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An SP1-like *cis*-Element Is the Major DNA Motif for Differential Expression Regulation of the Adipocyte Amino Acid Transporter

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Adipocyte amino acid transporter (AAAT) is induced during the 3T3-L1 preadipocyte differentiation process. In the −1819-bp 5′-upstream flanking region of the AAAT genomic gene, six DNase I protected sites were identified by using the 3T3-L1 adipocyte nuclear extract. Results of chloramphenicol acetyltransferase (CAT) expression from the chimeric AAAT promoter-driven CAT reporter gene indicated that one protein binding site, from −68 to −26, was essential for the promoter activity. However, this protein binding site does not contain recognition sites of the transcription factors important for adipocyte differentiation, i.e., the C/EBP or PPAR family. Further analysis revealed that the DNA sequence, TTCAAGTCCCGCCCTCCGCT from −65 to −46, was the *cis*-element essential and partially sufficient for inducible activity of the AAAT gene promoter. © 2000 Academic Press

Key Words: **adipocyte differentiation; Sp1-like *cis*-element; amino acid transporter; 3T3-L1 adipocyte.**

The major adipose tissue expansion in mammalian animals occurs at birth, when the adipose tissue is needed for the newborn to survive the periods of energy deprivation as well as to insulate from the low temperature of the environment. On the base of this adipose tissue expansion is adipocytes differentiation. Established preadipocyte cell lines, e.g., 3T3-L1 preadipocytes that can be induced to differentiate into adipocytes in cell culture, provide models with which to investigate the adipocyte differentiation program (1–4). When exposed to the appropriate differentiation inducers, including IGF-1 (or insulin at a nonphysiologically high concentration), dexamethasone (DEX, a glucocorticoid) and 3-isobutyl-1-methylxanthine (MIX, a cAMP phosphodiesterase inhibitor that increases intracellular cAMP), 3T3-L1 preadipocytes differentiate into adipocytes.

Differentiated adipocyte phenotype is the result of coordinated expression of a large number of adipocyte specific genes, which has been a focus for studying adipocyte differentiation. Large body of evidence has demonstrated that transcription factors belonging to C/EBP family and PPAR family are the key transcription factors regulating this coordinated adipocyte specific gene expression program (1, 4–7). In these two-transcription factor families, C/EBPα and PPARγ are two of the most important transcription factors for regulating adipocyte differentiation. Most of the adipocyte specific genes, such as aP2/422, Glut4, SCD1, OB, insulin receptor, etc. have functionally critical C/EBPα and/or PPARγ binding sites in their transcriptional regulatory promoter regions and their expression is transactivated by C/EBPα both *in vitro* and *in vivo* (8–14). In addition, transgenic expression of C/EBPα is sufficient to induce 3T3-L1 preadipocyte differentiation without hormonal stimulation (15, 16). C/EBPα antisense mRNA blocks hormonal induced 3T3-L1 preadipocyte differentiation (17). Furthermore, ectopic expression of PPARγ and/or C/EBPα is capable to convert several fibroblast cell lines and muscle cell line into preadipocyte cell lines (18, 19). Thus, C/EBPα and PPARγ act synergistically to induce adipocyte gene expression and promote adipocyte differentiation.

AAAT is an adipocyte amino acid transporter gene previously isolated from a 3T3-L1 adipocyte cDNA library. It is preferentially expressed in 3T3-L1 adipocytes and also in mouse adipose tissue (20). The expression of AAAT is induced during 3T3-L1 preadipocyte differentiation process. To understand AAAT gene expression regulation, we isolated and analyzed the promoter region of AAAT genomic gene. In the present paper, we report that a proximal Sp1-like GC rich *cis*-element is a crucial motif for activating

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AAAT gene expression during differentiation induction. Site-directed mutations in this GC-rich motif inactivate its promoter function.

MATERIALS AND METHODS

Materials. [^{3}H]Acetyl-CoA and Poly(dI–dC) (dI–dC) were from Amersham–Pharmacia Biotech. G418 was from Gibco BRL. pCATbasic and pCATpromoter 3 vectors were from Promega.

Cell culture. The 3T3-L1 preadipocytes were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% calf serum and allowed to reach confluence. Differentiation of two-day post-confluent preadipocytes (designated as day 0) was initiated with 1 μg/ml insulin, 1 μM dexamethasone (DEX) and 0.5 mM 3-iso-butyl-1-methylxanthine (MIX) in DMEM supplemented with 10% fetal bovine serum (21, 22). After 48 h (day 2), the culture medium was replaced with DMEM supplemented with 10% fetal bovine serum and 1 μg/ml insulin for additional 48 h, and the cells were then fed every other day with DMEM containing 10% fetal bovine serum. The cytoplasmic triglyceride droplets are visible by day 4 and the cells are fully differentiated by day 6.

Isolation and analysis of genomic clones. 5′ 560 bp of AAAT cDNA fragment (20) was labeled with [α-^{32}P]dATP by random-priming method (23), and then used to screen ~1.5 million phage plaques of a genomic DNA library constructed in λEMBL3 cloning vector containing inserts of approximately 20 kb from Sau3A partial digested 3T3-L1 DNA (11). The hybridization was carried out in 25% formamide, 4× SSC, 1× Denhardt's solution, 1% SDS, 50 mM sodium phosphate, pH 7.0, 0.5 mg/ml sodium pyrophosphate, and 50 μg/ml denatured and fragmented ssDNA at 42°C overnight and the membranes were washed in 1× SSC and 0.1% SDS at 50°C twice and 55°C once. Seven positive clones were isolated. A positive clone with a 16-kb insert containing the longest 5′ upstream region was used for further studies. The 16-kb insert of isolated λ phage was digested with *Eco*RI and then cloned into pBluescript. Each *Eco*RI restriction fragment was further digested with restriction enzymes to generate a set of subclones. Exons were identified by Southern analysis of these subclones using different fragments of AAAT cDNA as probes. The borders of intron-exon were determined by sequencing the exon-containing subclones. The 5′ upstream flanking sequence was determined by sequencing a 1.9-kb *Eco*RV–*Sma*I fragment.

Isolation of RNA and primer extension analysis. Total RNA was isolated from non-differentiated 3T3-L1 preadipocytes (day 0) and differentiated adipocytes (day 6) by the guanidine isothiocyanate method (24). A synthetic oligonucleotide (5′-CGAGCGTTTCGGGCTCTGG-3′ designated TA) which is complementary to nucleotides 104–122 of the cDNA sequence was end-labeled by [γ-^{32}P]ATP (5000 Ci/mmol) and T4 polynucleotide kinase to a specific activity of 4.5×10^7 cpm/μg. RNA sample (6 μg of total RNA) and 6×10^5 cpm of ^{32}P-labeled TA oligonucleotide primer (13 ng) were annealed at 65°C for 45 min followed by 45°C for 45 min in buffer containing 50 mM Tris–HCl, pH 8.3, 75 mM KCl, and 3 mM $MgCl_2$. After cooled down to room temperature, the extension reaction was started by addition of a mixture to give final concentrations of 50 mM Tris–HCl, pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT, 500 μM dNTPs, and 100 units RNase H$^-$ reverse transcriptase (SuperScriptII, Gibco BRL). The reaction mixture was incubated at 37°C for 5 min and 42°C for 1 h; and was terminated by heating at 70°C for 15 min. The DNA was obtained by ethanol precipitation and analyzed by 6% polyacrylamide sequencing gel. To obtain the length of the primer extension reaction product, DNA fragment containing the first exon was sequenced by the labeled TA oligonucleotide primer using Sanger's dideoxynucleotide method (25).

Nuclear extracts and DNase I footprinting. Nuclear extracts from 3T3-L1 cells were prepared using urea extraction method. 3T3-L1 cell monolayers were washed twice with cold PBS and once with hypotonic lysis buffer containing 20 mM Tris–HCl, pH 7.5, 10 mM NaCl, 3 mM $MgCl_2$, 1 mM DTT, 1 mM sodium vanadate, 30 mM β-glycerol phosphate and 2 μl/ml protease inhibitors cocktail 1 and 2 (PIC 1 and 2) (26). Cells were then scraped from plate into the same hypotonic buffer and incubated on ice for 5 min. NP-40 was added to the cell suspension to a final concentration of 0.1% for preadipocytes and 0.15% for adipocytes. After homogenizing the cells, nuclei were pelleted by centrifuging at 500*g* for 5 min. The nuclear pellet was then washed once with hypotonic buffer and once with nuclear storage buffer containing 40% glycerol, 50 mM Tris–HCl, pH 8.0, 3 mM $MgCl_2$, 1 mM DTT, 1 mM sodium vanadate, 30 mM β-glycerol phosphate and 2 μl/ml PIC 1 and 2. The washed nuclei were pelleted by centrifugation at 6000*g* for 20 s and resuspended in 10 pellet volumes of extraction buffer containing 1.1 M urea, 0.33 M NaCl, 1.1% NP-40, 17.5 mM Hepes, pH 7.6, 1.1 mM DTT, 1 mM sodium vanadate, 30 mM β-glycerol phosphate and 2 μl/ml PIC 1 and 2. After incubated on ice for 30 min, the mixture was centrifuged at 12,000*g* for 10 min. The supernatant was collected and glycerol was added to final concentration of 10%. The nuclear extract was aliquoted, quick-frozen and stored at −80°C. The protein concentration of the nuclear extract was determined by Lowry method.

DNase I footprinting was carried out as described before (10). AAAT promoter DNA (−1819 to +67) was fragmented by restriction enzymes or BAL-31 exonuclease digestion into suitable length (−1819 to −1443, −1443 to −1116, −1116 to −716, −843 to −455, −475 to +67, −372 to +67 and −182 to +67). To label DNA for footprinting assay, plasmid containing the targeted DNA fragment was digested with appropriate restriction enzyme(s) and labeled by fill-in method to specific activity of 1.0×10^7 cpm/μg. One-end labeled DNA fragment was obtained by digesting the DNA with a second restriction enzyme and purified by polyacrylamide gel electrophoresis. In each DNase I digestion reaction, 6 ng labeled DNA fragment was mixed with 5 μg Poly(dI–dC) (dI–dC) and nuclear extract in 20 mM Hepes, pH 7.4, 5 mM DTT, 1 mM $MgCl_2$, and 60 mM KCl, incubated on ice for 20 min and digested with appropriately diluted DNase I for 60 seconds. The DNA was analyzed on 6% polyacrylamide sequence gel. The protein binding site(s), which was protected from the DNase I digestion, was identified by sequencing the labeled DNA fragment using Maxam-Gilbert chemical cleavage sequencing reactions (27).

Construction and transfection of chimeric promoter-CAT gene vector. The 1.9-kb (−1819 to +67) *Eco*RV–*Sma*I fragment of the AAAT gene promoter was inserted into promoter-less pCATbasic vector to make a chimeric promoter–CAT reporter vector, p-1819CAT. This vector was then digested with nuclease BAL-31 to generate a series of 5′ deletions from which p-372CAT, p-182CAT, p-91CAT, and p-55CAT vectors were made. Fine deletions within −74 and site-directed mutations in −74 to +67 promoter were made by PCR with mutated primers; and all the mutants were confirmed by sequencing. pP1-CAT vector was constructed by inserting P1 oligonucleotide, corresponding to the promoter sequence from −65 to −46, into pCATpromoter vector. To make stably transfected 3T3-L1 cells a *Neo* resistant gene was inserted into these chimeric promoter-CAT vectors.

Chimeric promoter–CAT vectors and control pCAT vector were transfected into 30% confluent low passage 3T3-L1 preadipocytes to make stably transfected cell lines by the calcium phosphate precipitation method (28). Briefly, 20 μg CAT reporter vector DNA in 250 mM $CaCl_2$ solution was added to equal volume NaCl–Hepes–Na_2HPO_4 solution (280 mM NaCl, 50 mM Hepes, pH 7.12, and 1.5 mM Na_2HPO_4) to form DNA–calcium phosphate coprecipitate and the mixture was added directly to the culture medium. After 8 h at 37°C in CO_2 incubator, cells were shocked with 10% dimethyl sulfoxide–PBS for 3 min and then returned to incubator for 24 h in DMEM containing 10% calf serum. G418 was added to select the *Neo*-resistant cells. The antibiotic resistant foci were pooled and propagated to make stable cell line for CAT activity analysis.

Chloramphenicol acetyltransferase (CAT) assay. The cells of each stably transfected cell line were plated into 3.5-cm plates at 2×10^4

cell/plate and cultured as described for normal 3T3-L1 cells. After reaching 2-day postconfluence, the cells were subjected to differentiation induction protocol. At indicated times during differentiation process, cells were harvested for CAT analysis.

The CAT assay followed the protocol developed by Neumann *et al.* (29). Cell monolayers were washed twice with PBS and overlaid with TEN buffer (40 mM Tris–HCl, pH 7.5, 1 mM EDTA, and 150 mM NaCl) at room temperature for 5 min. The cells were scraped, pelleted by microfuging and resuspended in 0.1 mM Tris–HCl, pH 7.8. After three cycles of freeze/thaw, supernatant was collected by microfuging the mixture for 10 min. The collected supernatant was then incubated at 70°C for 15 min followed by at room temperature for 5 min. The precipitated proteins were removed by microfuging the heat-treated supernatant for 10 min. One hundred and fifty micrograms of the clarified supernatant was mixed with 50 μl of the CAT assay reaction mixture containing 0.1 M Tris–HCl, pH 7.8, 1.5 mg/ml chloramphenicol, 0.2 μCi [^{3}H]acetyl-CoA and 250 μM acetyl-CoA in a liquid scintillation vial and carefully overlaid with 4 ml scintillation fluid. The reaction was counted for 10 cycles over 6 to 10 h. The CAT activity was calculated from the linear rate of acetylchloramphenicol formation and the results were normalized to 3.5-cm plate.

RESULTS AND DISCUSSION

Analysis of AAAT gene genomic clone. From total of 7 isolated positive clones, a clone containing 16 kb genomic DNA insert, which has the longest 5′ upstream region of AAAT gene was purified and the *Eco*RI digested fragments (5, 8, and 3 kb) of this insert were subcloned into plasmid vector for further characterization. Three exons were identified in this 16-kb genomic DNA fragment by restriction enzyme mapping and Southern blot analysis; and the intron-exon boundaries were determined by sequencing. Two exons, 1110 and 134 bp, respectively, were in an 8-kb fragment and the third one, only 160 bp, was in a 3-kb fragment. Southern blot analysis of 3T3-L1 cell genomic DNA digested with *Eco*RI indicated that there were an 8- and a 7-kb *Eco*RI fragments hybridized by AAAT total cDNA probe and the 3′ 0.9-kb cDNA probe (from 1865 to 2720, Ref. 20) hybridized only the 7-kb fragment (results not shown). Thus, it is likely that the 3-kb fragment from λ phage insert is part of the 7-kb DNA.

To determine the transcription initiation site, primer extension assay was carried out using a primer complement to AAAT cDNA sequence from 104 to 122 (20). As shown in Fig. 1B, the primer extended bands were about 130 bp long, and RNA sample of adipocyte produced much stronger bands than RNA of preadipocyte. tRNA sample did not produce any detectable band. The transcription initiation site was determined by comparing the primer extended product with DNA sequencing which used the same primer. The result was summarized in Fig. 1A. A putative TATA box sequence, TTTAAT, was found at −22 bp and the cDNA sequence previously reported (20) started at +11. The 1.9-kb *Eco*RV–*Sma*I fragment contained a 1819-bp 5′ upstream flanking region and 67 bp untranslated cDNA sequence upstream of *Sma*I site was isolated and used for promoter analysis (Fig. 1A).

DNase I footprinting analysis of AAAT gene 5′ flanking sequence. To determine the potential regulatory DNA elements in AAAT gene promoter, DNase I footprinting analysis was conducted to find protein binding sites. Nuclear extracts from 3T3-L1 adipocytes and preadipocytes were used to carry out DNase I footprinting analysis. Six DNase I protected sites were identified in the 1.9-kb AAAT promoter DNA (−1819 to +67) with 3T3-L1 adipocyte nuclear extract (Fig. 2). These DNase I protected sites were clustered in two regions. Two sites, −1272 to −1249 and −1017 to −1002, were located relatively far away from the transcription initiation site (Figs. 2A and 2B). The −1272 to −1249 site appeared to be protected by nuclear extracts from both 3T3-L1 preadipocytes and adipocytes, while the −1017 to −1002 site appeared to be more adipocyte specific. The other four sites were located in the proximal promoter region (Figs. 2C–2E). The sites at −259 to −234, −222 to −200 and −68 to −26 were protected only by the nuclear extract of 3T3-L1 adipocytes. The site at −146 to −121 was predominantly protected by adipocyte nuclear extract and to some extends by preadipocyte nuclear extract as well. Within the −1819-bp promoter region, no DNase I footprinting site was found only protected (or predominantly protected) by nuclear extract of preadipocyte. All six footprinting sites were preferentially protected by nuclear extract of adipocyte. Thus, AAAT promoter was unlikely to be activated by de-repression during differentiation induction. Sequence comparison of these sites with database of transcription factor recognition sites revealed that the site at position −222 to −200 was for transcription factor NF-1. Sequence homologous to the complement sequence of GC box/SP1-binding site was found in the footprinted region at −259 to −234, −146 to −121 and −68 to −26, while the footprinted region at −258 to −234 also contained a C/EBPβ site. A notable aspect for the site at position −68 to −26 is that it has two repeat sequences, CCTCCGCCTTTTCAAGTCCCGCCCTCCGCTTTTTCAAGTC.

Functional analysis of AAAT promoter by activating CAT reporter gene expression during 3T3-L1 cell differentiation induction. To identify which of these protein-binding sites were essential for differentially inducing AAAT expression during 3T3-L1 preadipocytes differentiation, DNA fragments of AAAT promoter with a series of 5′-deletion or site-directed mutations were inserted into promoter-less pCATbasic vector. These AAAT promoter CAT reporter chimeric vectors were stably transfected into 3T3-L1 preadipocytes, and CAT activity in these stable cell lines during differentiation induction was measured (Figs. 3A and 3B).

A

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-1819    GATATCAGA  CACATATGGC  ACACAGACAC  AGATGCCTGT  GAAACACCCA
-1770    TACATGTAAA  ATAATAAGAT  GAAAATAAAA  TAAAATGAAA  TAAAACACAG
-1720    AAGCAAAGGT  TTATTGCTCA  GCCATTCGTG  TTATGTCGGA  TTCTAATAGA
-1670    TCTGCCCTGC  CTCCTCTCCT  CCTTGCTTCT  TCCTTCCCTT  CCTTCCTTCT
-1620    CCCCACTCTC  TCCCTTCTTT  GAGAAAAGAT  AATAATACAT  TCTTTTCCAG
-1570    TAAATTCAAA  AATTAACAAC  ACTATCCTTC  AAAAGCTATG  ATGGAGCCGG
-1520    GTGGTGGTGG  TGGTGGTGGT  GGTGGTGGCG  GCACATGCCT  TTAATACCAG
-1470    CAGGGAGGCA  GAGGCAGGGT  GGATTTTGGA  GTTCAAGGCC  AGTCTGGTCT
-1420    ACAGAGCAAG  TTCTAGGATA  GTCAGAGTCA  CACGGAGAAA  CCCTGCTTCC
-1370    TTCTCCAACA  AGCCCTCCCT  GCCCTCTCTC  AGGCTAGCCT  TTCCCAGCAG
-1320    CCCCTCATTC  CCATCTTTAC  TCTGATGTCT  GTGGCCAGGC  TGGTCATGAC
-1270    CTTGGAATCC  TTCCATAGTT  GGGATTGCAG  TCCTGAGAGC  TGCGGGTGAG
                     Ds1
-1220    CGCCTGGTAG  AACAGACTGA  GCCCTGTAGC  CTCCTTTGCT  CGACCTCCAG
-1170    GCAAGCTCCA  GACTTTCCAA  AAGAGCCATT  CTTCAGGGCG  TTTCTCAAGT
-1120    GGTCAACAGA  GTTCAGGAAG  GGACTCTTGG  CTGTCCTCCT  CCTTCAGCCC
-1070    AGCCTCTCAC  TACCCTGCTT  CACCCTTTGG  GAAGCCCCAG  ACCTTTCTCC
-1020    CACTGATGAA  ATCACAAGGA  ATCCGGGTAG  GAGGTTGGGC  CAAAGAGCTG
                     Ds2
-970     GCTGGAACCA  GTTTGAGTAT  TTCTCTTACT  AGGGGCAGGC  AGTGGGGCAG
-920     AACGCTCCTA  GCCAATCCTG  ATCCTGATCC  ACAAACTTCA  GCCTCATTTT
-870     CCCCACCCTA  CATGTCCCAA  AGATTCTGGA  TTCAGCAGAC  CTCTTTCCTC
-820     CTCCTCTTCC  TCCTCCTCCT  CCTCCTCCTC  TCTAAGTCTG  TTATTCTTAC
-770     AGTTGATGGG  TAACAGTGTC  TAAACTAAGA  ATGTTTGTGG  TGAACTGGCC
-720     ATTTAAATAT  TTTTTGCTAC  ATTTATTTAT  ATATTTGTTT  ATTTGTGTGT
-670     GTTGCATGTT  GCATGCTCCT  GTGGGGAGGA  CCCAATCTGT  GACTATCTGA
-620     GGAAAGAGAC  TGGGTTGCAG  CTCAAGCATG  AGGGCCTGAG  TTCAAATCCC
-570     CCACATTCAA  TGTTGGGGCC  TACTGGCCCC  TCCTCCCTCC  CTTTCCCCAA
-520     TTTGTTTCTA  CAGACTGGGG  TTCTTTTTCT  TTATGTAGCC  CTGGCAGCCC
-470     TAGAACTCGC  TCTGTAGACC  AACCAAGGCT  GATCTTGAAC  TCACAGAGAT
-420     CTATCTGCCT  GCCTGCCTCT  GCCTCCTGAG  AGCAGGGATT  AAAGGTGTGT
-370     GCCACTCATC  TGTCTGCCCT  TTCTAGGGGT  CCTTCTGTGG  AATATCAGAT
-320     ATGATCCACA  ATTAGATTCT  TTTGACCAGT  GGTGAGGTGC  GTGTGGGTGG
-270     GTGTGGGTTG  GATAGTTTCC  GAACGCCCGC  CCCTTTTGGA  GGCTTTGCAC
                                 Ps3
-220     CCTTCTGGCT  CGATGCCCTT  GCCCCGGCCC  CGCCCCTGGC  TATGCATTGT
                     NF-1
-170     TATTCACCCT  AGGTTGCCCA  CTGTGATCTT  GGCTCCGCCC  CTCGCTCGCC
                                           Ps2
-120     ACCTGGCGCC  GAATGTTCCA  TAACCATTCA  GCGCTTGCGG  GACGGCCTCC
-70      GCCTTTTCAA  GTCCCGCCCT  CCGCTTTTTC  AAGTCACGCC  CCTCCGCCTT
                                 Ps1
-20      TAATCCTGGT  CGCTTCCGTC-1
                    +1TAGCAGCCTC  AGAGACACGC  GGCGCGGCCT
                                  SmaI
 31      GGGAGGTTTC  TCCTTGGCGC  CACTTTCCAT  ATCCCCCGGG  CTGCTTCCTA
 81      TTTGCATCTG  GAAATTTGAT  TTTTCTCTCC  AGAGCCCGAA  ACGCTCGGTC
                                 3'-GG      TCTCGGGCTT  TGCGAGC-5'
                                            TA primer
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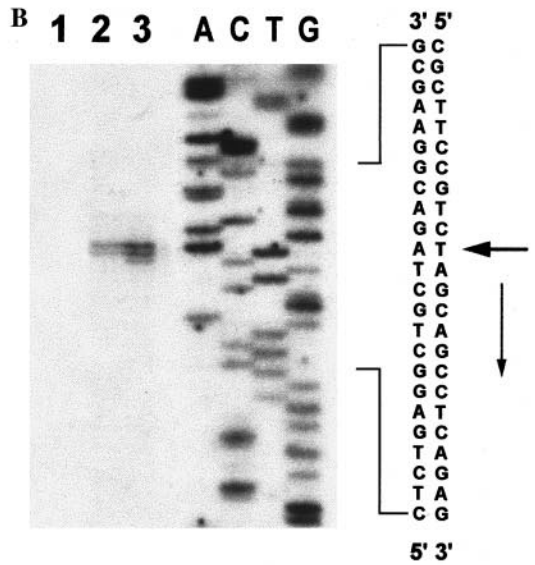
B



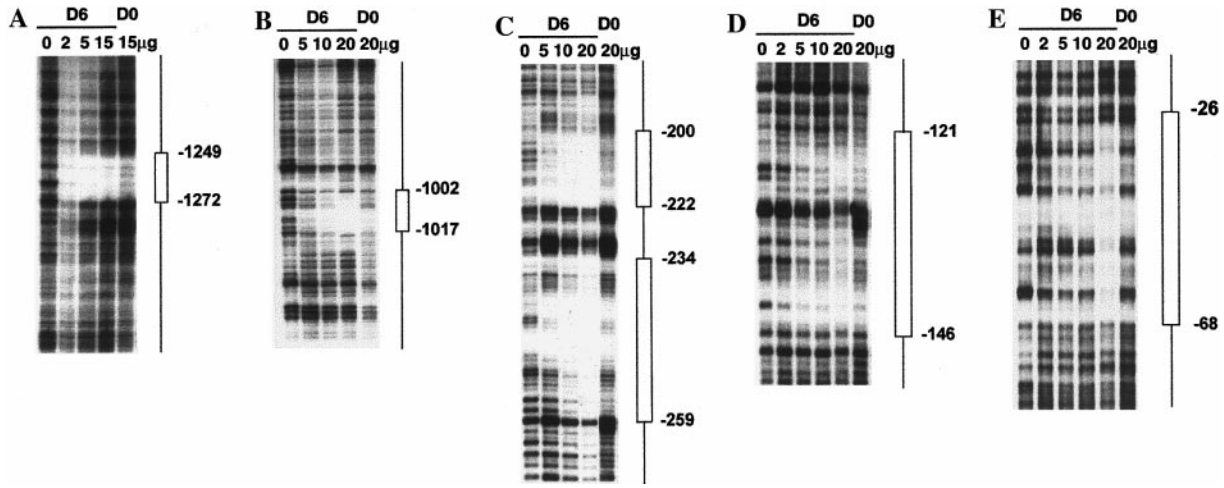
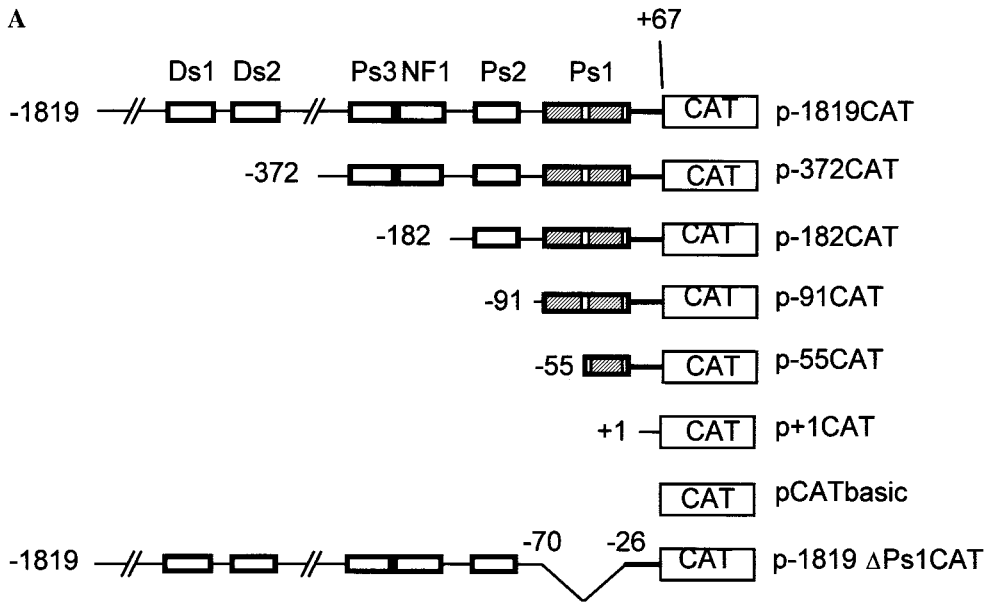


FIG. 2. DNase I footprinting sites in AAAT gene promoter. The DNase I footprinting assay was described under Materials and Methods. Six protein-binding sites were identified within −1819 AAAT promoter. Two were in distal promoter region: (A) −1272 to −1249 and (B) −1017 to −1002. Four were in proximal promoter region: (C) −259 to −234 and −222 to −200, (D) −146 to −121 and (E) −68 to −26. D6 indicates the nuclear extract prepared from day 6 differentiated 3T3-L1 adipocytes and D0 indicates the nuclear extract from day 0 3T3-L1 preadipocytes. The numbers (0, 5, 10 μg, etc.) indicate the amount of nuclear extract proteins used in the reaction. Maxam-Gilbert chemical cleavage sequencing reaction was used to determine the DNase I protected positions.

Previously, we have reported that the expression of AAAT mRNA is dramatically induced after the differentiation induction in 3T3-L1 cells, although it has a low basal expression level in preadipocyte (20). This prompts us to investigate the promoter region important for the inducible promoter activity not for the low basal activity. To identify the inducible promoter region, we compared the CAT activities before and after differentiation induction in the same promoter-CAT vector stably transfected cell line. If the cell line shows an increased CAT activity after differentiation induction, it indicates that the promoter construct contains the inducible DNA element. Otherwise, the promoter construct is inactive no matter how much basal promoter activity it has. Therefore, all the comparisons were made within the same promoter-CAT construct and the same stably transfected cell line. This eliminates all the variations caused by different cell lines or vector constructs. As shown in Table 1, cell lines transfected with pCATbasic, p+1CAT or p-55CAT vector all had relatively low CAT activity. The most important point is that the CAT activities in these cell lines did not respond to the stimulation of differentiation inducers. They remained at relatively low level both before and after the differentiation induction. In contrast, the CAT activities in stable cell lines transfected with p-1819CAT, p-372CAT, p-182CAT, or p-91CAT vector were also relatively low before induction, but they went up dramatically after stimulation by differentiation inducers. The increase of CAT activity in these cell lines after hormonal induction was 10- to 25-fold over the non-stimulated basal level. Although there were some variations of basal CAT activities and induced CAT activities between these stable cell lines, the response of their promoter constructs during differentiation induction was similar.

The CAT activity induced by −1819 promoter construct started on day 2 after differentiation induction; and the high CAT activity was maintained through the differentiation process. This CAT expression pattern mimicked the endogenous AAAT gene expression. (Table 1). It appears that this −1819 promoter region includes most of the *cis* DNA elements important for differentially induced AAAT gene expression. Similar results were observed with the −3.3-kb AAAT promoter reporter vector (results not shown). However, the CAT activity induced by −372, −182, or −91 promoter construct was only transiently increased during differentiation induction. Their CAT activity levels fell down in terminally differentiated adipocytes. This phe-

FIG. 1. Structure and sequence of AAAT gene 5′ upstream flanking region and the transcriptional initiation site. (A) The sequence of 5′ untranscribed flanking region of AAAT genomic gene and part of the first exon from −1819 to +130. The underlined sequences indicate the DNase I protected sites identified in Fig. 2. A putative TATA box was in bold face letter at position −22 to −17. The transcription initiation site was indicated as position +1. Primer extension analysis was conducted using TA primer. (B) Result of primer extension analysis. Lane 1 was yeast tRNA, lane 2 was total RNA from day 0 3T3-L1 preadipocytes and lane 3 was total RNA from day 6 adipocytes. Lanes labeled ACTG were the sequencing analysis of the first exon using the same labeled TA primer. The big arrow points the transcription initiation site and the small arrow points the direction of transcription.



B

Wt	CTCCGCCTTTTCAAGTCCCGCCCTCCGCTTTTTCAAGTCACGCCCCTCCGCC**TTTAAT**---to +67
M1	CTCCGCCcccgtggaTCCCGCCCTCCGCTTTTTCAAGTCACGCCCCTCCGCC**TTTAAT**---to +67
M2	CTCCGCC---------TCCCGCCCTCCGCTTTTTCAAGTCACGCCCCTCCGCC**TTTAAT**---to +67
M3	CTCCGCCTTTTCAAGTCCCGCCCTCCGCTcccgtggaTCACGCCCCTCCGCC**TTTAAT**---to +67
M4	CTCCGCCTTTTCAAGTCCCGCCCTCCGCT---------TCACGCCCCTCCGCC**TTTAAT**---to +67
M5	CTCCGCCTTTTCAAGTCaataCCTCCGCTTTTTCAAGTCACGCCCCTCCGCC**TTTAAT**---to +67
M6	CTCCGCCTTTTCAAGTC----CCTCCGCTTTTTCAAGTCACGCCCCTCCGCC**TTTAAT**---to +67
M7	CTCCGCCTTTTCccagCCCGCCCTCCGCTTTTTCAAGTCACGCCCCTCCGCC**TTTAAT**---to +67

FIG. 3. Chimeric AAAT promoter-CAT reporter vector constructs. (A) A series of AAAT promoter fragments were inserted into pCATbasic vector to construct the reporter vectors. Ds1, Ds2, Ps3, NF1, Ps2, and Ps1 were six DNase I protected sites found in −1819 AAAT promoter. The promoter DNA fragments in chimeric vectors were started from the positions indicated and ended at +67 *Sma*I site. (B) Site-directed mutations were introduced into −74 to +67 promoter; and these mutated promoters were then inserted into pCATbasic vector. A Neo-resistant gene was inserted into these vectors to give a selection marker. Stable cell lines harboring these reporter vectors were made by transfecting 3T3-L1 preadipocytes.

nomenon was observed in all of these short promoter vectors transfected stable cell lines. These results suggested that some important *cis*-elements might locate in the promoter region between −1819 to −372, which is important for sustaining AAAT expression in adipocyte. However, these potential *cis*-elements could only be functional in the presence of the proximal *cis*-element Ps1 (−68 to −26). Without this proximal *cis*-element, −1819ΔPs1 promoter, containing all the other *cis*-elements, was inactive in response to inducers' stimulation (Table 1).

From these CAT activity analysis, the border of minimum functional inducible promoter was determined in position between −91 and −55, since p-91CAT vector transfected cells exhibited markedly increased CAT activity after induction while p-55 CAT transfected cells did not. The inducible *cis*-element was most likely the Ps1 site, which is located in the position of −68 to −26 (Fig. 1A). The result from p-1819ΔPs1CAT (−1819 to +67 with deletion between −70 to −26) confirmed the role of Ps1 site in activating gene expression after differentiation induction. It also indicated that the presence of Ps1 site was required for sustaining the gene expression in terminally differentiated adipocyte, even though itself was not enough to maintain the gene expression in adipocyte.

To find the core sequence of *cis*-element within this Ps1 site, promoter-CAT vectors with fine deletions and site-directed mutations were constructed and stably transfected into 3T3-L1 preadipocytes (Fig. 3B); and their ability to induce CAT expression was tested. To clarify the CAT analysis results, all the CAT activities

TABLE 1

CAT Activity Induced by the AAAT Promoter in Stably Transfected 3T3-L1 Cells during the Differentiation Induction Process

	Day 0	Day 1	Day 2	Day 3	Day 4	Day 6
pCATbasic	1.2 ± 0.2	2.4 ± 0.5	3.8 ± 0.8	4.4 ± 0.4	4.0 ± 1.0	4.2 ± 0.6
p+1CAT	0.6 ± 0.2	1.3 ± 0.3	1.9 ± 0.4	0.8 ± 0.1	2.1 ± 0.5	1.0 ± 0.1
p-55CAT	0.8 ± 0.1	1.2 ± 0.2	1.6 ± 0.4	2.1 ± 0.4	3.6 ± 0.2	3.3 ± 0.3
p-91CAT	2.2 ± 0.2	42.7 ± 6.6	50.9 ± 8.1	29.8 ± 3.2	5.8 ± 0.9	4.5 ± 1.0
p-182CAT	6.5 ± 0.5	45.5 ± 5.0	47.8 ± 3.2	33.8 ± 4.5	7.3 ± 2.0	7.4 ± 2.0
p-372CAT	7.6 ± 0.3	51.7 ± 3.0	71.8 ± 10.6	51.8 ± 4.9	24.6 ± 2.1	13.2 ± 4.7
p-1819CAT	1.8 ± 0.2	2.9 ± 1.6	9.9 ± 2.0	9.4 ± 1.1	19.6 ± 2.0	54.6 ± 4.8
p-1819 ΔPs1CAT	1.2 ± 0.1	1.1 ± 0.2	2.2 ± 0.3	3.4 ± 0.2	0.6 ± 0.1	1.3 ± 0.2
AAAT mRNA[a]	0.16	0.14	1.40	2.10	2.90	2.40

Note. The CAT activity was expressed in picomoles per hour of acetyl-chloramphenicol formation. For each vector, three independent stable transfections were carried out and three stable cell lines were obtained. All three stable cell lines exhibited similar CAT activity expression patterns during differentiation induction. The results in this table were averages of three samples from one stable cell line.

[a] The amount of mRNA was expressed as relative density determined by scanning the Northern blot film with a densitometer.

from these mutated promoter vectors were subtracted by corresponding CAT activities from pCATbasic vector. As shown in Table 2, all constructs with promoters longer than −65 could induce the CAT expression during the differentiation induction. Shorter than that, the promoters were inactive, i.e., CAT activities remained at basal level during the entire differentiation induction process. In addition, CAT expression analysis of mutated promoters indicated that the two repeated sequences were functionally different. Substitution (M1) or deletion (M2) in the first repeat largely inactivated the promoter, while substitution (M3) or deletion (M4) in the second repeat had no effect on the promoter activity (Fig. 3B and Table 3). Based on these results, it was concluded that the sequence of DNA *cis*-element essential for the inducible promoter activity is around −65 to −46, TTCAAGTCCCGCCCTCCGCT.

TABLE 2

CAT Activity Induced by the Fine-Deleted AAAT Promoter in Stably Transfected 3T3-L1 Cells during the Differentiation Induction Process

	Day 0	Day 1	Day 2	Day 3	Day 4	Day 6
p-74CAT	0.4	14.1	25.6	23.8	12.6	7.0
p-69CAT	2.8	5.2	24.0	18.2	11.5	7.5
p-65CAT	0.6	18.5	24.5	16.4	3.2	2.8
p-63CAT	—[a]	0.8	1.0	0.4	0.2	—
p-62CAT	—	—	0.1	0.6	—	—

Note. The CAT activity was expressed in picomoles per hour of acetyl-chloramphenicol formation. CAT activity from the promoterless pCATbasic vector transfected cell was used as basal activity and subtracted from each CAT analysis sample. The results in this table were averages of two samples from one stable cell line. Transfections and CAT analysis were repeated once to confirm the observation.

[a] — indicates that the CAT activity from this cell line is less than that of pCATbasic control at the corresponding time point.

TRANSFAC database search reveals that this sequence contains two potential transcription factor binding sites: a sequence from −65 to −58, 5′-TTCAAGTC-3′, is almost the same to Nkx-2.5 binding motif, which is 5′-TTCAAGTG-3′; and a sequence from −60 to −51, 5′-GTCCCGCCCT-3′, is closely related to the Sp1 motif, 5′-GGGGCGGGGC-3′. Based on this information, mutations in these two sequences were made (Fig. 3B). M5 and M6 were mutations in Sp1-like sequence, while M7 was mutation in Nkx-2.5 recognition sequence. CAT expression from these mutated promoters clearly indicated that the Sp1-like sequence is the functional *cis*-element as two mutants of Sp1-like sequence (M5 and M6) both inactivated the promoter while mutant of Nkx-2.5 sequence (M7) had little effect on the promoter function (Table 3).

TABLE 3

CAT Activity Induced by the Mutated AAAT Promoter in Stably Transfected 3T3-L1 Cells during the Differentiation Induction Process

	Day 0	Day 1	Day 2	Day 3	Day 4	Day 6
wt	0.4	14.1	25.6	23.8	12.6	7.0
M1	—[a]	—	0.3	0.2	0.6	0.3
M2	—	0.3	—	0.5	0.4	0.3
M3	0.6	11.6	15.6	12.0	7.0	1.6
M4	1.0	3.0	23.0	17.2	9.6	2.0
M5	—	0.4	0.3	—	0.1	0.7
M6	0.1	0.3	0.2	—	0.5	—
M7	2.2	12.8	31.2	28.0	2.6	4.0

Note. The CAT activity was expressed in picomoles per hour of acetyl-chloramphenicol formation. CAT activity from the promoterless pCATbasic vector transfected cell was used as basal activity and subtracted from each CAT analysis sample. The results in this table were averages of two samples from one stable cell line. Transfections and CAT analysis were repeated once to confirm the observation.

[a] — indicates that the CAT activity from this cell line is less than that of pCATbasic control at the corresponding time point.

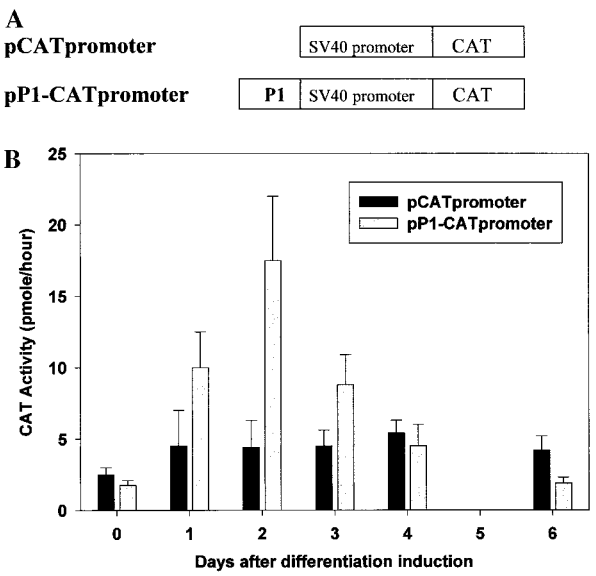


FIG. 4. CAT expression induced by P1 *cis*-element during 3T3-L1 cell differentiation process. (A) CAT reporter vectors. P1 oligonucleotide was inserted into pCATpromoter vector. A neo resistant gene was constructed into the vectors for selection. (B) Stable cell lines transfected with pCATpromoter or pP1-CAT promoter vector were obtained and subjected to differentiation induction protocol. The CAT expression during differentiation process was measured. The CAT activity is measured as picomoles per hour of acetylchloramphenicol formation.

CAT expression induced by P1 DNA element. All the results indicated that Sp1-like sequence was the essential *cis* DNA motif. An oligonucleotide named P1, corresponding to −65 to −46, was inserted into CAT promoter vector to test if P1 element was also sufficient for promoter function. Compared with pCATpromoter control vector, P1 sequence did not affect the CAT expression level before differentiation induction. It increased the CAT activity expression only after differentiation induction (Fig. 4). Similar to the previous CAT analysis with AAAT promoter constructs, the P1 sequence induced CAT activity was decreased in the differentiated 3T3-L1 adipocytes. Nevertheless, compared to pCATpromoter control, P1 element significantly increased CAT expression from the vector during early differentiation induction. Based on this result, P1 DNA element is at least partially sufficient for the AAAT promoter.

In summary, our present work has demonstrated that a Sp1-like *cis*-element is the important motif for AAAT gene differential expression. This Sp1-like motif is sufficient for AAAT promoter to induce gene expression during early differentiation induction. No DNA motif similar to recognition sequence by transcription factors belonging to C/EBP or PPAR families was found essential for AAAT promoter function. The present study suggested that Sp1 family transcription factors might be involved in the regulation of adipocyte specific gene expression during adipocyte differentiation induction.

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REFERENCES

1. MacDougald, O. M., and Lane, M. D. (1995) *Annu. Rev. Biochem.* **64,** 345–373.
2. Spiegelman, B. M., and Flier, J. S. (1996) *Cell* **87,** 377–389.
3. Smas, C. M., and Sul, H. S. (1995) *Biochem. J.* **309,** 697–710.
4. Gregoire, F. M., Smas, C. M., and Sul, H. S. (1998) *Physiol. Rev.* **78,** 783–809.
5. Cao, Z., Umek, R. M., and McKnight, S. L. (1991) *Genes Dev.* **5,** 1538–1552.
6. Tontonoz, P., Hu., E., Graves, R. A., Budavari, A. I., and Spiegelman, B. M. (1994) *Genes Dev.* **8,** 1224–1234.
7. Landschulz, W. H., Johnson, P. F., Adashi, E. Y., Graves, B. J., and McKnight, S. L. (1988) *Genes Dev.* **2,** 786–800.
8. Spiegelman, B. M., Frank, M., and Green, M. (1983) *J. Biol. Chem.* **258,** 10083–10098.
9. Bernlohr, D. A., Angus, C. W., Lane, M. D., Bolanowski, M. A., and Kelly, T. J., Jr. (1984) *Proc. Natl. Acad. Sci. USA* **81,** 5468–5472.
10. Christy, R. J., Yang, V. W., Ntambi, J. M., Geiman, D. E., Landschulz, W. H., Friedman, A. D., Nakabeppu, Y., Kelly, T. J., Jr., and Lane, M. D. (1989) *Genes Dev.* **3,** 1323–1335.
11. Ntambi, J. M., Buhrow, S. A., Kaestner, K. H., Christy, R. J., Sibley, E., Kelly, T. J., Jr., and Lane, M. D. (1988) *J. Biol. Chem.* **263,** 17291–17300.
12. Kaestner, K. H., Christy, R. J., and Lane, M. D. (1990) *Proc. Natl. Acad. Sci. USA* **87,** 251–255.
13. Zhang, Y., Proenca, R., Maffei, M., Barone, M., Leopold, L., and Friedman, J. M. (1994) *Nature* **372,** 425–432.
14. Hwang, C. S., Mandrup, S., MacDougald, O. M., Geiman, D. E., and Lane, M. D. (1996) *Proc. Natl. Acad. Sci. USA* **93,** 873–877.
15. Freytag, S. O., Paielli, D. L., and Gilbert, J. D. (1994) *Genes Dev.* **8,** 1654–1663.
16. Lin, F. T., and Lane, M. D. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 8757–8761.
17. Lin, F. T., and Lane, M. D. (1992) *Genes Dev.* **6,** 533–544.
18. Tontonoz, P., Hu, E., and Spiegelman, B. M. (1995) *Curr. Opin. Genet. Dev.* **5,** 571–576.
19. Tontonoz, P., Hu, E., and Spiegelman, B. M. (1994) *Cell* **79,** 1147–1156.
20. Liao, K., and Lane, M. D. (1995) *Biochem. Biophys. Res. Commun.* **208,** 1008–1015.
21. Reed, B. C., and Lane, M. D. (1980) *Proc. Natl. Acad. Sci. USA* **77,** 285–289.
22. Student, A. K., Hsu, R. Y., and Lane, M. D. (1980) *J. Biol. Chem.* **255,** 4745–4750.
23. Feinberg, A. P., and Vogelstein, B. (1983) *Anal. Biochem.* **132,** 6–13.
24. Chirgwin, J. M., Przybyla, A. E., MacDougald, R. J., and Rutter, W. J. (1979) *Biochemistry* **24,** 5294–5299.

25. Sanger, F., Nicklen, S., and Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. USA* **74,** 5463–5467.
26. Ronnett, G. V., Knutson, V. P., Kohanski, R. A., Simpson, T. L., and Lane, M. D. (1984) *J. Biol. Chem*. **259,** 4566–2575.
27. Sambrook, J., Fritsch, E. F., and Maniatis, T. Molecular Cloning, 2nd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
28. Graham, F. L., and Van der Ed, A. J. (1973) *Virology* **52,** 456–457.
29. Neumann, J. R., Morency, C. A., and Russian, K. O. (1987) *Biotechniques* **5,** 444–447.