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STUDY OF THE ALZHEIMER'S A4 PRECURSOR GENE PROMOTER REGION BY GENOMIC SEQUENCING USING TAQ POLYMERASE

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The β-amyloid protein has been identified as the prominent component of the fibrillary aggregates of the neuritic plaques found in Alzheimer's disease (AD). In this paper, the DNA methylation pattern of the promoter region of the Alzheimer's disease amyloid precursor gene (PAD) was assessed using the recently developed genomic sequencing technique with Taq polymerase. We analyzed seven potential methylation sites between position -460 and -275 of the PAD promoter. Three of the CpG dinucleotides we analyzed are located in the flanking regions of the AP-1 binding site and heat-shock response element consensus sequences. Of the seven CpG dinucleotides present in this region, we found none to be methylated. This finding indicates that, in healthy brain tissue, cytosine methylation of this binding motif seems not to affect protein/DNA interaction. However, it remains to be determined whether methylation of these sites is significant in AD patients.

The amyloid A4 protein has been identified as the prominent component of the fibrillar aggregates of the senile neuritic plaques and cerebrovascular amyloidosis that represent the pathological hallmark of Alzheimer's disease (1,2). Recently, the promoter region of the Alzheimer's disease A4 amyloid precursor gene (PAD) was isolated and its sequence determined (3). Sequences homologous to the binding site of the transcriptional factor AP-1 and the heat-shock element binding protein were found in the PAD promoter. In addition, this promoter contains six copies of the 9-bp GGGCGC repetitive elements and several CpG dinucleotides, known to be potential methylation sites, but lacks the canonical TATA box. These features of the PAD promoter strongly resemble those found in the promoters of the housekeeping genes.

The AP-1/jun family of transcripton factors is known to respond to cellular signal transduction pathways and has also been found in the enhancer of many

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cellular and viral genes (4). The heat shock (HSE) motif is a rapidly induced element that responds to a variety of environmental stresses which include heat and toxic organic and inorganic chemicals (5). The existence of methyl groups on cytosines of CpG doublets is known to have profound effects on the expression of eukaryotic genes. To date the methylation status of the cytosine has been studied with methylation-sensitive restriction enzymes such as HpaII, MspI or SalI (6) or genomic sequencing (7). However, restriction enzymes can only detect approximately 10% of the methylation sites, whereas genomic sequencing allows quantitative detection of all methylated cytosine. Recently a high resolution method to study DNA methylation and protein/DNA interaction in vivo was developed by Saluz and Jost (8); this procedure, genomic sequencing with Taq polymerase, is the most simple and rapid technique now available and, therefore, has been used in this study.

Three possible regulatory elements are present in a 200 bp region of the PAD gene promoter: the CpG dinucleotides, the consensus sequence for the AP-1 transcription factor, and the heat-shock binding protein. In this paper, we analyze the methylation status of this region of the A4 amyloid precursor gene.

MATERIAL AND METHODS

Preparation of Genomic DNA. Total genomic DNA from normal brain tissue was prepared according to Saluz and Jost (8). Briefly, tissue frozen in liquid nitrogen was pulverized. 10 ml of a 0.5 M EDTA pH 8, 0.01 M EGTA, 1% sarcosyl, proteinase K 300 µg/ml,was added to the ground tissue and incubated overnight at 37°C. 10 ml of phenol was added to the incubation mixture and DNA extracted. Phenol extraction was repeated 5-6 times for 5 min. After one additional phenol/chloroform extraction, the DNA was dialyzed overnight against 10 L of distilled water. The DNA was then precipitated with 1 volume of isopropanol washed with 70% ethanol and resuspended in 1 ml of 10 mM Tris HCl pH 7.5. Total genomic DNA was then digested with HindIII and treated by the cytosine base modification reaction according to Maxam and Gilbert (9).

Labeling of the Oligonucleotide Primer. A synthetic 7 mer oligonucleotide fragment of the sequencing primer (5'-GACGGCC-3') was annealed to the 3' end of a 33-mer fragment (5'-CACTGTTCACGAAGCCCAGGTGGCCGTCGGCCG-3') and extended with polymerase I Klenow fragment as follows: 1 µl of 33-mer (0.5 µg/µl), 1 µl of the 7-mer (0.14 µg/µl), 2 µl of water and 3 µl of 10x buffer (500 mM sodium cloride; 100 mM Tris HCl, pH 7.5; 100 mM $MgCl_2$; 10 mM DTT). The reaction was heated to 75°C for 2 min followed by cooling at room temperature and then placed on ice. After adding 1 µl of 10 mM each of dTTP, dGTP, dCTP and 20 µl (200 µCi) of ^{32}P alpha-dATP (3000 Ci/mmole), 3 µl (15 units) of E. coli DNA polymerase I (Klenow fragment), the sample was incubated at 23°C for 20 min.. The 27-mer labeled, single-stranded fragment was separated on a 15% sequencing gel. The band containing the 27-mer was removed from the gel and the primer eluted for 4 h at room temperature in 300 µl of CH3COONH4, pH 7.5. 10 µg of E. coli carrier DNA was added to the sample and DNA precipitated by adding 2.5 volumes of ethanol and 0.5 volume of 3 M sodium acetate. The 27-mer primer used in this study is homologous to the upper strand of the PAD promoter from position -199 to -232.

Linear Amplification of DNA. 69 µl (50 µg) of digested and chemically-cleaved genomic DNA, 17 µl of labeled primer, 10 µl of 10x buffer (166 mM [NH4] $2SO_4$, 670 mM Tris, pH 8.8, 67 mM $MgCl_2$, 100 µM 2-mercaptoethanol, bovine serum albumin at 2 mg/ml) and water to 100 ml were incubated 95°C for 5 min in a Perkin-Elmer/Cetus DNA Thermal Cycler. Samples were then chilled in ice water and 3 µl of each dNTP, to a final concentration of 300 µl, and 1-5 U of Taq polymerase were added to the reaction. Samples were then placed in the Thermal Cycler and denatured for 1 additional minute at 95°C. The amplification procedure was performed as follows: 30 cycles of 1 min of denaturation at 95°C, 2 min of annealing at 65°C and 3 min of extension at 72°C. After purification with 10 µl of 1% N-cetyl-N-N-N-cetyl-N-N-N-trimethylammonium bromide carried out as described (8),samples were dissolved in 8 µl of 50 mM NaOH, 0.5mM EDTA, 4 M urea, 0.02% bromophenol blue, and electrophoresed on an 8% PA gel. The gel was dried and autoradiographed 1-24 h at -80°C. Procedures for DNA methylation study are summarized in Figure 1.

RESULTS

We analyzed a region approximately 200 bp from position -460 to -275 in the 5' end of the PAD promoter to determine the methylation pattern of the CpG

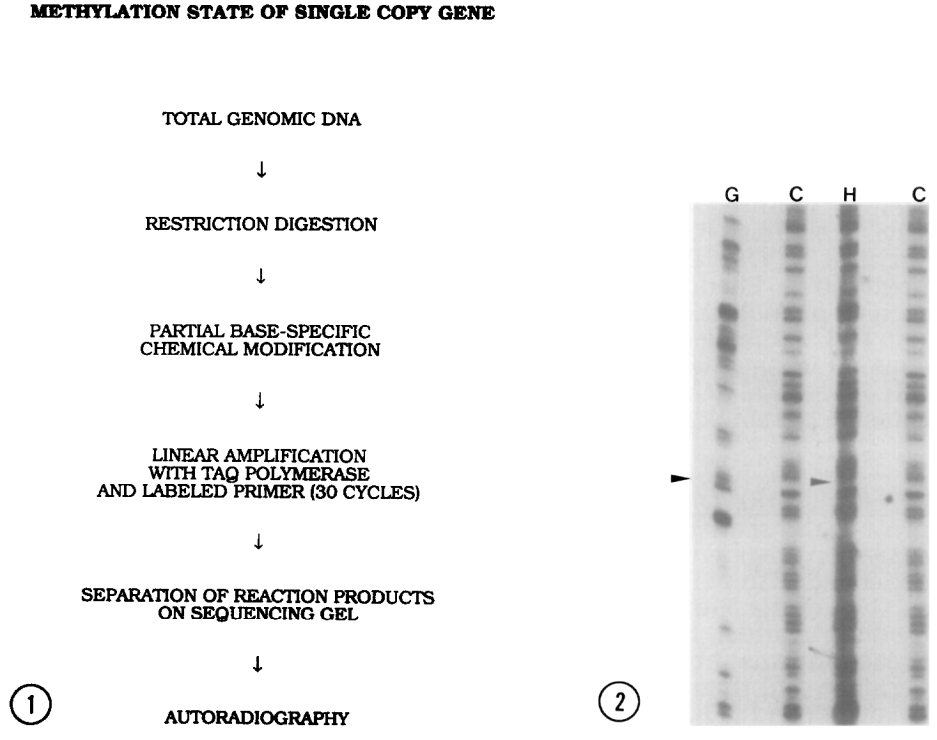

Figure 1. Flow diagram summarizing procedures for DNA methylation study.

Figure 2. Autoradiogram depicting the methylation state of the upper strand of the promoter of Alzheimer's disease amyloid A4 precursor gene. Arrowhead marks position -290 from the transcription initiation site. Cytosine-specific reaction of lineary amplified human genomic DNA (Lane H) and control cytosine and guanine reactions are shown. For the cytosine-specific reaction, 50 µg of genomic DNA, digested with HindIII, was used.

nucleotides present in this sequence. This region, having a GC content of 75%, contains the AP-1 binding site motif (TGACTCA) at position -350 and the heat-shock response element (CT-GAA-TTC-AG) at position -317 and 7 CpG dinucleotides. Three of the CpG doublets analyzed are located in the region flanking the consensus sequence of the AP-1 and heat shock binding site at positions -354, -337 and -319. The remaining four are located at positions -456, -423, -406, -379. Figure 2 illustrates our finding. The modified base 5 methyl-cytosine is distinguished from cytosine by its lack of reaction with hydrazine resulting, in turn, in the absence of a band in the cytosine sequencing lane when compared with the cloned control sequence. As shown in Figure 2, the sequence pattern of the plasmid control and the genomic DNA (Lane H) are identical, suggesting that all the cytosine residues of the PAD gene promoter in the region we analyzed are unmethylated.

DISCUSSION

The etiology of the Alzheimer's disease is unknown. It has been recently suggested that the β-amyloid precursor protein, which is abnormally deposited in the brain of Alzheimer's patients (10), has a normal function in this organ, possibly acting as an intercellular adhesion molecule that makes direct connection between neurons. Recent studies further indicate that an alteration of the mRNA level of the PAD gene occurs in brains of patients with Alzheimer's disease (11,12). These findings imply that alteration in the regulatory mechanism(s) of the PAD gene could contribute to the rate of amyloid formation and, therefore, play a role in the etiology of AD.

In the present study, we utilized the newly developed genomic sequencing procedure with Taq polymerase to analyze the methylation pattern of DNA from normal brain tissue in a region of the PAD promoter between position -460 and -275. This region also includes the AP-1/jun transcription binding site and the heat-shock response element. The Ap-1/jun transcription factor, a phorbol-ester-inducible enhancer binding protein, shares its binding site with the product of oncogenes c-jun and c-fos. Recent experiments also show that DNA binding affinity of the c-jun for its recognition site is greatly increased by association with c-fos and the domain responsible for its protein-protein interaction is the "leucine zipper", a region of the DNA binding domain probably playing an important role in DNA recognition via these proteins (13,14).

The HES motif is an inducible element present in eukaryotic and prokaryotic genes. Several factors can induce a heat-shock promoter response in cells, and the proteins induced by this response seem to have a very general protective function, and may play a role in normal growth and development. Apart from heat, this response has been observed in organisms undergoing hypoxia or exposure to organic and inorganic chemicals such as ethanol or

cadmium cloride (15). Recent studies indicate that the binding activity of these recognition sequences could be influenced by methylation status of the flanking CpG site (16). Of the seven sites we examined in our study, three were located at position -354, four nucleotides away from the 5' end of the AP-1 binding site; -337, six nucleotides away from the 3' end of the same motif; -319, located within the HSE consensus sequence. None of the CpG sites we analyzed were methylated.

The results this study suggest that cytosine methylation does not seeem to play a role in controlling protein/DNA interaction at these binding elements in the brain of healthy individuals. However, further studies are required to determine whether methylation of these sites occurs in AD patients and to ascertain the protein/DNA interaction patterns in the affected binding regions.

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