of the oligonucleotide competitor DNA.

We conclude from this study of the *PAD* gene promoter

sequence that at least four mechanisms could act to control the regulation of the *PAD* gene: the stress-related HSE; the 'oncogene-related' AP-1/Fos binding site; the potential protein binding at the GC-rich element; and the possible methylation of the CpG region. A disturbance in the regulation of *PAD* gene products over a long period of time could well contribute to the rate of amyloid formation. Control of *PAD* gene expression in the clinical conditions of DS and familial and sporadic AD might have therapeutic potential.

Materials and methods

Cloning and DNA sequencing

Clone H1.30 was isolated from a chromosome 21 library (*Hin*dIII fragments in Charon 21A, courtesy of Dr M.Van Dilla) using the *Bam*HI–*Acc*I fragment (−47 to +227; Kang *et al.*, 1987) of the PreA4$_{695}$ cDNA as probe. The human chromosome-21-specific gene library used in this work was constructed at the Lawrence Livermore National Laboratory, Livermore, CA, under the auspices of the National Laboratory Gene Library Project, which is sponsored by the US Department of Energy. Hybridizations were performed in 5 × SSPE, 50% formamide, 1 × Denhardt's solution, 1 mM EDTA at 42°C with 10^6 c.p.m./ml of randomly primed probe (Feinberg and Vogelstein, 1984). A 593-bp *Hin*dIII–*Bam*HI fragment of clone H1.30 was subcloned into M13 vectors and sequenced on both strands. This fragment also was used to screen a human genomic library (vector L47.1, courtesy of Dr B.Horsthemke, University of Essen) and to isolate clone PN.1. A 3.8-kb *Bam*HI fragment was subcloned into a Bluescript vector (pKS+, Genofit, Heidelberg). A set of ordered deletions along the DNA was constructed with the help of exonuclease III and mung bean nuclease (Genofit). DNA from 12 deletion plasmids was purified on CsCl gradients and used for DNA sequencing. The chain termination method (Sanger *et al.*, 1977) was performed using T7 DNA polymerase (Sequenase, USB) on single-stranded as well as on denatured plasmid DNA templates (Chen and Seeburg, 1985).

Nuclease S1 protection and primer extension analysis

The nuclease S1 protection assay was performed as described (Ruppert *et al.*, 1986). A uniformly labelled single-stranded DNA probe was synthesized by annealing an oligonucleotide primer 5′-GCCGCGTCCTTGCTCTGC-3′ to mha6 template DNA (a M13mp19 clone of the 593-bp *Hin*dIII–*Bam*HI *PAD* gene 5′ end fragment) and extension with Klenow polymerase (Boehringer Mannheim) to the *Hin*dIII site. The probe was hybridized to 10 μg of total human fetal brain RNA, digested with S1 nuclease (Boehringer Mannheim) and the resulting products analysed on a 6% sequencing gel.

For primer extension analysis, the same annealing mix as for the S1 probe was prepared. Extension of the oligonucleotide with Klenow polymerase was performed with dGTP, dTTP and [α-^{32}P]dCTP in the absence of dATP. The resulting 29-bp labelled primer was purified on a 6% denaturing (8 M urea) polyacrylamide gel. 10^5 c.p.m. of primer were annealed to 20 μg of total fetal brain RNA for 30 min at 42°C in 40 mM Tris–HCl, pH 8.3, 80 mM NaCl, 6 mM $MgCl_2$, 2.5 mM DTT. After addition of all four dNTPs at 0.5 mM and 20 U AMV reverse transcriptase (Genofit) the reaction was incubated for 30 min at 42°C. After ethanol precipitation, products were separated on a 6% sequencing gel and subjected to autoradiogrpahy.

In vivo promoter assay

In order to assay *in vivo* promoter activity, CAT assays in HeLa cells transfected with the appropriate constructs were performed as described (Schöler and Gruss, 1985). The 593-bp *Hin*dIII–*Bam*HI fragment was cloned into pBLCAT3 (Luckow and Schütz, 1987). To generate a construct with the *PAD* gene promoter in reverse orientation to the CAT gene, the 593-bp *Hin*dIII–*Bam*HI fragment was treated with Klenow polymerase and cloned via blunt ends into the *Hin*cII site of pUC19. The insert of a properly oriented clone was cut out with *Hin*dIII and *Bam*HI and cloned into pBLCAT3. pSV2CAT and the hMTII promoter (Karin *et al.*, 1984) cloned into pBLCAT3 were used as controls.

Gel-retardation assays

Nuclear extracts from HeLa cells were prepared as described (Dignam *et al.*, 1983; Staudt *et al.*, 1986). The 593-bp *Hin*dIII–*Bam*HI fragment was digested with *Msp*I, the resulting fragments were end-labelled with Klenow polymerase and [α-^{32}P]dCTP and purified on a 10% polyacrylamide gel. Binding reactions (10 μl) contained 5000 c.p.m. of probe fragment, 1 μg poly[d(I-C)] (Boehringer Mannheim), 10 mM Hepes, pH 8.0, 90 mM NaCl, 2 mM DTT, 0.1 mM EDTA and 1 μl nuclear extract (~5–10 μg of protein). Reactions were incubated for 30 min at room temperature. Protein–DNA complexes were separated from free DNA on 3.5% polyacrylamide gels in 0.5 × TBE at 4°C and 10 V/cm. Gels were dried onto Whatman DE81 ion-exchange paper and subjected to autoradiography with two intensifying screens at −70°C.

Acknowledgements

We thank B.Horsthemke for the human genomic library, T.Herget for providing fetal brain RNA and A.Sippel and R.Rupp for discussion during the course of these studies. This work was supported by the Fritz Thyssen Stiftung (to J.M.S.), the Deutsche Forschungsgemeinschaft through SFB 74/A2 and 317, the Bundesministerium für Forschung und Technologie, the Fonds der Chemischen Industrie and the National Health and Medical Research Council of Australia.

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Received on May 5, 1988; revised on June 10, 1988