Acetylcholinesterase in man is encoded by a single gene, ACHE, located on chromosome 7q22. In this study, the transcription start sites and major DNA promoter elements controlling the expression of this gene have been characterized by structural and functional studies. Immediately upstream of the first untranslated exon of the gene are GC-rich sequences containing consensus binding sites for several transcription factors, including Sp1, EGR-1 and AP2. In vitro transcription studies and RNase protection analyses of mRNA isolated from human NT2/D1 teratocarcinoma cells reveal that two closely spaced transcription cap sites are located at a consensus initiator (Inr) element similar to that found in the terminal transferase gene. Transient transfection of mutant genes shows that removal of three bases of the initiator sequence reduces promoter activity by 98% in NT2/D1 cells. In vitro transcription studies and transient transfection of a series of deletion mutants of the ACHE promoter linked to a luciferase reporter show an Sp1 site at −71 to be essential for promoter activity. Purified Sp1 protein protects this site from DNase cleavage during in vitro footprinting experiments. A conserved AP2 consensus binding site, located between the GC box elements and the Inr, is protected by recombinant AP2 protein in DNase footprinting experiments, induces a mobility shift with AP2 protein and AP2-containing cell extracts, and fosters inhibition of transcription by AP2 as measured by transient transfection in mouse and human cell lines and in in vitro transcription reactions. These results indicate that AP2 functions as a repressor of human ACHE and mouse AChE transcription.

A defining feature of neurotransmitter systems is the coordinate use of disparate molecular elements to achieve a specific functional goal. Cholinergic neurons exemplify this by utilizing nicotinic and muscarinic receptors and the enzymes choline acetyltransferase and acetylcholinesterase (ACHE, EC 3.1.1.7) to modulate the effects of the neurotransmitter acetylcholine. ACHE is a serine hydrolase that is located in a variety of tissue environments by linkage of diverse anchoring subunits or carboxyl-terminal sequences to the constant catalytic domain of the enzyme (reviewed in Massoulie et al., 1993; Taylor and Rade´, 1994). In humans ACHE activity is encoded by a single gene, ACHE, located within 6 kilobases of DNA sequence on chromosome 7q22 (Getman et al., 1992; Ehrlich et al., 1992). Multiple forms of ACHE protein arise from alternative splicing of exons found at the 3′ end of the open reading frame (Li et al., 1991). The alternative splicing gives rise to carboxyl termini which differ in their capacity to disulfide bond with other catalytic and structural subunits or associate with the plasma membrane (cf. Massoulie et al., 1993; Taylor and Rade´, 1994).

The necessity for coordinated expression of each component of cholinergic neuronal systems for the maintenance of homeostasis is illustrated by the loss of receptor function and skeletal muscle toxicity in myasthenia gravis and the profound symptoms associated with ACHE inhibition from organophosphate poisoning. Many components of cholinergic neural systems have not been investigated at the level of the gene, and the nature of regulatory mechanisms which foster coordinated expression of cholinergic macromolecules remains obscure.

One approach useful for investigating gene structure and expression relies on interspecies comparison of a single genetic locus. The genomic structure, alternative RNA splicing, and amino acid sequence of genes encoding ACHE have been investigated in a wide variety of species (Rachinsky et al., 1990; Li et al., 1991, 1993). Promoter elements controlling expression of ACHE genes in mammalian cells have been identified and studied functionally in Torpedo (Ekström et al., 1993) and mouse muscle (Mutero et al., 1995). Partial sequences of the human ACHE 5′-flanking region have been reported (Ben Aziz-Aloya et al., 1993), but little is known about functional promoter elements or transcriptional regulation of ACHE in mammalian cells. We have cloned and characterized putative promoter sequences for the human ACHE gene and have determined the transcription initiation sites. From functional studies we find that GC-rich sequence elements containing consensus binding sites for Sp1 are essential for transcription of ACHE, while the transcription factor AP2 acts as a repressor of human ACHE and mouse ACHE transcription in vitro.

**EXPERIMENTAL PROCEDURES**

Cell Culture—Human cell lines and the mouse fibroblast cell line C3H10T1/2 (10T1/2) were obtained from the American Type Culture Collection. The human cholinergic teratocarcinoma subline NTera2/D1, SK-N-SH neuroblastoma, and embryonic kidney (HEK) lines were maintained in Dulbecco's modified Eagle's medium containing 4.5 g sucrose/liter and 10% fetal bovine serum. The human hepatocarcinoma cell line Hep-G2 was grown in Earl's minimal essential medium with 10% fetal bovine serum, 1 mM sodium pyruvate, and non-essential amino acids. The myoblast cell line C3H10T1F2L-2B (10T1L2-3), a clone of 10T1/2 cells that constitutively expresses myogenin (described previously in Brennan et al., 1991), was a generous gift of Dr. Eric.
Acetylcholinesterase Transcriptional Regulation

Olson, The University of Texas M. D. Anderson Cancer Center, Houston, TX. The 10TFL2–3 cells were differentiated into myotubes by switching the culture medium to Dulbecco's modified Eagle's medium containing 2% horse serum and 0.5% fetal bovine serum. All cells were grown at 37°C in a humidified atmosphere containing 5% CO2.

Library Screening and Construction of 5'-Flanking Region Deletion Mutants—Genomic clones of the human ACHE gene were obtained by screening a pWE15 cosm id library (gift from Dr. Glen Evans, University of Texas, Dallas, TX) containing human placental genomic DNA. The library was screened from duplicate filter lifts with a 357-bp PCR fragment amplified from exon 2 of the human ACHE gene cDNA sequence (Soreq et al., 1990). The sequences of PCR primers were: (5') AACHGAGGCAGCCGAGACT (HindIII) and 5' TACGCGCTCAGTGATGCGCTCG (HUMX1–5). Taq polymerase (Perkin Elmer) and an EriCopter Therm Cycler were used to amplify human genomic DNA in a 50-μl reaction using 10 cycles of 94°C for 1 min, 64°C for 2 min, and 72°C for 2 min. This was followed by 25 cycles of 94°C for 1 min and 72°C for 2 min, and a final extension reaction at 72°C for 7 min. A 4-6 kb NotI DNA restriction fragment from cosmid clone 18D1–1 was subcloned into pSK II (Stratagene, La Jolla, CA). Deletions of 5'-flanking DNA sequences were accomplished by both restriction digestion (constructions A and F) and PCR amplification (constructions B-E). The 5' primers used to construct the latter four clones contained the following sequences: (B) CCGGAGGGAGGGAGGG; (C) CACTGGGGCGGCGCGG; (D) CGGGCGGGGCGGCGTG; and (E) CGGGCGGGGCGGCGTG. All amplifications used the same 3' primer (CGTCTGGCGGCTCGAGG) located 90 bp downstream of the KpnI site in the intron region between exons one and two and were achieved by using five cycles at 94°C for 30 s, 57°C for 4 min, followed by 25 cycles of 94°C for 30 s, 59°C for 4 min, and a final extension reaction at 94°C for 7 min. After band isolation and end polishing with Klenow fragment of DNA polymerase 1, the PCR products were digested with KpnI and cloned into Smal and KpnI sites in pSK II Bluescript. Plasmid inserts were then removed by digestion with BamHI and KpnI and ligated into the luciferase reporter vector pXP1 (a generous gift of Dr. Steven Nordeen, University of Colorado) containing human ACHE gene sequences consisting of 1–2 intron DNA from the KpnI site to an NsilI site 16 bases downstream of the 5' splice junction of exon 2. Clones A and B were chosen for sequencing by digesting the primer (CGTCTGGCGGCTCGAGG) located 90 bp downstream of the KpnI site by BamHI and Saci or Bsu36I, respectively, and ligating to the same KpnI-NsilI fragment in pXP1. The integrity of ligation junctions was confirmed by sequencing. The mouse Ache promoter fragments were obtained from genomic clones described previously (Li et al., 1993).

Sequencing—DNA sequencing employed the dye deoxy method (Sanger et al., 1977) using fragments cloned into both M13 and pBluescript SKII+ phagemid vectors as templates and primed with sequence-specific oligonucleotides. The immediate promoter region is especially GC-rich, and to obtain consistent sequences in both the 3' and 5' directions required the use of Taq polymerase at 70°C. Gene 32 protein, and deaza dGTP to prevent formation of secondary structure.

Cell Culture, Transfection, and Assays—Cells were transfected by the calcium phosphate method as described previously (Ekstrom et al., 1993). Transfection reactions contained a total of 25 μg of DNA/10 cm plate, consisting of the reporter plasmid and 5 μg of pCMV-βgal for normalization of transfection efficiencies. DNA precipitates were incubated with cell cultures overnight in serum-containing medium in a 3% CO2 atmosphere. After transfection, cells were incubated in fresh medium for an additional 24 h, followed by lysis of harvested cells by sonication. Luciferase activity in cell lysates was determined by autoinjecting 30 μl of lysate containing 70 μM luciferin (Analytical Luminescence, La Jolla, CA) and 0.25 mM ATP in a Turner Design luminometer. AChE enzyme activity was determined by the method of Ellman et al. (1961).

Electrophoretic Mobility Shift Assays—Nuclear extracts were prepared as described (Schreiber et al., 1989). Protein concentrations in the extracts were estimated using BCA reagent (Pierce). For AP2, double-stranded oligonucleotides containing the intact or mutated AP2 site and associated flanking sequences corresponded to the following sequences: 5'-TGGGGCGGCGGGGTGCGTCTA-3' and 5'-TGGGGCGGCGGGGTGCGTCTA-3'. These fragments were labeled with the Klenow fragment of DNA polymerase I in the presence of [32P]dCTP. Oligonucleotides containing the mutated (m) and wild-type (wt) AP2 consensus sequence of the mouse Ache promoter were used as unlabeled competing oligonucleotides. The wt competing oligonucleotide contained the sequence 5'-GATCGAATCTGCGCGGCAAGCCGCTGTC-3'. Recombinant AP2 was expressed from Promega. Antisense antibody was from the sequence 5'-TGGGGCGGCGGGGTGCGTCTA-3'. The antibody was from Cruz Biotechnology. Mobility shift reactions contained 5 μg of nuclear extract protein, antibodies, or unlabeled competing oligonucleotides, 5 μg of bovine serum albumin, 4 μg of poly(dI-dC) in 12% glycerol, 12 μM HEPES, pH 7.9, 60 μM KCl. After a 10-min incubation on ice, 20,000 counts/min of the [32P]-radiolabeled probe was added. The mixture was then incubated for 15 min on ice. The complexes were resolved from free DNA on 6% polyacrylamide gel in 1 X TGE buffer (Tris-glycine-EDTA, pH 8.5) at 4°C.

In vitro Transcription and RNase Protection—In vitro transcription was performed as described previously (Dignam et al., 1983), using DNA fragments containing varying lengths of sequence upstream of exon 1 of the ACHE gene and extending down to the KpnI site in the 1–2 intron. RNase protection experiments were conducted by doing fragment from the ACHE genomic cosmid clone 18D1–1 in pBluescript SKII+. Antisense RNA probes were made with these templates by in vitro transcription essentially as described by Ausubel et al. (1987). RNase digestions conducted 25,000 units/ml RNase A, 225,000 units/ml RNase T1, and 50 μg of total RNA.

Site-specific Mutagenesis—A 493-bp fragment extending from 113 bases upstream of the 3' cap site to the KpnI site in the 1–2 intron was mutated (Kunkel et al., 1987) after single strand rescue of the insert, contained in pSK Bluescript II, with helper phage VCSM13, then inserted into the ACHE-luciferase reporter construct. The sequence of the mutant sense oligonucleotide was GTTGGCGGAGCCAGAAGTGCGCGGCTGTC. The mutated region in the AP2 site and the integrity of the entire DNA insert were ascertained by DNA sequencing.

DNase Footprinting—Footprinting experiments were performed as described previously (Dignam et al., 1989). Purified AP2 and Sp1 proteins were obtained from Promega (Madison, WI). Transcription factors were incubated with end-labeled DNA probes for 30 min at 0°C. DNase I digestions were conducted at room temperature for 45–60 s.

RESULTS

Isolation of ACHE Genomic Clones and Characterization of 5'-Flanking Region—To identify regulatory elements of ACHE, a human genomic cosmid library was screened with a DNA fragment amplified by PCR from human genomic DNA obtained from a blood sample from one of the authors. This fragment corresponded to 357 bp of exon 2, the first translated exon of both the human ACHE and mouse Ache genes (Li et al., 1991). Using this fragment as probe, eight distinct cosmid clones were obtained, with inserts averaging 30 Kbp in size. To locate potential regulatory regions of ACHE, restriction digests of cosmids inserts were Southern blotted and probed with mouse Ache cDNA clones representing upstream regions of the gene (Li et al., 1993). Cross-hybridization revealed a region of similarity between the two species that is located 1–2 kb upstream of exon 2 (data not shown). Sequence analysis of 2.6 kb of human genomic DNA upstream of the 5' end of exon 2 of ACHE revealed a short region of DNA with 91% sequence identity to a 216 bp portion of the 5' terminus of mouse cDNA clones (Li et al., 1993) (Fig. 1). Like the mouse mouse Ache clones, this region is approximately 1.5 kb upstream of exon 2. Part of this human sequence corresponds to exon 1 of in the mouse gene, an untranslated sequence that splices to the open reading frame of Ache that begins in exon 2 (Li et al., 1993). Substantial sequence identity between mouse and human genes was also noted upstream of exon 1, encompassing what has previously been defined as a promoter for mouse Ache expression (Li et al., 1993). This putative human promoter region contains a preponderance of G and C bases, lacks a TATA box, and contains four consensus binding sites for the transcription factor Sp1 and three sites corresponding to the early growth response (EGR-1) transcription factor (Wang et al., 1993). Also present are two consensus binding sites for the transcription factor AP2 (Williams et al., 1991) and a consensus binding site for NF-κB (Kunsch et al., 1992) in the intronic region 21 bp downstream of the conserved RNA splice donor site corresponding to the 3' junction of exon 1 in mouse Ache.

Assessment of Promoter Activity of 5'-Flanking Regions of the ACHE Gene—Potential promoter activities of ACHE gene fragments were ascertained by constructing fusion genes of the 5'-flanking region of ACHE linked to luciferase as a reporter. A
2.6-kb AChE genomic DNA fragment was subcloned into the luciferase reporter vector pXP1. This fragment consisted of approximately 1.1 kb of DNA sequence upstream of the region similar to mouse AChE exon 1, exon 1, and 1.5 kb of the putative intronic region between sequences corresponding to exons 1 and 2 in AChE. The 3' end of this and all subsequent promoter fragments constructed includes the first 16 bases of exon 2 upstream of the ATG start codon to ensure that the RNA splice acceptor site at the 5' end of exon 2 facilitates proper splicing of exon 1 sequences to the luciferase open reading frame. Transient transfection of this construct, denoted A, into various established human cell lines revealed substantial promoter activity when luciferase activity was normalized to β-galactosidase activity (Table I). The magnitude of this promoter activity correlated roughly with the amount of AChE enzymatic activity expressed by the cell lines. Human embryonic kidney (HEK) and human hepatocarcinoma Hep-G2 cell lines fostered enzymatic activity expressed by the cell lines. Human embryonic kidney (HEK) and human hepatocarcinoma Hep-G2 cell lines fostered the highest AChE enzyme activity, with both cell lines achieving greater enzymatic activity than the HEK and Hep-G2.

### Table I

| Cell line | AChE promoter-luciferase activity | AChE enzyme activity |
|-----------|----------------------------------|---------------------|
| Light units/β-Gal | n mole/min/mg protein |
| HEK | 72 ± 4.4 | Not detected* |
| Hep-G2 | 45 ± 3.1 | Not detected* |
| SK-N-SH | 123 ± 23 | 1.05 ± .07 |
| NTera2/D1 | 586 ± 89 | 7.65 ± 1.7 |

*Activity less than 0.05 n mole/min/mg protein.

Although the NT2/D1 cell line displayed greater AChE enzyme activity when luciferase activity was normalized to β-Gal than HEK and Hep-G2, the amount of AChE enzyme activity was not detected in these cell lines. When construction A was transfected into the human cell line NTera2/D1, an AChE-expressing cholinergic subline of the teratocarcinoma cell line NTera 2 (NT2), luciferase activity was 8-13-fold higher than HEK and Hep-G2.

Since the NT2/D1 cells displayed greater AChE enzyme activity and promoter activity of AChE upstream sequences relative to other human cell lines, this cell line was chosen for defining the location of the promoter region of the AChE gene. Given the sequence similarity between the human and mouse genes upstream of exon 1, and the known promoter activity of the mouse AChE gene in this region (Li et al., 1993), we constructed 5’ deletion mutants by PCR amplification of construction A to analyze the promoter potential of this region. The locations of the upstream primers used for amplification of constructions B through E were chosen so that the Sp1 consensus sites in the putative promoter region upstream of exon 1 would be sequentially deleted (Fig. 2). An additional 5’ deletion mutant (construction F) was prepared by restriction digestion of construction A with Bsu36I. This resulted in the removal of the GC-rich region and most of the sequence similar to mouse exon 1. Transient transfection of constructions A through F in NT2/D1 cells revealed no significant difference in promoter activity between constructions A, B, and C. However, construction D was almost 5-fold lower in promoter activity than A and B, suggesting that the second upstream Sp1 consensus sequence (Fig. 1) is essential for activated transcription of AChE.

Mapping of the Transcription Start Sites—Previous studies have revealed that transcription of endogenous AChE in mouse erythroleukemia and myoblast cell lines is characterized by the use of multiple transcription start sites (Li et al., 1993) (cf. Fig. 1). Attempts at determining the transcription start sites of human AChE by primer extension from sequence-specific primers in exon 2 yielded RNA products which lacked the clear definition of cap sites seen in mouse. To define the cap site and regulatory elements, in vitro transcription experiments were conducted using HEK cell nuclear extract and linear DNA templates with 5’ termini corresponding to constructions B through E. To obtain RNA transcripts that were of sufficient size for analysis by polyacrylamide gel electrophoresis, constructions B-E were cut with KpnI, yielding templates (B′-E′) that were 495–410 bp in size (Fig. 3). These templates were expected to yield RNA transcripts that were 380–395 bases long if human AChE utilized the same cap sites as mouse AChE. Transcription in vitro with these reagents revealed that a RNA transcript approximately 385 bases long was produced in equal amounts by both constructions B′ and C′. Constructions D′ and E′ were totally inactive in these assays (Fig. 3, A and B). Hence, the 12 bp which comprise the difference between C′ and D′ are essential for transcription of AChE in HEK nuclear extracts. Loss of this region, which contains the second upstream Sp1 site, abolishes transcription completely. These data agree with the transient transfection experiments with constructs B through E, which showed this Sp1 consensus sequence to be important for promoter activity in NT2/D1 cells (cf. Fig. 2).

The size of the RNA transcript produced in these in vitro transcription reactions suggested that, unlike mouse AChE RNA transcripts isolated from myoblast and erythroleukemia cell lines, a dominant transcription start site is used by human AChE. The approximate location of this cap site corresponds to the most 3′ site used by mouse AChE and coincides with a consensus DNA sequence for an initiator (Inr) element similar to one found in the terminal deoxynucleotidyl transferase gene.
promoter (Smale et al., 1990). To confirm the location of the mRNA cap site used by ACHE, RNase protection analysis was performed with RNA isolated from NT2/D1 cells. A 750-bp antisense cRNA probe was generated by transcribing a human DNA fragment containing sequences corresponding to exon 1. This probe was then used to protect NT2/D1 total cellular RNA. Two closely spaced bands, 73 and 76 bases in length, were protected from digestion (Fig. 4A). The size of these bands is consistent with RNA transcripts resulting from the use of two different cap sites at the 5' end of exon 1. Similar to the ACHE in vitro transcription product seen in Fig. 3A, the 5' end of these two bands coincides with the terminal deoxynucleotidyl transferase Inr element consensus site present at the 5' end of exon 1. However, the in vitro transcription assays revealed only one band, suggesting a single cap site was used in the HeLa cell nuclear extract environment. To resolve this apparent discrepancy, ACHE RNA products from in vitro transcription reactions with HeLa cell nuclear extract were electrophoresed on a higher percentage polyacrylamide gel. Under these conditions, the band representing the ACHE-specific transcript resolved into two bands as well (Fig. 4B). Thus evidence from experiments using distinct methodologies and two different human cell lines indicates that two mRNA cap sites are present at the 5' end of exon 1 in ACHE and that these sites reside within a consensus sequence for an initiator element (Fig. 4C).

In order to ascertain the importance of the initiator element consensus site in ACHE transcription, three bases within this site were deleted by digesting construction B with Bpu1102I, blunting the ends with S1 nuclease, and ligating the ends. This resulted in the removal of three bases, TCA, in the putative Inr sequence GGCTCAGCC, yielding the Inr element deletion mutant BΔ3. Insertion of this mutant Inr construction, which was otherwise identical to construction B, into the luciferase reporter vector and transfection into NT2/D1 cells revealed that these three bases are essential for promoter activity. The BΔ3 construction displayed less than 2% of the promoter activity compared to the wild-type construction B (Table II). This result is in accord with the RNase protection data described above which tentatively placed the two mRNA cap sites for ACHE at base 3 (C) and base 6 (A) of this putative Inr element.

Repression of ACHE Expression by AP2—AP2 binds to DNA as a homodimer, with an aggregate molecular mass of 104 kDa (Williams et al., 1991). Promoters which have been reported to be activated by AP2 contain consensus binding sites for the factor several hundred bp upstream of core promoter elements (Haslinger and Karin, 1985; Lee et al., 1987). The presence of two AP2 consensus binding sites between essential GC box elements and the transcription cap sites in the ACHE promoter (cf. Fig. 1) suggested that AP2 might influence expression of the gene by interfering with activated or basal transcription. To assess the effect of AP2 on ACHE transcription, construction B was co-transfected into NT2/D1 cells with an expression

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**Fig. 2.** Promoter activity of ACHE-luciferase (Lucif) gene chimeras transiently transfected into NT2/D1 teratocarcinoma cells. Transfection efficiencies were normalized by co-transfection with the lacZ gene driven by the CMV early promoter. Data represent the means and standard errors of three separate experiments, each performed in triplicate.

**Fig. 3.** In vitro transcription of ACHE gene 5'-flanking fragments in HeLa cell nuclear extracts. A, representative autoradiograph of one in vitro transcription experiment. B, map of DNA templates used for in vitro transcription experiments. ACHE mRNA levels were normalized to a transcript produced from a DNA fragment containing the CMV promoter included in each reaction. Data represent the means and standard deviations of three separate in vitro transcription experiments.

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Acetylcholinesterase Transcriptional Regulation
Acetylcholinesterase Transcriptional Regulation

Acetylcholinesterase (ACHE) is a crucial enzyme involved in the hydrolysis of acetylcholine, which is a neurotransmitter at the cholinergic synapse. The expression of the ACHE gene is tightly regulated, and studies have shown that the transcriptional repression of ACHE is mediated by the AP2 transcription factor. This repression is critical for the prevention of AchE (Acetylcholinesterase) gene expression in muscle cells, ensuring that AchE is not expressed in the nerve terminal, thereby preventing autolysis.

**Fig. 4. Determination of transcription start sites of ACHE.** A, RNase protection analysis of NT2/D1 cell total RNA. The antisense cRNA probe consisted of 750 bases extending from the KpnI site in the 1–2 intron to base –318 upstream of exon 1. Lane 1, NT2/D1 total RNA; lane 2, trNA; lane 3, digested probe; lane 4, undigested probe. B, in vitro transcription of ACHE in HeLa nuclear extract. a, CMV template; b, ACHE template; c, CMV transcript; d, ACHE transcripts. Reaction was as in Fig. 3, except RNA transcripts were separated on an 8% polyacrylamide gel. C, location of cap sites at the consensus Inr element of human ACHE.

Vector containing a cDNA encoding the open reading frame for wild-type AP2. Transient transfection experiments showed that AP2 has an inhibitory effect on ACHE promoter activity when normalized against β-galactosidase. When the AP2 site proximal to exon 1 was deleted by site-directed mutagenesis (construction BmtAP2), the repressive effect of AP2 was abolished (Table I).

To examine the possibility that increased levels of AP2 protein expressed in the transfected cells were repressing ACHE promoter function by competition with endogenous transcription factors (squelching), an amino-terminal deletion mutant of the AP2 open reading frame was cotransfected into untreated NT2/D1 cells with construction B. This AP2 mutant, ΔN165AP2, lacks the transcriptional activation domain of the wild-type factor but retains the dimerization and DNA-binding domains. ΔN165AP2 can therefore still homodimerize and bind to DNA but is unable to form protein–protein interactions with other members of the RNA polymerase transcription complex (Williams et al., 1991). Similar to wild-type AP2, co-transfection of ΔN165AP2 decreases ACHE promoter activity approximately 6-fold (Table I). These data suggest AP2 specifically represses ACHE transcription by sterically interfering either with the function of the transcription complex at the cap sites 19 bp downstream, or with the binding of Sp1 transcription factors 21 bp upstream.

To assess the universal nature and functional significance of AP2-mediated repression of ACHE gene expression, two mouse cell lines that express AP2 were used to test the effect of this transcription factor on a DNA fragment containing the mouse AChe promoter. RNase protection experiments revealed that fibroblast cell line 10T1/2 and myoblast cell line 10TFL2–3 (a 10T1/2-derived line with transfected myogenin stably integrated into its genome) contain mRNA for AP2, indicating the factor is expressed in these cell lines (data not shown). Transient transfection of 10T1/2 and 10TFL2–3 cells with AChe promoter–luciferase reporter constructions containing either a native or mutated AP2-binding site showed that the reporter gene containing a mutated AP2 site exhibited 5-fold higher promoter activity than the wild-type promoter (Table I). Thus mouse cell lines which express AP2 are able to repress mouse AChe expression in a manner similar to the repression of human ACHE transcription in recombinant AP2 co-transfection experiments in human NT2/D1 cells.

Induction of Gel Mobility Shifts by AP2-containing Nuclear Extracts—To confirm the specificity of endogenous AP2 activity interacting with the AP2-binding site of the mouse AChe promoter, nuclear extracts prepared from 10T1/2 fibroblasts and differentiated 10TFL2–3 myocyte cells were used in band shift experiments containing end-labeled double-stranded oligonucleotides corresponding to the conserved AP2 element in the mouse AChe promoter region. Fig. 5 shows that nuclear extract from 10T1/2 cells induces a mobility shift of the wild-type AP2 oligonucleotide (lane 2), while anti-AP2 antibody induces a supershift of the complex (lane 4). In addition, unlabeled oligonucleotides containing the wild-type, but not the mutated, AP2 consensus binding site can compete for binding to the endogenous factor in 10T1/2 cells (lanes 6–8). Nuclear extracts from 10TFL2–3 myocyte cells induce a mobility shift of oligonucleotides containing the AP2 consensus binding site of light intensity (lane 3); the band intensity is increased slightly when anti-AP2 antibody is present (lane 5). This result is consistent with the finding that these myocyte cells contained much lower levels of AP2 mRNA compared to 10T1/2 and undifferentiated 10TFL2–3 cells, as assessed by RNase protection (data not shown). Purified AP2 protein also induced a mobility shift of the wild-type oligonucleotide, while oligonucleotides containing the mutated AP2 sequence did not (Fig. 5, lanes 9–11). Together, these results indicate AP2 binds in a specific manner to the conserved AP2 element found in the mouse and human ACHe gene promoters.

AP2 Represses Transcription of ACHE In Vitro—If AP2-induced transcriptional repression of ACHE is the result of steric interference with proximal downstream elements, factors binding to the Inr element may undergo competition with AP2 for access to binding sites. Alternatively, bound AP2 homodimer may force the use of a less efficient cryptic Inr element further downstream in exon 1. To distinguish between these alternatives, recombinant AP2 protein was added in vitro transcription reactions containing HeLa cell nuclear extract and construction B′ as DNA template. Addition of AP2 protein selectively decreased ACHE-specific transcription in a concentration-dependent manner compared to an internal control template driven by the CMV promoter (Fig. 6). Furthermore,
Effect of AP2 on activity of human ACHE and mouse Ache promoters-luciferase reporter genes

Specific activity of ACHE promoter (light units/β-Gal) was normalized to the wild type construction B. Activity of mouse Ache promoter (light units/β-Gal) containing a mutated AP2 site (MmtAP2) was normalized to the wild-type promoter construction (MwtAP2). Values represent the mean ± S.E. of two to three separate experiments performed in triplicate.

| Cell line                  | Cotransfected expression vector | Human ACHE promoter-luciferase | Mouse Ache promoter-luciferase |
|----------------------------|--------------------------------|-------------------------------|-------------------------------|
|                            |                                 | B          | BmtAP2     | B.13       | MwtAP2     | MmtAP2     |
| Human NT2/D1 teratocarcinoma | pRSV                          | 1.0 ± 0.12 | 0.96 ± 0.14 | 0.013 ± 0.01 |               |             |
|                            | pRSV/AP2                       | 0.34 ± 0.04 | 1.06 ± 0.04 | ND          |               |             |
|                            | pRSV/ΔN165AP2                  | 0.15 ± 0.02 | ND          | ND*         |               |             |
| Mouse 10T1/2 fibroblast    |                                |             |             |             | 1.0         | 5.8 ± 1.2   |
| Mouse 10TFL2–3 myotube     |                                |             |             |             | 1.0         | 5.1 ± 1.4   |

* ND, not determined.

The size of the ACHE RNA transcript produced in the in vitro transcription reaction was not affected by addition of recombinant AP2. These experiments indicate AP2 competes for access to binding sites on the DNA template with factors involved in mediating transcription; repression of ACHE transcription by AP2 is not due to the obligate use of a less efficient cryptic cap site downstream.

Location of Transcription Factor-binding Sites by DNase Footprinting—Transient transfection and in vitro transcription experiments described above using 5’ deletion mutants of the ACHE 5’-flanking region showed that the second upstream Sp1-binding site in the 5’-flanking region of exon 1 is essential for promoter activity. To determine whether the inhibitory effects of AP2 on ACHE transcription are due to interference with binding of Sp1, DNase footprinting experiments were conducted using 32P-end-labeled construction B and recombinant AP2 and Sp1 proteins. Sp1 protein protected regions of the DNA template from DNase I digestion that correspond to the fourth and second upstream Sp1 consensus sites in the GC-rich region (Fig. 7A). This result is consistent with evidence obtained from functional studies shown in Figs. 2 and 3, where the second upstream Sp1 site is essential for promoter activity in vivo and in vitro.

Labeling of the sense or antisense strand of B’ and incubation with AP2 protein yielded two footprints that correspond to the distal and proximal AP2 consensus binding sites downstream of the Sp1 sites in the ACHE promoter (Fig. 7, B and C). AP2 also protected a region of exon 1 from DNase digestion, indicating that AP2 binding may occur 45 bp downstream of the transcription cap sites. However, evidence from transfection experiments indicates this downstream site is not involved in AP2-dependent transcriptional repression of ACHE. ACHE promoter constructs of smaller size were produced by digesting construction B with the restriction enzyme EcoRI. This resulted in the removal of all sequences downstream of base +20 in exon 1, including the downstream AP2-binding site identified in the footprinting experiments. When this construction was done.

**Fig. 5. Electrophoretic mobility shift analysis of the mouse Ache AP2 sequence in mouse fibroblast and myotube nuclear extracts.** Double-stranded oligonucleotides containing the wild-type (wt) or the mutated (m) AP2 site and the flanking sequences as found in the mouse Ache promoter were end-labeled with [32P]dCTP and incubated with nuclear extracts prepared from undifferentiated 10T1/2 (1/2) fibroblasts (lanes 2, 4, 6–8) and 10TFL2-3 (TF) myotubes (lanes 3 and 5), or with purified recombinant AP2 protein. A specific anti-AP2 antibody was used for supershift experiments (lanes 1, 4, 5, and 10). Competition with unlabeled oligonucleotides containing the AP2 consensus sequence (lane 7) and a mutated AP2 sequence (lane 8) are also shown.

**Fig. 6. Repression of in vitro ACHE transcription by AP2.** Representative autoradiograph of an in vitro transcription experiment with AP2 protein. Recombinant AP2 protein was incubated with ACHE construction B’ in HeLa cell nuclear extracts. The ratios of ACHE RNA transcripts to control CMV promoter-driven RNA transcripts are as follows: lane 1, 1.0; lane 2, 0.62 ± 0.04; lane 3, 0.67 ± 0.06; lane 4, 0.32 ± 0.05. Data represent the means and standard deviations of four separate experiments.
into the luciferase reporter vector and co-transfected into NT2/D1 cells with the AP2 expression vector, promoter activity was decreased approximately 70% compared to cultures that were co-transfected with the expression vector alone (data not shown). This result is consistent with the ability of AP2 to repress promoter activity of the full-length constructs in transient transfection and in vitro transcription experiments, indicating the AP2-binding site present in exon 1 is not involved in the observed repression of ACHEx promoter activity.

To determine if binding of AP2 and Sp1 proteins to the ACHEx promoter is mutually exclusive, both proteins were assayed together in footprinting experiments after each factor was incubated alone with the DNA template end-labeled in the sense strand. Subsequent addition of Sp1 protein to the reaction had no effect on AP2 binding, nor did AP2 alter binding of Sp1 (Fig. 7C, lanes 4–6). These experiments show AP2 does not influence binding of Sp1 to the essential second upstream Sp1-binding site. Interestingly, AP2 and Sp1 also prevented DNase I digestion of a small region upstream of the Sp1 sites (bases –136 to –118), but only when present together in the footprinting reaction. Neither protein alone protected this region. As the DNA base sequence of this region does not correspond to any known binding determinant for either of these transcription factors, the significance of this finding remains to be assessed.

**DISCUSSION**

Definition of ACHEx Promoter Region and Cap Sites—Herein we report the identification of the core promoter region and attendant Inr element and transcription start sites of human ACHEx using structural and functional studies. There is a marked structural similarity to mouse AChex in the promoter region with an absence of TATA and CAAT boxes and a high frequency of guanosine and cytosine residues in the promoters of both genes. Each gene also contains multiple binding sites for the transcription factor Sp1, which overlap with those for the EGR-1 transcription factor, a zinc finger protein (Wang et al., 1993). Three EGR-1 sites are found in the ACHEx promoter region 58, 68, and 85 bp 5′ of the upstream transcription cap site. Also present are two AP2 consensus binding sites located between the Sp1/EGR-1-binding site region and the cap sites and a binding site for the p50-p65 NF-kB heterodimer downstream of the 3′ splice junction of exon 1. This consensus sequence is not conserved in mouse AChex.

Other gene promoter regions with GC-rich characteristics have formerly been regarded as housekeeping entities, whereby gene products involved in maintaining the economy of the cell are constitutively expressed. However, a closely regulated genes have been found to possess promoters with a high frequency of G and C residues (Lusky et al., 1987; Mumula et al., 1988), thus weakening an artificial promoter classification system based solely on DNA base sequence. One consistent finding in studies of genes with GC-rich promoters has been the use of multiple transcription start sites, presumably due to the variability in binding sites available to proteins involved in activating the RNA polymerase II transcription complex (Kollmar et al., 1994; Lu et al., 1994). Mouse AChex is representative of this, where transcription start sites of the gene in murine erythroleukemia and myoblast cell cultures range over a 20-bp region upstream of the Inr element. Evidence presented here shows that human ACHEx transcription in NT2/D1 and HeLa cells differs from mouse AChex by having only two closely spaced RNA cap sites, both of which originate at the Inr element. Given the high degree of similarity between the promoter and Inr element regions of the two genes, preference for cap site utilization may be tissue-specific. Future investigation into ACHEx cap site usage in human muscle and hematopoietic cell lineages should be informative in this regard.

Evidence from both transfection and in vitro transcription experiments shows the second upstream Sp1 site 71 bp 5′ of the first cap site in ACHEx is essential for activated transcription. Analysis of deletion constructs revealed this single site with attendant downstream sequences confers promoter activity equivalent to that observed from over 1 kilobase pair of sequence upstream of exon 1. Sp1 protein also protects this site from DNase I digestion in footprinting experiments. These results suggest the presence of a compact locus for regulating activated transcription of ACHEx, wherein EGR-1, Sp1, and other factors might compete for overlapping binding sites located within a limited portion of the promoter region of ACHEx. Although the effect of EGR-1 on human ACHEx transcription is unknown, the overlapping Sp1 and EGR-1 consensus binding sites in the mouse AChex promoter foster competition for binding between EGR-1 protein and Sp1 protein (Mutero et al., 1995). Similarly, the NF-kB site present in the intron region downstream of exon 1 of ACHEx appears to be non-functional. Co-transfection of expression vectors containing the p50 and p65 subunits of this transcription factor had no effect on construction A in NT2/D1 cells (data not shown).

Regulation of ACHEx Transcription by AP2—The DNA sequence encoding the region spanning the ACHEx promoter has been reported previously (Ben Aziz-Aloya et al., 1993). Our sequence differs from this report; an additional two G bases are present, 17 and 18 bp upstream of the 5′ cap site, revealing the AP2 consensus sequence GCCGGAAGGC. This consensus site
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was not noted by Ben Aziz-Aloya and colleagues (1993). We find identical sequences in clones from separate genomic libraries obtained from two individuals. Importantly, the studies reported here show this sequence represents a functional binding site for AP2. The presence of an identical AP2 consensus sequence in the mouse Ache promoter argues for evolutionary conservation of this gene regulatory element.

Functional AP2-binding sites that mediate transcriptional activation are often found upstream of core promoter regulatory regions, as has been reported for human genes encoding collagenase, growth hormone, keratin K14, metallothionein IIa, and proenkephalin, and for the murine major histocompatibility complex H-2 kb gene and the SV40 virus regulatory region (Haslinger and Karin, 1985; Lee et al., 1987; Hyman et al., 1989; Williams et al., 1988; Leask et al., 1991; Mitchell et al., 1987). The presence of two consensus sites for AP2 located between the Sp1 sites and the Inr element of the ACEE promoter region is unusual and suggests a novel role for AP2 in transcriptional regulation.

The ability of AP2 to repress human ACEE and mouse AcehE transcription in the transfection and in vitro transcription experiments presented in this study is remarkable, since this factor has previously been described only in an activating capacity. The proximity of the AP2 consensus binding sites to the essential Sp1 site in the promoter and to the Inr element presented the possibility that the factor disrupts transcription of ACEE. DNase I protection studies showed Sp1 and AP2 are able to bind to the ACEE promoter simultaneously (Fig. 7C), indicating that AP2 does not interfere with binding of activating elements to the GC box region of the promoter.

Several lines of evidence indicate AP2-mediated repression is due to a specific steric interference with the basic transcription factor machinery. First, mutation of the AP2-binding site proximal to the cap sites in the ACEE promoter eliminated AP2 repression of human ACEE promoter activity in transient transfection experiments using human NT2/D1 cells. Similarly, mutation of the AP2 site in the mouse AcehE promoter increased promoter activity in two mouse cell lines that express AP2. These results indicate DNA binding is essential for inhibition of transcription by AP2, a fact confirmed by band shift analysis with nuclear extract prepared from the AP2-expressing mouse cell lines. Second, addition of AP2 protein to in vitro transcription reactions selectively decreased transcription from the ACEE DNA template, while the internal control template containing the CMV immediate early promoter was unaffected (Fig. 3). If AP2-mediated repression was the result of squelching, i.e. sequestration of components of the RNA polymerase II transcription complex away from the DNA (Gill and Ptashne, 1988), then transcription from both templates would have been inhibited. Third, deletion of the activating domain of AP2 (construction ΔN165AP2) did not alter the transcriptional repression effect on ACEE promoter constructions. Retention of repressor activity after removal of the protein-protein interaction domains from the homodimer argues that AP2 sterically hinders some aspect of RNA polymerase II-directed transcription and does not repress transcription through either specific or non-productive protein binding.

Together these results suggest AP2 interferes with some aspect of RNA polymerase II-mediated transcription, perhaps by blocking access of Inr binding elements or members of the RNA polymerase II transcription complex to the cap sites. This idea is supported by the fact that mutation of the proximal AP2 site alone in the human ACEE promoter is sufficient for abolishing repression by AP2 protein. AP2 bound to the second upstream binding site closer to the GC-rich region of human ACEE apparently does not interfere with the function of the transcription complex at the cap sites.

Recently, the promoter region of the neuronal nicotinic acetylcholine receptor α3 subunit gene has been characterized and reported (Yang et al., 1995). It is of interest to note that the promoter of this gene is highly similar to the ACEE promoter; no TATA box is present, an essential Sp1 site resides at −70, and AP2 binds to a site between −22 and −30. It is not known whether AP2 functions as a repressor of the nicotinic receptor α3 subunit gene; however, the proximity of bound AP2 to the transcription start sites of the gene suggests that, like ACEE, the α3 subunit gene is also susceptible to repression by AP2. This finding raises the intriguing possibility that AP2 functions as a common regulatory element in coordinating expression of certain members of the cholinergic neurotransmitter system.

A steric hindrance model for AP2-mediated repression of ACEE transcription is reminiscent of the most frequently observed mechanism for transcriptional repression in prokaryotes, which involves competition between DNA-binding proteins and general transcription factors at or near gene transcription start sites (reviewed in Levine and Manley, 1989). Examples of eukaryotic repressors that function in a similar manner include the repression of bovine prolactin and human glycoprotein hormone α subunit genes by glucocorticoid receptor (Sakai et al., 1988; Akerblom et al., 1988). Repression of both genes requires the presence of the DNA-binding domain but not the activating domain of the receptor, indicating competition for binding sites in the promoter is involved. Binding of SV40 large T antigen near the transcriptional initiation site in the SV40 early promoter inhibits transcription, a result thought to involve steric blockade of RNA polymerase II in the region (Myers et al., 1981). In addition, a cellular protein, LBP-1, has been shown to block transcription in vitro from an HIV-1 long terminal repeat promoter by occlusion of both the TATA box and transcription start sites following binding of the factor to the DNA template (Kato et al., 1991). Thus, AP2 joins a growing family of eukaryotic proteins that serve to attenuate gene expression by interfering with the intricate milieu fostering transcriptional events. Future analysis of mutant ACEE transgenes will enable assessment of the physiological importance of these interactions in vivo.

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