Nucleic Acids Research, 1995, Vol. 23, No. 12 **2229–2235**

DNA binding and regulatory effects of transcription factors SP1 and USF at the rat amyloid precursor protein gene promoter

Peter W. Hoffman and Jeffrey M. Chernak*

Molecular Physiology and Genetics Section, Laboratory of Cellular and Molecular Biology, Gerontology Research Center, National Institute on Aging, 4940 Eastern Avenue, Baltimore, MD 21224, USA

Received February 7, 1995; Revised and Accepted May 9, 1995

ABSTRACT

Two DNA elements which we have termed SAA and GAG have been shown to control expression of the rat amyloid precursor protein (APP) gene, and the region containing the SAA element has been shown to interact with nuclear proteins [Hoffman and Chernak (1994) *Biochem. Biophys. Res. Commun.* 201, 610–617]. In this report we study DNA sequences and proteins which influence the activity of the SAA element. An oligonucleotide containing the SAA element is specifically bound by nuclear proteins derived from rat PC12 cells, consistently forming four complexes designated C25, C30, C35 and C40 in electrophoretic mobility shift assays (EMSAs). We demonstrate that the C25, C30 and C40 complexes involve the binding of nuclear proteins to an SP1 consensus sequence located within the SAA element and that the C25 complex contains a protein antigenically related to the human SP1 protein. We establish further that the C35 complex requires a USF recognition site located within the SAA element and contains a protein antigenically related to the human upstream stimulatory factor (USF) protein. Using APP promoter/luciferase reporter gene constructs, we demonstrate that both the SP1 and the USF sites can play a role in the transcriptional activity of the SAA element. Finally, we show that complexes similar to the C25, C30 and C35 complexes are formed by rat cortex nuclear extracts and the SAA element in EMSA experiments, suggesting the relevance of our *in vitro* observations to the *in vivo* functioning of the rat APP promoter.

INTRODUCTION

One of the primary features of Alzheimer's Disease (AD) is the deposition of fibrillar amyloid within senile plaques in certain areas of the brain (1). The major constituent of these deposits is the Aβ peptide, which is derived from the larger amyloid precursor protein (APP) by proteolytic cleavage. The level of total APP mRNA has been reported to be increased in AD and Down's Syndrome brains relative to age-matched controls (2). Since the APP gene is located on chromosome 21 in the region of the trisomy in Down's Syndrome, the presence of amyloid plaques in these pateints may be related to gene dosage effects. Furthermore, hereditary forms of AD have been linked to mutations in the APP gene (3). Therefore, regulation of the expression of the APP gene may prove critical to the initiation or clinical progression of AD, and to attempts to control the disease. We have chosen to study the control of expression of the APP by structural analysis of the rat gene promoter and analysis of the transcriptional regulatory factors which interact with it. Previously, we cloned a DNA fragment starting at the first base pair 5′ of the translational start and extending 375 bp 5′ of the gene (4). This fragment was shown to direct high levels of reporter gene expression in rat PC12 cells (5). We also showed that these expression levels are dependent upon the presence of two sequence elements within the promoter, which we have termed GAG and SAA based upon the presence of consensus sequences for several DNA-binding transcription factors (Fig. 1). Deletion of the GAG element caused an ~85% reduction in activity while deletion of the SAA element caused an ~30% reduction in activity. Two DNA fragments and a 36 bp oligonucleotide containing the SAA element were shown to interact with rat brain nuclear proteins (5). To investigate interactions at the SAA element further, we have used a combination of electrophoretic mobility shift assays (EMSAs), supershift experiments and *in vitro* mutagenesis. We present evidence that several different nuclear proteins are capable of interacting with this element and that two of these proteins appear to be the transcription factors SP1 and upstream stimulatory factor (USF), or proteins closely related to these factors.

MATERIALS AND METHODS

Transfections and enzyme activity assays

PC12 cells were grown to 70% confluence on 10 cm tissue culture plates in DMEM media supplemented with 10% horse serum and 5% fetal bovine serum. Transfections were performed using Transfectam (Promega) according to the manufacturer's instructions. The appropriate luciferase gene reporter plasmid construct (2.5 μg) and 2.5 μg β-galactosidase reporter plasmid

* To whom correspondence should be addressed

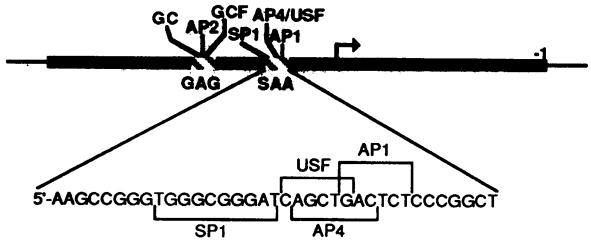

Figure 1. The location of the GAG and SAA elements of the rat APP promoter. The positions of two clusters of potential transcription factor binding sites termed GAG and SAA are shown. The thick line represents the 375 bp sequence of the region immediately 5′ of the rat APP coding region. Striped portions represent the bases included in the GAG or SAA elements. Selected potential regulatory sites are indicated above these sequences (4). The most prominent transcription start site is indicated by the directional arrow (5). The expanded sequence represents the SAA oligonucleotide with putative binding sites delineated.

(pCMV-βgal, Clontech) were used to transfect each plate of cells. Forty-eight hours after transfection the cells were pelleted and resuspended in Reporter Lysis Buffer (Promega). β-Galactosidase and luciferase assays were performed using commercially available reagents according to manufacturer's instructions (Promega and Analytical Luminescence Laboratory, respectively). For each plate, triplicate assays were performed and the values averaged. Activity levels were calculated as luciferase activity above background divided by β-galactosidase activity above background, and normalized to wild-type activity.

Nuclear extracts and EMSAs

Rat cortex nuclear extracts were prepared from freshly-dissected brains obtained from 6 month old male Wistar rats as described by Gorski *et al.* (6). PC12 nuclear extracts were prepared following the method of Dignam *et al.* (7). End-labeling of double-stranded DNA oligonucleotides with ^{32}P was accomplished using standard methods (8). Nuclear extracts (4 μg) were incubated for 5 min at room temperature in 1 × binding buffer (10 mM Tris–HCl pH 7.5, 50 mM NaCl, 1 mM $MgCl_2$, 4% glycerol, 0.5 mM EDTA, 0.5 mM DTT and 50 μg/ml polydI–dC). Where indicated, competitor DNA was included during the preincubation period. ^{32}P-labeled DNA oligonucleotide was then added, and the mixture was incubated for 20 min at room temperature. For supershift experiments, antibody was added following 5 min of the incubation with ^{32}P-labeled DNA, and the incubation was then continued for an additional 20 min. Final reaction volumes were 10 μl. Following incubation, 1/10th volume loading dye (250 mM Tris–HCl pH 7.5, 40% glycerol, 0.2% bromophenol blue, 0.2% xylene cyanol) was added and the samples were electrophoresed on 4% non-denaturing polyacrylamide gels. Gels were analyzed by autoradiography. DNA oligonucleotides used as probes or competitors were as follows, with bases varying from the APP promoter wild-type sequence emboldened and underlined: SAA = 5′-AAGCCGGGTGGGCGGGATCAGCTGACTCTCCCGGCT; GAG = 5′-CGCTCGGGTGCAGCTCCCCGGGGCTCCGCT; OCT1 consensus = 5′-TGTCGAATGCAAATCACTAGAA; NFκB consensus = 5′-AGTTGAGGGGACTTTCCCAGGC; SP1 consensus = 5′-ATTCGATCGGGGCGGGGCGAGC; AP1 consensus = 5′-CGCTTGATGAGTCAGCCGGAA; USF consensus = 5′-CACCCGGTCACGTGGCCTACACC; AP4 consensus = 5′-GAAAGAACCAGCTGTGGAATGT; MSP1 = 5′-AAGCCGGGT**ATAT**GGGATCAGCTGACTCTCCCGGCT; MUSF1 = 5′-AAGCCGGGTGGGCGGGAT**GTTAGA**ACTCTCCCGGCT. OCT1, NFκB, SP1 and AP1 consensus oligonucleotides were obtained from Promega. SP1-specific, AP1-specific and USF-specific antibodies were obtained from Santa Cruz Biotechnology.

Promoter constructs and DNA mutagenesis

The wild-type APP promoter/luciferase reporter gene construct has previously been described (5). Site-directed DNA mutations in the promoter were produced by the technique of Kunkel (9). The oligonucleotides used to create the MSP1, MUSF1, MAP1, MUSFu1, and MUSFu2 mutants were 5′-GCAAGCCGGGT**ATAT**GGGATCAGCTG, MUSF1 (described above), 5′-GAT-CAGCTGA**AGA**TCCCGGCTGC, 5′-CGGGATCA**CG**TGACTCTCCCGGCTGC and 5′-CGGG**G**TCA**CA**TGACTCTCCCGGCTGC, respectively, with all mutagenic changes emboldened and underlined.

RESULTS

The SAA oligonucleotide forms specific complexes with rat PC12 nuclear proteins

To assay for protein binding activity at the SAA element, a 36 bp double-stranded oligonucleotide covering bases –223 to –188 was produced and termed the SAA oligonucleotide (5; Fig. 1). When the ^{32}P-labeled SAA oligonucleotide was incubated without added protein extract, no bands of retarded mobility were observed (Fig. 2, lane 1). When labeled SAA oligonucleotide was incubated with PC12 nuclear extract, four complexes were apparent in EMSAs (Fig. 2, lane 2). These complexes have been termed C25, C30, C35 and C40 based on their relative mobility in our gel system. All bands were specifically competed by increasing amounts of unlabeled SAA oligonucleotide (Fig 2, lanes 3–5), but not by comparable molar amounts of non-specific competing oligonucleotides (lanes 6–14).

SP1 and USF recognition sequences and proteins are involved in complex formation

The SAA element contains multiple and overlapping potential binding sites for nuclear transcription factors (4; Fig. 1), including SP1 (10), AP4 (11), USF (12) and AP1 (13). To test if these sites and the corresponding factors are involved in formation of the observed complexes, we performed EMSAs with unlabeled SP1, AP4, USF and AP1 consensus oligonucleotides as competitors, and supershift assays with antibodies specific for some of these proteins.

Figure 3 shows the results of competition and supershift studies employing SP1 consensus oligonucleotides and SP1-specific antibodies. Complexes C25, C30 and C40 are specifically competed by unlabeled SP1 competitor DNA, while complex C35 is not (lanes 3–5). With increasing amounts of SP1-specific antibody, a higher molecular weight species appears, indicating that the SP1 antibody can interact with some of the original DNA–protein complexes to form a higher molecular weight complex (lanes 6–8). The appearance of this new species correlates with a decrease in the amount of C25 complex observed, while the other complexes do not appear to be significantly affected. The reaction of the SP1 antibody is specific

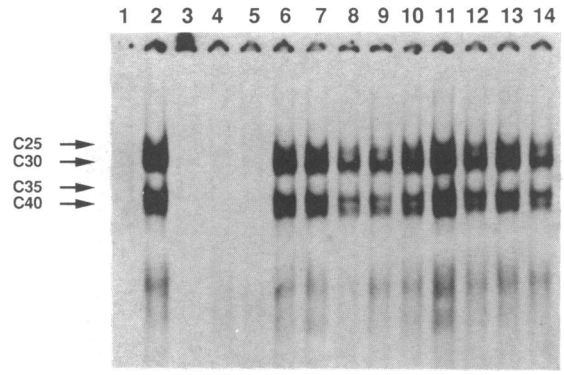

Figure 2. The SAA oligonucleotide forms specific complexes with rat PC12 nuclear proteins. EMSAs were performed using PC12 nuclear extracts. ^{32}P-labeled SAA oligonucleotide was present in all lanes. Details of the assay are described in the Materials and Methods. Lane 1, no extract; Lanes 2–5, extract plus 0× (lane 2), 10× (lane 3), 50× (lane 4), 150× (lane 5) molar excess of unlabeled SAA competitor DNA; lanes 6–8, extract plus 10× (lane 6), 50× (lane 7), 150× (lane 8) molar excess of unlabeled GAG competitor DNA; lanes 9–11, extract plus 10× (lane 9), 50× (lane 10), 150× (lane 11) molar excess of unlabeled Oct 1 competitor DNA; lanes 12–14, extract plus 10× (lane 12), 50× (lane 13), 150× (lane 14) molar excess of unlabeled NFκB competitor DNA.

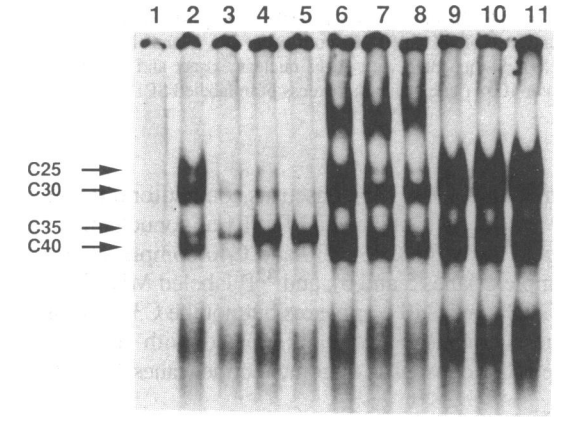

Figure 3. SP1 consensus sequences and SP1-related proteins are involved in formation of the C25, C30 and C40 complexes. EMSAs were performed as described in Figure 2. Lane 1, no extract; lane 2, extract alone; lanes 3–5, extract plus 50× (lane 3), 100× (lane 4) or 250× (lane 5) molar excess of unlabeled SP1 competitor DNA; lanes 6–8, extract plus 0.01 μg (lane 6), 0.1 μg (lane 7), or 1 μg (lane 8) SP1 antibody; lanes 9–11, extract plus 1.2 μg (lane 9), 12 μg (lane 10) or 120 μg (lane 11) of rabbit preimmune serum total protein.

since increasing amounts of rabbit preimmune serum (containing a variety of antibodies to proteins other than SP1) had no effect on the binding (lanes 9–11).

The results of competition and supershift studies employing AP1 consensus oligonucleotides and AP1-specific antibodies are shown in Figure 4. The addition of ≤250× molar excess of unlabeled AP1 oligonucleotide (lanes 3–5) or ≤1 μg of AP1-specific antibody (lanes 6–8) had no effect in EMSA experiments. In lanes 10–12 unlabeled SP1 competitor DNA was added in addition to AP1 competitor DNA in order to eliminate SP1-related complexes and thereby make changes to other complexes more easily visible. In addition, this experiment could

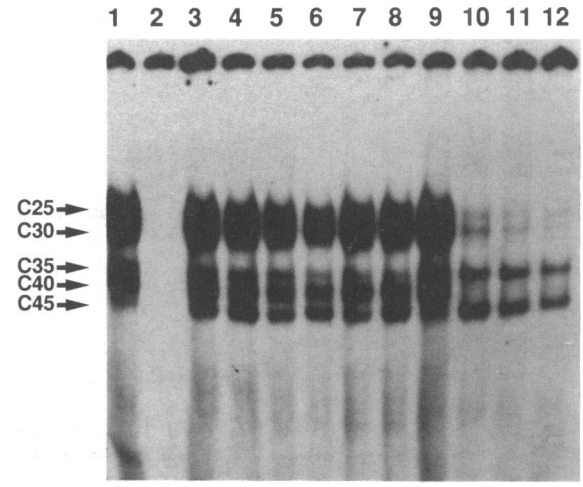

Figure 4. Transcription factor AP1 does not contribute to complex formation. EMSAs were performed as described in Figure 2. Lane 1, extract alone; lane 2, extract plus 250× molar excess of unlabeled SAA oligonucleotide; lanes 3–5, extract plus 50× (lane 3), 100× (lane 4) or 250× (lane 5) molar excess of unlabeled AP1 competitor DNA; lanes 6–8, extract plus 0.01 μg (lane 6), 0.1 μg (lane 7) or 1 μg (lane 8) AP1 antibody; lane 9, extract alone; lanes 10–12, extract plus 50× (lane 10), 100× (lane 11) or 150× (lane 12) molar excess of both unlabeled AP1 and unlabeled SP1 competitor DNAs.

provide information on possible interactions between the two sites. Addition of the SP1 oligonucleotide did not affect the reaction other than to eliminate the SP1-related complexes. In this figure an additional complex, designated C45, is observed. This complex appears only sporadically in our assays and has not yet been extensively studied.

Competition and supershift experiments designed to test for the involvement of transcription factor USF in complex formation are illustrated in Figure 5A. As in Figure 4, some lanes (4–6 and 11–14) contained unlabeled competitor SP1 oligonucleotide to eliminate the SP1-related bands and thus make the remaining bands more easily visible. This also enabled us to test for potential interactions between the two sites. However, no additional effect other than the elimination of the SP1-related complexes was noted. Complex C35 is specifically competed by unlabeled USF competitor DNA, while bands C25, C30 and C40 are not (lanes 2 and 3 and 5 and 6). With increasing amounts of USF-specific antibody, the appearance of the C35 complex is specifically eliminated, while the other complexes are unaffected (lanes 8–10 and 12–14).

While the observed complexes C25, C30, C35 and C40 appear to be accounted for by interactions of nuclear proteins at the SP1 and USF DNA recognition sites, there is also an AP4 recognition site which overlaps the USF site within the SAA oligonucleotide. Therefore, the AP4 protein could potentially contribute to formation of the observed complexes. We used unlabeled USF and AP4 consensus oligonucleotides in parallel reactions over a wide range of concentrations in order to detect and compare their ability to interfere with formation of the observed complexes. As shown in Figure 5B, the unlabeled USF oligonucleotide specifically competed the formation of the C35 complex in molar excess concentrations as low as 10-fold (lanes 2–7), while the AP4 oligonucleotide had little effect at concentrations as high as 200-fold molar excess (lanes 8–13). The other complexes were unaffected by either oligonucleotide.

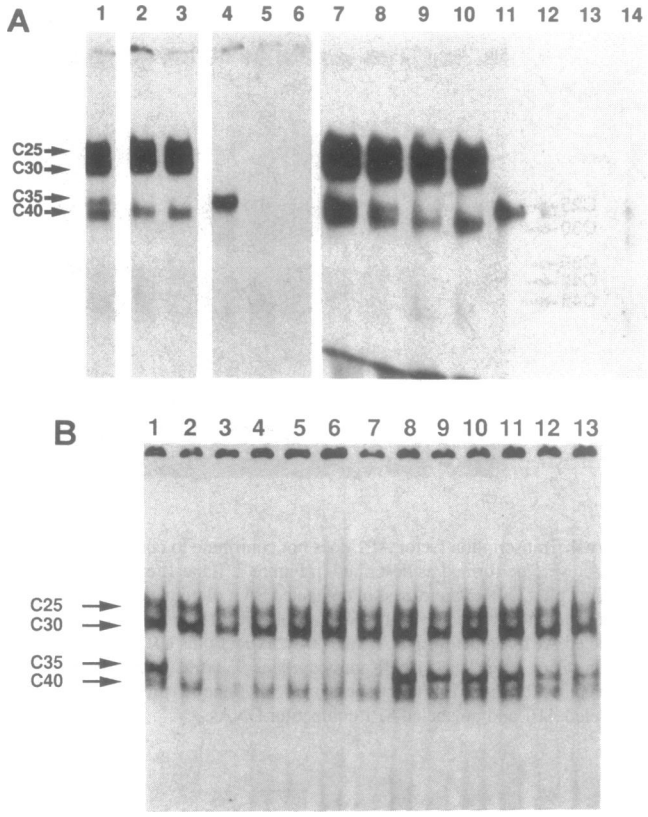

Figure 5. USF recognition sequences and USF-related proteins are involved in formation of the C35 complexes. EMSAs were performed as described in Figure 2. (**A**) Lane 1, extract alone; lanes 2 and 3, extract plus 10× (lane 2) or 100× (lane 3) molar excess of unlabeled USF competitor DNA; lanes 4–6, extract plus 125× molar excess unlabeled SP1 oligonucleotide plus 0× (lane 4), 10× (lane 5) or 100× (lane 6) molar excess of unlabeled USF competitor DNA; lanes 7–10, extract plus 0 μg (lane 7), 0.01 μg (lane 8), 0.1 μg (lane 9) or 1 μg (lane 10) USF antibody; lanes 11–14, extract plus 100× molar excess unlabeled SP1 consensus oligonucleotide and 0 μg (lane 11), 0.01 μg (lane 12), 0.1 μg (lane 13) or 1 μg (lane 14) USF antibody. (**B**) Lanes 1–7, extract plus 0× (lane 1), 10× (lane 2), 25× (lane 3), 50× (lane 4), 100× (lane 5), 150× (lane 6) or 200× (lane 7) molar excess unlabeled USF consensus oligonucleotide; lanes 8–13, extract plus 10× (lane 8), 25× (lane 9), 50× (lane 10), 100× (lane 11), 150× (lane 12) or 200× (lane 13) molar excess unlabeled AP4 consensus oligonucleotide.

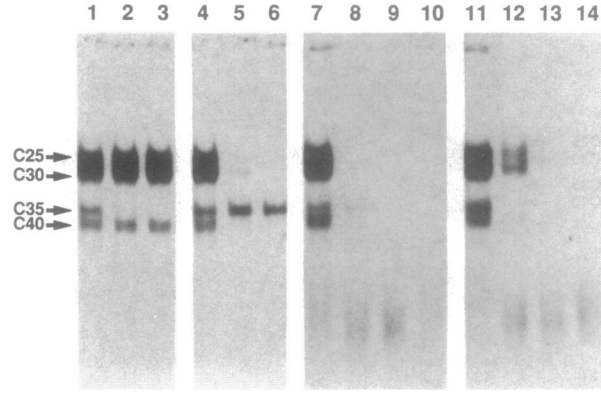

Figure 6. Mutant SAA oligonucleotides define sequences involved in complex formation. EMSAs were performed as described in the Materials and Methods, using ^{32}P-labeled SAA (lanes 1–7 and 11), MSP1 (lanes 8–10) or MUSF1 oligonucleotides (lanes 12–14). As indicated, varying molar amounts of unlabeled MSP1 (lanes 2 and 3), MUSF1 (lanes 5 and 6), USF consensus (lanes 9 and 10) or SP1 consensus (lanes 13 and 14) oligonucleotides were added as competitor DNA. Lanes 1, 4, 7 and 11, ^{32}P-SAA oligonucleotide incubated with PC12 nuclear extract; lanes 2 and 3, ^{32}P-SAA incubated with PC12 nuclear extract and 10× (lane 2) or 100× (lane 3) molar excess of unlabeled MSP1 competitor DNA; lanes 5 and 6, ^{32}P-SAA oligonucleotide incubated with PC12 nuclear extract and 10× (lane 5) or 100× (lane 6) molar excess of unlabeled MUSF1 competitor DNA; lanes 8–10, ^{32}P-MSP1 oligonucleotide incubated with PC12 nuclear extract and 0× (lane 8), 10× (lane 9) or 100× (lane 10) molar excess of unlabeled USF competitor DNA; lanes 12–14, ^{32}P-MUSF1 oligonucleotide incubated with PC12 nuclear extract and 0× (lane 12), 10× (lane 13) or 100× (lane 14) molar excess of unlabeled SP1 competitor DNA.

Mutant SAA oligonucleotides define sequences involved in complex formation

To test the sequence specificity of the proteins forming complexes with the SAA oligonucleotide further, two mutant oligonucleotides were synthesized which contained the same sequences as the SAA except that one, MSP1, contained sequence changes which eliminated the SP1 recognition site and the other, MUSF1, contained sequence changes which eliminated the USF recognition site. The results of experiments utilizing these oligonucleotides are shown in Figure 6. When the MSP1 oligonucleotide was used as an unlabeled competitor the C35 complex was eliminated in EMSA experiments, but the C25, C30 and C40 complexes were not (lanes 2–3). Furthermore, when MSP1 was ^{32}P-labeled and incubated with PC12 nuclear extract, only the C35 complex appeared (lane 8). This complex could be eliminated with the addition of unlabeled USF consensus competitor oligonucleotide (lanes 9 and 10). The unlabeled MUSF1 oligonucleotide was able to compete away the C25, C30 and C40 complexes but not the C35 complex (lanes 5 and 6), and ^{32}P-labeled MUSF1 produced the C25, C30 and C40 complexes but not the C35 complex (lane 12). These complexes were eliminated with the addition of unlabeled SP1 consensus oligonucleotide (lanes 13 and 14).

Mutant promoter/luciferase constructs demonstrate the functional significance of transcription factor binding sites

In order to test the contributions of each of the transcription factor binding sites to promoter activity we created mutations of our wild-type APP promoter/luciferase reporter gene construct (5) which incorporated site-specific base pair changes within the SP1, USF or AP1 sites. Two constructs termed MSP1 and MUSF1 were produced which incorporated the same mutations as the MSP1 and MUSF1 oligonucleotides, respectively. Another construct, MAP1, was created which carried mutations in the AP1 site. Two additional mutations termed MUSFu1 and MUSFu2 were produced which modified the USF site to make it more similar to the published consensus sequence (12). PC12 cells were transfected with each of these constructs and promoter activity was assessed by measuring relative luciferase activity in cell extracts. As shown in Figure 7, promoter activity was reduced in MSP1 by 30% relative to the wild-type construct. In contrast, the activity of the MUSF1 and MAP1 constructs was about the same as that of the wild-type construct, while the activity of the MUSFu1 and MUSFu2 constructs was increased by 60–80% relative to the wild-type construct.

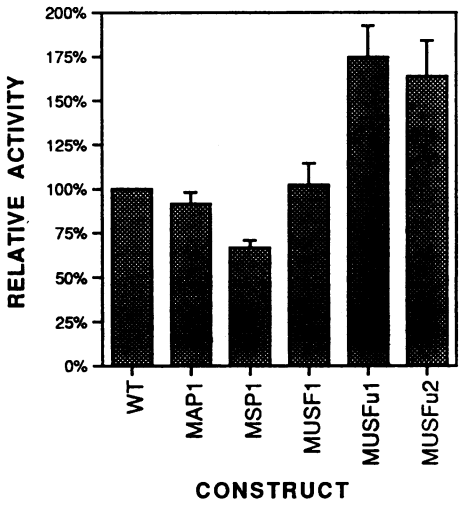

Figure 7. Functional activity of mutant promoter/luciferase constructs. Constructs containing mutant and wild-type rat APP promoters linked to a luciferase reporter gene (see Materials and Methods) were tested for activity in PC12 cells. Activity values represent luciferase activity relative to that of the wild-type construct. Error bars represent standard error of the mean. In columns lacking error bars, the standard error is <1%. $N > 15$ for MUSFu1 and MUSFu2, $N > 27$ for MUSF1 and MAP1, $N > 43$ for MSP1 and WT.

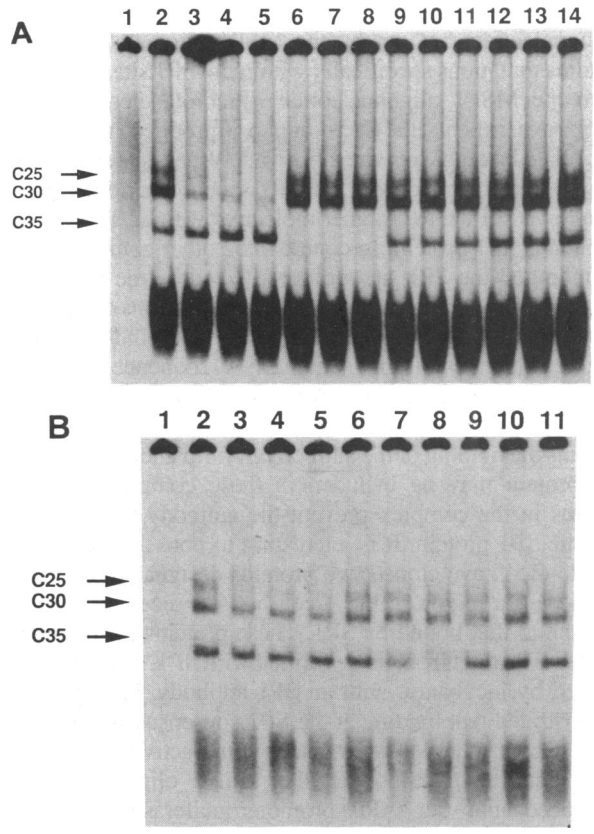

Figure 8. Rat cortex nuclear proteins form C25, C30 and C35 complexes with SAA oligonucleotide. EMSAs were performed as described in the Materials and Methods, using ^{32}P-labeled SAA and extracts derived from rat cortex. (**A**) All lanes except lane 1 contain 12 μg of rabbit preimmune serum total protein. Lane 1, no extract; lane 2, extract only; lanes 3–5, extract plus 50× (lane 3), 100× (lane 4), 150× (lane 5) molar excess of unlabeled SP1 competitor DNA; lanes 6–8, extract plus 50× (lane 6), 100× (lane 7), 150× (lane 8) molar excess of unlabeled USF competitor DNA; lanes 9–11, extract plus 50× (lane 9), 100× (lane 10), 150× (lane 11) molar excess of unlabeled AP4 competitor DNA; lanes 12–14, extract plus 50× (lane 12), 100× (lane 13), 150× (lane 14) molar excess of unlabeled AP1 competitor DNA. (**B**) All lanes contain 12 μg of rabbit preimmune serum total protein. Lane 1, no extract; lane 2, extract alone; lanes 3–5, extract plus 0.01 μg (lane 3), 0.1 μg (lane 4) or 1 μg (lane 5) SP1 antibody; lanes 6–8, extract plus 0.01 μg (lane 6), 0.1 μg (lane 7) or 1 μg (lane 8) USF antibody; lanes 9–11, extract plus 0.01 μg (lane 9), 0.1 μg (lane 10) or 1 μg (lane 11) AP1 antibody.

Rat cortex nuclear proteins form C25, C30 and C35 complexes with the SAA oligonucleotide

We investigated next whether the binding activities we observed in PC12 cells are also present in adult rat brain. The results of EMSA experiments using nuclear extracts from adult rat cortexes are shown in Figure 8. When ^{32}P-labeled SAA oligonucleotide was incubated with rat cortex extract in EMSA experiments, bands with mobilities similar to those of the C25, C30 and C35 PC12 complexes were observed (Fig. 8A, lane 2), but there is no evidence for formation of a complex analogous to the C40 PC12 complex. The cortex C25 and C30 complexes are specifically competed by the unlabeled SP1-specific oligonucleotide (lanes 3–5), while the cortex C35 complex is specifically competed by the unlabeled USF-specific oligonucleotide (lanes 6–8). Consensus oligonucleotides for AP4 (lanes 9–11) and AP1 (lanes 12–14) have no effect on complex formation. As seen in Figure 8B, antibodies directed against SP1 (lanes 3–5) and USF (lanes 6 and 7) interfered with the formation of the C25 and C35 complexes, respectively, while AP1 specific antibodies had no effect on complex formation. The SP1 antibody also produced a small amount of a higher molecular weight species, probably by complexing with some of the C25 complex (lanes 3–5). Consistent with these observations, we have also observed that when ^{32}P-labeled MSP1, which is mutated at the SP1 site, is incubated with rat cortex extract, only the C35 complex is formed, and when ^{32}P-labeled MUSF1, which is mutated at the USF site, is incubated with rat cortex extract, only the C25 and C30 complexes are formed (data not shown).

DISCUSSION

Recent research in our laboratory (5) and that of others (14) has demonstrated that two elements in the APP promoter are responsible for transcriptional regulation of the APP gene. In the rat genome we have termed these the GAG and SAA regulatory elements. The SAA element contributes ~30% of the transcriptional activity of the rat APP promoter when expressed in PC12 cells (5). When a 36 bp oligonucleotide containing the SAA element is incubated with a nuclear protein extract from PC12 cells, a specific pattern of four DNA–protein complexes is produced in EMSA experiments (Fig. 2). We have termed these complexes C25, C30, C35 and C40, based on their mobility in our gel system. In this paper we have investigated the activity of the SAA element by mapping DNA sequences within it which bind nuclear proteins, by characterizing some of these DNA-binding proteins, and by showing how mutations within each binding site affect complex formation and transcriptional activity.

The C25, C30 and C40 complexes involve protein binding at the SP1 consensus sequence located between bases –215 and

–206. These complexes can be competed by an SP1 consensus oligonucleotide, but not by the MSP1 oligonucleotide, which contains mutations specifically within the SP1 site. Furthermore, when the MSP1 oligonucleotide is radiolabeled and used in EMSA experiments, the C35 complex is produced but none of the SP1-related complexes are observed. When supershift experiments are performed with an SP1-specific antibody, the C25 complex is shifted to a higher molecular weight, suggesting that it contains SP1 protein. In contrast, the other complexes related to binding at the SP1 site are not affected by the SP1 antibody (Fig. 3). There are several possible explanations for this result. The C30 and C40 complexes may represent binding by protein species which bind the same DNA sequence but are not antigenically related to SP1. Alternatively, the SP1 protein may be present in these complexes but modified in a way which prevents the binding of the antibody. A third alternative is that the SP1 protein may be included in these complexes, but other proteins in the complex prevent the antibody from interacting with the SP1 protein. It is interesting to note that Kingsley and Winoto (15) have cloned two proteins designated SP2 and SP3 which bind a region in the T-cell antigen receptor α promoter identical to that bound by SP1. These proteins form complexes which are distinct from those formed with SP1, since they are not affected by incubation with an SP1 antibody. Finally, we have demonstrated that binding at the SP1 consensus site in the SAA element contributes to its transcriptional activity. When mutations identical to those in the MSP1 oligonucleotide are introduced into our APP promoter/luciferase gene reporter constructs, transcriptional activity is decreased by 30%. This decrease is comparable with that seen when the entire SAA element is deleted (5).

Several lines of evidence suggest that formation of the C35 complex involves binding of the transcription factor USF, or a closely related protein, to a USF recognition sequence between positions –206 and –199. This complex can be competed by a USF consensus oligonucleotide, but not by the MUSF1 oligonucleotide which contains mutations within the USF site. When the MUSF1 oligonucleotide is used as the probe in EMSA experiments, SP1-related complexes are formed but the C35 complex is not. When a USF antibody is included in the binding reactions, the formation of the C35 complex is specifically eliminated, although there is no concomitant appearance of a more slowly migrating species. This may be because binding of the antibody to the USF protein disrupts the complex.

In contrast to the straightforward results obtained with respect to the binding of USF to the SAA element, analysis of the contribution of USF to the functional transcriptional activity of the promoter has proved to be complicated. When the USF site is mutated to make it more closely resemble the published consensus sequence, the activity of the promoter/luciferase construct is increased 60–80% in transient transfections of PC12 cells. This result argues strongly for the possible involvement of the USF protein in the transcriptional regulation of the rat APP promoter. However, when the MUSF1 mutations are introduced into our promoter/luciferase constructs, the activity is not significantly altered from that of the wild-type construct. A possible explanation for these observations is that an additional regulatory protein can activate the promoter in lieu of USF binding, or that the mutation causes an increase in activity through an alternative pathway which compensates for the decrease in activity through the USF pathway.

The consensus DNA binding sequence for AP1 which is located within the SAA element does not appear to contribute to complex formation with PC12 nuclear proteins. An AP1 consensus oligonucleotide does not compete away any of the observed complexes, even when present at a 250-fold molar excess concentration. Furthermore, inclusion of an AP1-specific antibody had no effect on complex formation or migration. Taken together, these data suggest that AP1 is not active in binding the SAA oligonucleotide in our assay system. Additionally, a mutation which alters the AP1 site but does not affect the other sites has no effect on transcriptional activity (Fig. 7). Similarly, our data in PC12 cells do not support an important regulatory role for the AP4 site within the SAA element. The AP4 site at positions –204 through –198 overlaps the USF site at positions –206 through –199. The latter DNA sequence has been shown by oligonucleotide competition experiments to be involved in the formation of the C35 complex with USF or a related protein, but not in the formation of any other complex. Furthermore, addition of excess AP4 consensus oligonucleotide had no effect upon complex formation (Fig. 5B). In addition, the MUSFu1 and MUSFu2 mutations which make the USF recognition site more like the published USF consensus sequence also make the AP4 recognition site less like its consensus sequence. These mutations both improve the transcriptional activity of our APP promoter/luciferase constructs, and therefore suggest that AP4 does not play a positive role in regulating the APP promoter under our conditions. The explanation for these observations may be that the AP1 and AP4 sites within the SAA element are incapable of binding these factors. We have tested our PC12 nuclear extracts and found that they do contain protein factors which specifically bind AP1 and AP4 consensus oligonucleotides (data not shown), suggesting that AP1 and AP4 transcription factors are present in the extracts, but do not interact with these recognition sites under our conditions. It is possible, however, that these factors play a regulatory role either in other cell types or following treatment of the cells with agents such as phorbol esters, which activate AP1 gene expression (16).

We have investigated the relevance of our results with PC12 cells to the *in vivo* situation in rat brain using EMSA experiments with rat cortex nuclear extracts. Based upon several lines of evidence, the complexes produced by rat cortex nuclear extracts incubated with the SAA oligonucleotide appear to be the same as the C25, C30 and C35 complexes formed with PC12 nuclear proteins. First, the mobilities of the cortex complexes are similar to those of the PC12 complexes, and formation of the cortex C25 and C30 complexes is affected by the SP1 consensus oligonucleotide, while formation of the cortex C35 complex is affected by the USF consensus oligonucleotide (Fig. 8A). Secondly, SP1 antibody interferes with formation of the cortex C25 complex and USF antibody interferes with formation of the cortex C35 complex, just as they do with the PC12 complexes (Fig. 8B). Finally, the mutant oligonucleotides MUSF1 and MSP1 form the same complexes with cortex extracts as they do with PC12 extracts (data not shown). In contrast, we do not observe formation of a cortex C40 complex similar to that seen with the PC12 extracts. Since the PC12 cells used in these experiments were undifferentiated, the proteins required for formation of this complex may be specific to the undifferentiated state and therefore absent in cortex. Additional experiments are required to confirm or disprove this hypothesis. It is interesting to point out that Izumi *et al.* (17) noticed that a DNA–protein complex was

formed by incubating the murine APP SP1 site with liver or kidney nuclear extracts but not with brain nuclear extracts.

Several other groups have studied the binding of nuclear proteins to the APP promoter. Quitschke has described binding activity to the human equivalent of the rat SAA region, which he termed the APBα region (14). Although this study did not include experiments to identify the specific protein factor(s) involved, the DNA sequence defined by mutagenesis studies suggests that the USF recognition site is the target. Similarly, Kovacs *et al.* recently identified the interaction of USF at the overlapping AP1/AP4 sites in the human APP promoter (18). Each of these studies examined only one DNA–protein complex, and neither study identified an SP1 binding activity. Pollwein identified two different complexes formed by HeLa nuclear proteins, and one of these complexes was shown to involve binding at the human SP1 recognition site (19). However, supershift studies were not done to conclusively identify the protein involved in formation of this complex. Therefore, we cannot be certain whether this complex is analogous to the C25 complex or to another complex. Izumi *et al.* noted several complexes which bound an oligonucleotide containing the mouse equivalent of the rat SAA element (17). These complexes could be competed with SP1 consensus oligonucleotide but were not further investigated. Thus far no group working with the human or mouse APP promoters has reported more than two DNA–protein complexes or identified more than one of the factors involved. We have observed at least four different DNA–protein complexes involving the SAA element, and we have presented evidence suggesting that at least three different types of DNA binding activities are involved. The differences between our observations and those of others may be due to differences between the cell lines or nuclear extracts used, the use of different oligonucleotide probes, and/or sequence or structural differences between the human and rat SP1 sites (4). It is particularly interesting to point out that one of the SP1-related activities we observe, complex C40, may be differentially regulated in different cells or tissues, since it appears to be present in extracts made from PC12 cells but not in extracts made from rat cortex.

This work has identified two DNA sequences, an SP1 site and a USF site, which interact with the transcription factors SP1 and USF at the SAA element of the rat APP promoter. Additional factors which interact with the SP1 site but not with an SP1 antibody have also been observed. We have demonstrated that each of these transcription factor binding sites can play a role in the regulation of the rat APP gene in PC12 cells. Additionally, we have shown that one of the DNA-binding proteins which interacts with the SAA element may be missing from rat cortex extracts, suggesting the possibility of tissue-specific transcriptional control. Future experiments will be directed towards understanding the individual and combined contributions of the SAA and the GAG elements to APP gene expression *in vivo* and *in vitro* under conditions which induce APP transcription, such as treatment with phorbol esters, NGF, IL-1 and retinoic acid (16,20–22).

ACKNOWLEDGEMENTS

The authors wish to thank Drs John Kusiak, Guang Bai, C. L. Creasy and George Roth for helpful discussion, and Mr Michael Prenger for technical support.

REFERENCES

1 Hardy, J. and Allsop, D. (1991) *Trends Pharmacol. Sci.* **12**, 383–388.
2 Joachim, C. and Selkoe, D.J. (1992) *Alz. Dis. Assoc. Disord.* **6**, 7–34.
3 Hardy, J. (1992) *Nature Genet.* **1**, 233–234.
4 Chernak, J.M. (1993) *Gene* **133**, 255–260.
5 Hoffman, P.W. and Chernak, J.M. (1994) *Biochem. Biophys. Res. Commun.* **201**, 610–617.
6 Gorski, K., Carneiro, M. and Schibler, U. (1986) *Cell* **47**, 767–776.
7 Dignam, J.D., Lebovitz, R.M. and Roeder, R.G. (1983) *Nucleic Acids Res.* **11**, 1475–1489.
8 Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
9 Kunkel, T.A., Roberts, J.D. and Zakar, R.A. (1987) *Methods Enzymol.* **154**, 367–382.
10 Dynan, W. and Tjian, R. (1983) *Cell* **35**, 79–87.
11 Mermod, N., Williams, T.J. and Tjian, R. (1988) *Nature* **332**, 557–561.
12 Sawadogo, M. and Roeder, R.G. (1985) *Cell* **43**, 165–175.
13 Lee, W., Haslinger, A., Karin, M. and Tjian, R. (1987) *Nature* **325**, 368–372.
14 Quitschke, W.W. (1994) *J. Biol. Chem.* **269**, 21229–21233.
15 Kingsley, C. and Winoto, A. (1992) *Mol. Cell. Biol.* **12**, 4251–4261.
16 Yoshikai, S., Sasaki, H., Doh-ura, K., Furuya, H. and Sakaki, Y. (1990) *Gene* **87**, 257–263.
17 Izumi, R., Yamada, T., Yoshikai, S., Sasaki, H., Hattori, M. and Sakaki, Y. (1992) *Gene* **112**, 189–195.
18 Kovacs, D.M., Wasco, W., Felsenstein, K.M. and Tanzi, R.E. (1994) *Abstracts of the 24th Annual Meeting of the Society of Neuroscience* **20**, 848.
19 Pollwein, P. (1993) *Biochem. Biophys. Res. Commun.* **190**, 637–647.
20 Mobley, W.C., Neve, R.L., Prusiner, S.B. and McKinley, M.P. (1988) *Proc. Natl. Acad. Sci. USA* **85**, 9811–9815.
21 Goldgaber, D., Harris, H.W., Hla, T., Maciag, T., Donnelly, R.J., Jacobsen, J.S., Vitek, M.P. and Gajdusek, D.C. (1989) *Proc. Natl. Acad. Sci. USA* **86**, 7606–7610.
22 Konig, G., Masters, C.L. and Beyreuther, K. (1990) *FEBS Lett.* **269**, 305–310.