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Cell-free transcription directed by the 422 adipose P2 gene promoter: Activation by the CCAAT/enhancer binding protein

(3T3-L1 preadipocyte/differentiation/cyclic AMP/stearoyl-CoA desaturase/glucose transporter)

DOMINIQUE CHENEVAL, ROBERT J. CHRISTY, DEBORAH GEIMAN, PETER CORNELIUS, AND M. DANIEL LANE

Department of Biological Chemistry, The Johns Hopkins University School of Medicine, Baltimore, MD 21205

Contributed by M. Daniel Lane, July 9, 1991

ABSTRACT **Previous investigations have shown that CCAAT/enhancer binding protein (C/EBP) can function as a trans-activator of the promoters of several adipocyte-specific genes—i.e., the 422 adipose P2 (422/aP2), stearoyl-CoA desaturase 1 (SCD1), and glucose transporter 4 (GLUT4) genes, in 3T3-L1 mouse preadipocytes. We now describe a cell-free system prepared from nuclei of 3T3-L1 cells that carries out transcription directed by these promoters. To measure transcript formation, we employed a polymerase chain reaction-assisted analysis. Nuclear extract from 3T3-L1 adipocytes that express C/EBP supports a higher rate of transcription of chimeric 422(aP2) promoter–chloramphenicol acetyltransferase (CAT) reporter gene constructs than nuclear extract from preadipocytes that lack C/EBP. A competitor oligonucleotide containing the C/EBP binding site sequence and antibodies raised against C/EBP inhibit transcription directed by the 422(aP2) promoter. The factor limiting transcription by nuclear extract from preadipocytes appears to be C/EBP, since recombinant C/EBP (rC/EBP) markedly activates transcription of the 422(aP2) promoter–CAT gene with preadipocyte extract but not with adipocyte extract. rC/EBP also activates cell-free transcription of SCD1 promoter–CAT and GLUT4 promoter–CAT chimeric genes. Point mutations within the C/EBP binding site in the 422(aP2) promoter markedly decrease transcription activated by rC/EBP. Consistent with activation by cAMP of the 422(aP2) promoter in intact preadipocytes, cAMP-dependent protein kinase activates transcription through this promoter with the cell-free system, this effect being independent of C/EBP. Thus, regulation of transcription directed by the 422(aP2) promoter in the cell-free system resembles that which occurs in intact 3T3-L1 cells.**

A large body of evidence indicates that differentiation of 3T3-L1 mouse preadipocytes in culture constitutes a faithful model of "adipose conversion" *in vivo* (1–3). During this process specific reprogramming of enzymatic and regulatory functions to those typical of adipocytes occurs through coordinated transcriptional activation of a family of adipose genes (2–5). The promoters of several of these genes have been analyzed (4–7), and their expression has been shown to be activated by agents used to induce adipose conversion (4, 8).

Several lines of evidence have implicated the CCAAT/enhancer binding protein (C/EBP) in the transcriptional activation of adipose-specific genes during the differentiation of 3T3-L1 preadipocytes. First, C/EBP was found to bind to the promoters of the 422 adipose P2 [422(aP2)], stearoyl-CoA desaturase 1 (SCD1), and glucose transporter 4 (GLUT4) genes (6, 9). Second, expression of the C/EBP gene is activated during preadipocyte differentiation just prior to the expression of the 422(aP2), SCD1, and GLUT4 genes (9, 10). Third, cotransfection into 3T3-L1 preadipocytes of a C/EBP expression vector with chimeric 422(aP2), SCD1, and GLUT4 promoter-chloramphenicol acetyltransferase (CAT) reporter genes results in trans-activation of the reporter gene (6, 9). Moreover, mutations in the C/EBP binding sites within the promoters of these genes disrupt trans-activation (9, 11).

To investigate the mechanism by which C/EBP activates expression through the promoters of adipocyte genes, a cell-free system was developed using extracts of nuclei from 3T3-L1 cells. With this cell-free system and polymerase chain reaction-assisted analysis to measure transcript formation, we show that purified recombinant C/EBP activates transcription through the promoters of the 422(aP2), SCD1, and GLUT4 genes.

EXPERIMENTAL PROCEDURES

Materials. The 1708-base-pair (bp) 422(aP2) promoter-CAT chimeric gene was obtained by subcloning the 5′ flanking region of a 4.3-kilobase (kb) 422(aP2) genomic clone (7, 8) in pSV0-CAT as described for the 858-bp 422(aP2) promoter–CAT and 248-bp 422(aP2) promoter–CAT constructs (8). The 363-bp SCD1 promoter–CAT and 785-bp GLUT4 promoter–CAT genes were prepared as described (4, 6). pHXTB, which contains 250 bp of the adenovirus major late promoter and 540 bp of the adenovirus type 2 (Ad2) gene sequence, was generously provided by J. Cordon (The Johns Hopkins University School of Medicine). Rabbit anti-C/EBP antibodies were raised against an internal peptide (amino acid residues 253–266) of C/EBP (12) and purified by passage over a staphylococcal protein A-Sepharose column.

Nuclear Extracts. 3T3-L1 preadipocytes were maintained and induced to differentiate into adipocytes as described (9, 13). Nuclear extracts were prepared by a modification of the protocol of Dignam (see refs. 14 and 15) from undifferentiated preadipocytes or from adipocytes 5 days after initiation of differentiation. The final protein concentration was 8–12 mg/ml.

Cell-Free Transcription. *In vitro* transcription was carried out by incubating 80 μg of nuclear extract; supercoiled DNA templates in a 5:1 ratio, experimental to Ad2 construct (1 μg:0.2 μg); ATP, GTP, CTP, and UTP at 0.6 mM each; 10 mM dithiothreitol; and 20 units of RNasin (Promega) in 40 μl of buffer D (14) for 60 min at 30°C. The reaction was terminated with 120 units of RNase-free DNase I (Boehringer Mannheim) and the mixture was incubated at 37°C for 30 min, after which 360 μl of a solution containing 50 mM Tris·HCl at pH 7.5, 1% SDS, 5 mM EDTA, and tRNA at 10 μg/ml was added. After extraction with phenol/chloroform, RNA was

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Abbreviations: C/EBP, CCAAT/enhancer binding protein; rC/EBP, recombinant C/EBP; 422(aP2), 422 adipose P2; SCD1, stearoyl-CoA desaturase 1; GLUT4, glucose transporter 4; CAT, chloramphenicol acetyltransferase; Ad2, adenovirus type 2; PKA, protein kinase A (cAMP-dependent protein kinase).

isolated by precipitation with ethanol. The washed pellet was then resuspended in 20 μl of reverse transcription buffer (10 mM Tris·HCl, pH 8.3/50 mM KCl/1.5 mM $MgCl_2$); dATP, dTTP, dCTP, and dGTP at 1 mM each; 0.3 unit of reverse transcriptase from avian myeloblastosis virus; and the downstream oligonucleotide primers, a 30-base oligonucleotide corresponding to the CAT gene sequence (16) ending at position +194 and a 21-base oligonucleotide corresponding to the Ad2 gene sequence (17) ending at position +170, and primer extension was allowed to occur. Samples were chilled and 80 μl of PCR buffer (10 mM Tris·HCl, pH 8.3/50 mM KCl/1.5 mM $MgCl_2$) containing 1.5 μCi of [α-^{32}P]dATP (3000 Ci/mmol; 1 Ci = 37 GBq) was added along with the upstream 21-base oligonucleotides starting at position +1 of the transcribed sequence for each gene. The PCR reaction was allowed to proceed for eight cycles (45 sec, 94°C; 45 sec, 55°C; 45 sec, 72°C) and the amplified products were analyzed on 6% acrylamide/8 M urea sequencing gels. The sizes of the PCR products were estimated by comparison with end-labeled *Hae* III-digested phage φX174 replicative form DNA standards and quantitated by laser densitometric scanning. Each experiment was repeated two–four times; the standard deviation varied ±12% from normalized values.

Recombinant C/EBP (rC/EBP). The construction of a nearly full-length bacterial expression vector for mouse C/EBP and the preparation of bacterial extracts are described elsewhere (18). The 6 M urea-containing extract of the final lysed cell pellet was dialyzed against 50 mM Tris·HCl, pH 8.0, containing 250 mM NaCl and loaded on a Whatman DE-52 anion-exchange column. The column was washed extensively with the same buffer and batch-eluted with 1 M NaCl. The eluate was dialyzed against 20 mM Tris·HCl, pH 8.0/250 mM NaCl and applied to a Mono Q FPLC column (Pharmacia). Fractions eluted with a linear NaCl gradient (0.25–1 M) were collected and assayed for C/EBP by Western blotting with anti-C/EBP antibodies, rC/EBP (≈42 kDa) being eluted as a single peak at about 0.5 M NaCl.

RESULTS AND DISCUSSION

PCR-Assisted Analysis of Cell-Free Transcription. To approximate the cellular context in which preadipocyte differentiation occurs, nuclei from 3T3-L1 cells (adipocytes or preadipocytes) were used to prepare extracts for *in vitro* transcription. Chimeric genes containing segments of the 5′ flanking regions and the transcription start sites of the 422(aP2), SCD1, and GLUT4 genes (4, 6–8) linked to the CAT reporter gene served as DNA templates for cell-free transcription. Because of the low transcriptional activity of the nuclear extracts, a PCR method was devised to measure transcript formation. After incubation of a chimeric gene with nuclear extract from fully differentiated 3T3-L1 adipocytes, RNA transcripts were isolated, reversed-transcribed, and amplified by PCR in the presence of [α-^{32}P]dATP, dTTP, dGTP, dCTP, and appropriate oligodeoxynucleotide primers. As illustrated in Fig. 1*A*, an amplified ^{32}P-labeled DNA product of the predicted size of 215 bp is generated. An internal standard consisting of 540 bp of the Ad2 gene driven by the Ad2 major late promoter, added to each transcription reaction, also gives rise to an amplified DNA product of the correct size (170 bp, Fig. 1*A*). Transcription is dependent upon the presence of both the chimeric gene template (Fig. 1*A*, lane 2) and nuclear extract (Fig. 1*A*, lane 3) and is

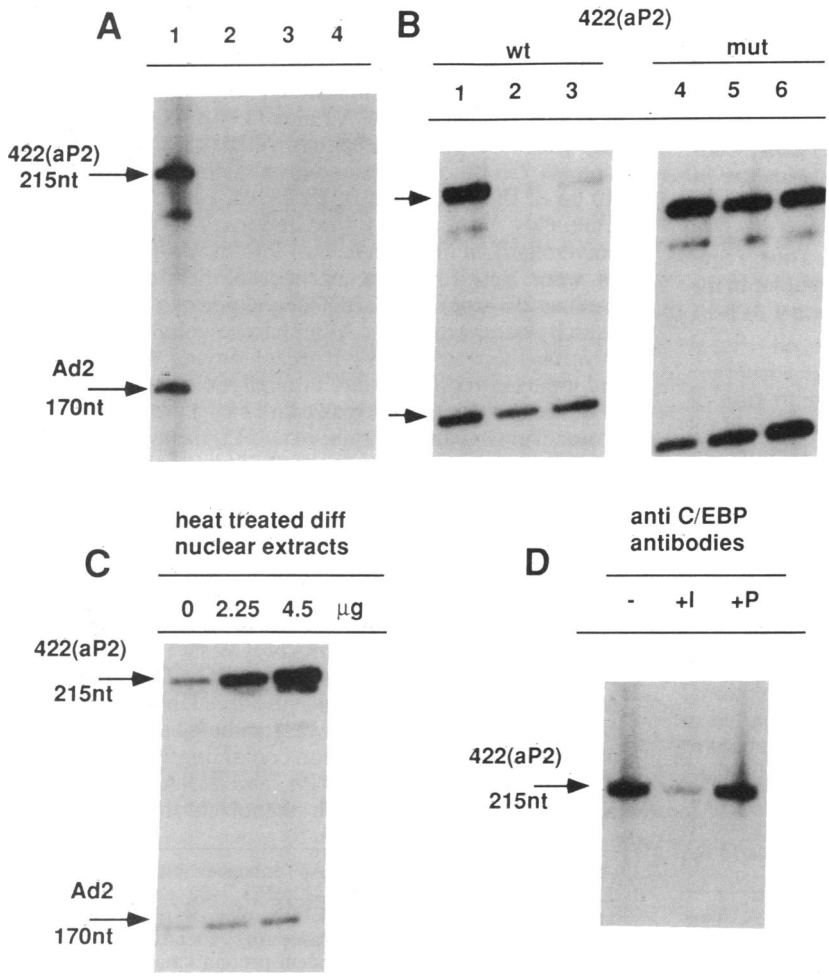

FIG. 1. Cell-free transcription of a chimeric 422(aP2) promoter–CAT gene. Cell-free transcription of a chimeric 1708-bp 422(aP2) promoter–CAT gene and an internal control gene (pHXTB, which contains the Ad2 major late promoter and gene) was conducted, using 80 μg of nuclear extract from fully differentiated 3T3-L1 adipocytes (in *A*, *B*, and *D*) or from undifferentiated 3T3-L1 preadipocytes (in *C*). PCR-assisted analysis of the isolated RNA transcripts and gel electrophoresis were performed as described in *Experimental Procedures*. (*A*) Lane 1, complete system; lane 2, gene templates deleted; lane 3, nuclear extract deleted; and lane 4, α-amanitin added at 2 μg/ml. (*B*) Lanes 1 and 4, complete system; lanes 2 and 3, a 50- and 100-fold molar excess, respectively, of a competitor oligonucleotide containing the wild-type (wt) C/EBP binding site (nucleotides −154 to −125) in the 422(aP2) promoter was added; lanes 5 and 6, a 50- and 100-fold excess, respectively, of an oligonucleotide containing a 5-base mutation (see text) in the C/EBP binding site of the 422(aP2) promoter was added. (*C*) Indicated amounts of heat-treated (95°C for 5 min, after which precipitated protein was removed) nuclear extract from fully differentiated 3T3-L1 adipocytes were added. (*D*) Ten micrograms of antibodies against a C-terminal C/EBP peptide (+I) or the preimmune antibody fraction (+P) was added. The positions of the [^{32}P]DNA PCR products of the 422(aP2)–CAT and pHXTB genes are indicated—i.e., 215 and 170 nucleotides (nt), respectively.

inhibited by a low concentration of α-amanitin (2 μg/ml), an inhibitor of RNA polymerase II (Fig. 1*A*, lane 4). Treatment of the reaction mixture with DNase I after transcription removes all traces of the promoter–CAT template, as no PCR-amplified product is detected in controls without nuclear extract. Possible formation of tandemly repetitive transcripts on the circular plasmid template without termination is ruled out, since replacement of the upstream primer by an oligonucleotide corresponding to nucleotides −50 to −29, just 5′ to the transcriptional start site, fails to generate a PCR-amplified product (results not shown).

To optimize conditions for measurement of the relative amounts of transcripts formed during the reaction, the dependence of the amount of [^{32}P]DNA amplification product upon the number of PCR cycles and the level of input RNA was assessed in experiments using nuclear extract from 3T3-L1 adipocytes and the 1708-bp 422(aP2) promoter–CAT construct as template. It was determined that the amount of [^{32}P]DNA product increased exponentially for approximately 15 PCR cycles (Fig. 2*A*). Eight cycles were used in all subsequent transcription assays, since under these conditions the amount of [^{32}P]DNA product is proportional to the input RNA transcript (Fig. 2*B*).

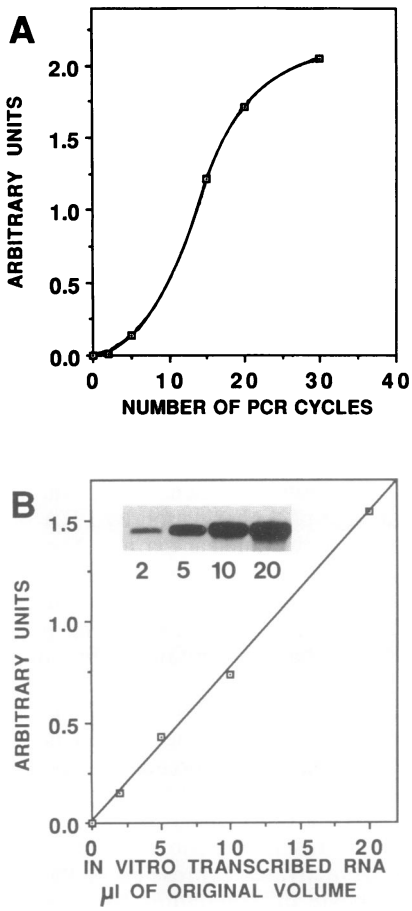

FIG. 2. PCR-assisted analysis of transcripts formed during cell-free transcription. Cell-free transcription of the 1708-bp 422(aP2) promoter–CAT gene was conducted with 80 μg of nuclear extract from 3T3-L1 adipocytes. RNA transcripts were isolated, reverse transcribed, and amplified by PCR using [α-^{32}P]dATP and unlabeled dTTP, dGTP, and dCTP, after which the ^{32}P-labeled DNA product was subjected to gel electrophoresis. (*A*) Number of PCR cycles was varied while using RNA isolated from the total (40 μl) cell-free transcription reaction mixture. (*B*) Eight PCR cycles were used to amplify RNA isolated from differing aliquot equivalents (in μl) of the cell-free transcription reaction mixture. The amount of amplified [^{32}P]DNA product (in arbitrary units) was determined by densitometric scanning of radioautograms (*Inset*).

Activation of Transcription by C/EBP. In a previous study it was shown that a nuclear factor expressed in differentiated 3T3-L1 adipocytes binds at a site within the promoter of the 422(aP2) gene between nucleotides −149 and −130 (9). As illustrated in Fig. 1*B*, an oligonucleotide containing the binding site sequence 5′-GATCCAAAGTTGAGAAATTTC-TATTAAAAA-3′ (nucleotides −154 to −125) blocks transcription driven by the 422(aP2) promoter with nuclear extracts from differentiated 3T3-L1 adipocytes. That this inhibition is specific is indicated by failure of a mutant oligonucleotide with a 5-base mutation in the C/EBP binding site 5′-GATCCAAAGTTGctcccTTTCTATTAAAAA-3′ to inhibit transcription (Fig. 1*B*).

Several lines of evidence suggested that the nuclear factor responsible for activation of cell-free transcription at this site might be C/EBP. Like C/EBP (19), the nuclear factor is heat stable. Thus, supplementation of nuclear extract from undifferentiated cells, which have no detectable C/EBP, with heat-treated nuclear extract from differentiated cells, which contains C/EBP (9), activates transcription (Fig. 1*C*). In addition, an antibody directed against a peptide corresponding to amino acid residues 253–266 (12) upstream of the DNA-binding domain in C/EBP inhibits cell-free transcription driven by the 422(aP2) promoter (Fig. 1*D*). This inhibition is prevented by prior exposure of the antibody to the peptide against which it was directed (results not shown).

Direct evidence that C/EBP *per se* activates cell-free transcription through the 422(aP2) promoter, as well as the promoters of two other differentiation-induced genes—i.e., the GLUT4 and the SCD1 genes (6, 9)—was derived from experiments using purified rC/EBP and nuclear extract from undifferentiated 3T3-L1 preadipocytes, which are deficient in this transcription factor (9, 10). Thus, by supplementation with rC/EBP, transcription through the promoters of all three genes* is markedly activated (Fig. 3*A*) and, as illustrated for the 422(aP2) promoter, this activation is concentration dependent (Fig. 3*B*). Furthermore, activation of the 422(aP2)–CAT gene by rC/EBP is blocked by an oligonucleotide containing the C/EBP binding site (nucleotides −154 to −125) of this promoter (Fig. 3*C*). It should be noted that transcription by nuclear extract from fully differentiated 3T3-L1 adipocytes, which express C/EBP, is not activated by rC/EBP (results not shown).

Consistent with these findings, site-specific mutations in the C/EBP binding site of the 422(aP2) promoter disrupt activation of transcription by rC/EBP with nuclear extract from undifferentiated cells. Mutation of one (mutant I-8) or two (mutant I-4) nucleotides within the C/EBP binding site markedly reduces transcription driven by this promoter (Table 1). Nuclear extract from differentiated 3T3-L1 adipocytes, which already contain a high level of endogenous C/EBP, supports a higher basal transcription rate than nuclear extract from undifferentiated preadipocytes (Table 1). The fact that mutations in the C/EBP binding site of the 422(aP2) promoter also reduce transcription by nuclear extracts from differentiated cells suggests that the factor present in these extracts and responsible for the higher rate of transcription is C/EBP.

Interpretation of these results, however, is complicated by two factors. First, homologues of C/EBP that possess similar DNA-binding domains are known to be present in the nuclear extracts of 3T3-L1 cells. It was recently shown that two other members of the C/EBP gene family, C/EBP-β [or LAP, liver activation protein (20)] and C/EBP-δ (21), bind at the same

*Similar results were also obtained with 848-bp 422(aP2) promoter–CAT and 248-bp 422(aP2) promoter–CAT constructs (results not shown).

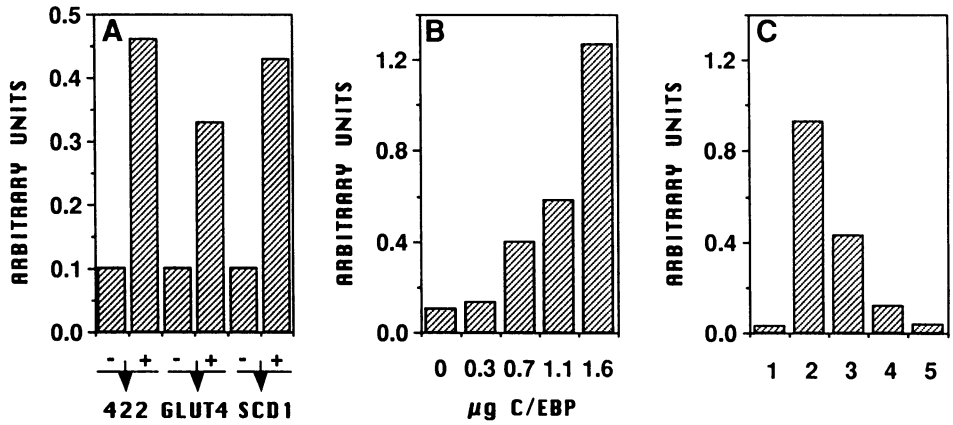

FIG. 3. Effect of rC/EBP on cell-free transcription of the chimeric 422(aP2) promoter–CAT, GLUT4 promoter–CAT, and SCD1 promoter–CAT genes. Cell-free transcription with nuclear extract from 3T3-L1 preadipocytes and PCR analysis of RNA transcripts was conducted as described in *Experimental Procedures*. (*A*) Chimeric 1708-bp 422(aP2)–CAT, 785-bp GLUT4–CAT, or 363-bp SCD1–CAT genes were used in the absence (−) or presence (+) of 0.8 μg of rC/EBP. (*B* and *C*) The 1708-bp 422(aP2) promoter–CAT gene was used as template. In *B*, the indicated amounts of rC/EBP were used. In *C*, 1.3 μg of rC/EBP was added in bars 2–5. A competitor oligonucleotide (containing the C/EBP binding site sequence as in Fig. 1) was added in 20- (bar 3), 50- (bar 4), and 100- (bar 5) fold molar excess.

site as C/EBP in the GLUT4 (K. H. Kaestner and M.D.L., unpublished results) and albumin (21) promoters and are expressed in the preadipocyte (ref. 21 and results not shown). Both C/EBP-β and C/EBP-δ possess homologous leucine zipper/basic dimerization domains and are capable of forming heterodimers with C/EBP (20, 21). It has been shown (ref. 21 and P.C. and M.D.L., unpublished results) that when differentiation is induced there is a transient rise in the expression of C/EBP-β and C/EBP-δ followed by a larger increase in the expression of C/EBP that persists in the fully differentiated 3T3-L1 adipocyte. It is possible, therefore, that a heterodimer(s) between C/EBP and one of its homologues activates expression of the 422(aP2) gene during preadipocyte differentiation. However, since the expression of the endogenous 422(aP2) gene is most closely correlated with the expression of C/EBP (9, 18), it is likely that C/EBP is the limiting factor whether hetero- or homodimers are involved.

Second, the 3′ end of the C/EBP binding site overlaps a negative regulatory element (Fig. 4) that has been implicated in repression of transcription of the 422(aP2) gene in the preadipocyte and derepression in the adipocyte (22). Both C/EBP and a nuclear protein(s) phosphorylated by cAMP-dependent protein kinase (PKA) appear to act, perhaps cooperatively, within this negative regulatory region (between nucleotides −149 and −122; see Fig. 4) in the 422(aP2) promoter. Thus, mutations in the C/EBP binding site might block activation by C/EBP and also promote derepression.

Table 1. Effect of mutations in the C/EBP binding site on transcription driven by the 422(aP2) promoter

Promotor	Sequence in the C/EBP binding site	Transcript formed, arbitrary units: Undiff. without rC/EBP	Undiff. with rC/EBP	Diff. without rC/EBP
	−143 −133			
wt	—TTGAGAAATTTCTAT—	0.07	0.63	0.97
I-8	—TcGAGAAATTTCTAT—	0.07	0.30	0.44
I-4	—TcGAGAgATTTCTAT—	0.07	0.14	0.43

Cell-free transcription was carried out as described in *Experimental Procedures*, using 858-bp 422(aP2) promoter–CAT constructs with or without site-specific mutations in the C/EBP binding site. Undiff., nuclear extract from undifferentiated 3T3-L1 cells supplemented or not with 1.2 μg of partially purified rC/EBP; Diff., nuclear extract from differentiated cells; wt, wild type.

Activation of Transcription by PKA. Previous studies in this laboratory have shown that cAMP, one of the cellular factors implicated in the induction of differentiation by 3T3-L1 preadipocytes, activates expression of both the endogenous 422(aP2) gene (D.G. and M.D.L., unpublished results) and a transfected reporter gene driven by the 422(aP2) promoter in 3T3-L1 preadipocytes (8, 22). The mechanism of this activation involves derepression through a negative regulatory element which, as illustrated in Fig. 4, overlaps the C/EBP binding site (22). Consistent with activation of the promoter by cAMP in the intact cell, preincubation of nuclear extract from undifferentiated 3T3-L1 preadipocytes with the catalytic subunit of PKA markedly activates cell-free transcription directed by the 422(aP2) promoter (Table 2). The specificity of this effect is shown by the fact that activation is blocked by an inhibitor (protein kinase inhibitor type II) of PKA added during the preincubation; moreover, transcription driven by the Ad2 major late promoter is not affected by PKA (results not shown). Hence, the activation mechanism appears to involve phosphorylation by PKA of a protein(s) present in preadipocyte nuclear extract. The possibility was considered that phosphorylation of the trace amount of endogenous C/EBP in the extract (results not shown) might be responsible for the activation. However, preincubation of rC/EBP with PKA had no additional effect on transcription beyond that of rC/EBP alone (Table 2). Moreover, incubation of rC/EBP with PKA and [γ-^{32}P]ATP does not give rise to a ^{32}P-labeled protein product, whereas incubation of nuclear extract from 3T3-L1 preadipocytes with PKA and [γ-^{32}P]ATP gives rise to several labeled proteins detectable by SDS/PAGE, none of which have a molecular mass similar to that of C/EBP (results not shown). Thus, the mechanism by which cAMP activates expression of the 422(aP2) gene during preadipocyte differentiation does not appear to involve PKA-catalyzed phosphorylation of C/EBP, but rather

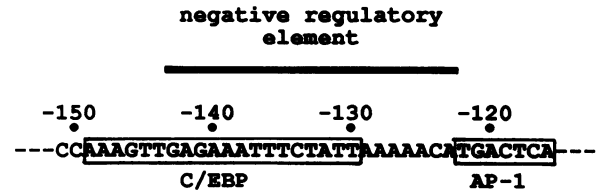

FIG. 4. Region of the 422(aP2) gene promoter containing a negative regulatory element and the binding sites for C/EBP and transcription factor AP-1.

Table 2. Effect of PKA and C/EBP on transcription driven by the 422(aP2) promoter

Preincubation	Cell-free transcription	Transcript formed, arbitrary units
NE	—	0.02
NE + Inhib. II	—	0.02
NE + PKA	Inhib. II	0.46
NE + PKA + Inhib. II	—	0.02
NE + rC/EBP	Inhib. II	0.68
rC/EBP + PKA	Inhib. II + NE	0.62
NE + PKA + Inhib. II	rC/EBP	0.45
NE + PKA	Inhib. II + rC/EBP	0.93

Preincubation was for 30 min at 30°C with ATP, GTP, CTP, and UTP at 600 μM each and 12 mM $MgCl_2$ in buffer D and the indicated additions. After preincubation cell-free transcription was carried out for 60 min at 30°C with the components of the standard assay including the 858-bp 422(aP2) promoter–CAT gene and the indicated additions. NE, nuclear extract (80 μg of protein) from 3T3-L1 preadipocytes; rC/EBP, 1.2 μg of rC/EBP; PKA, 60 units of catalytic subunit of the cAMP-dependent protein kinase; and Inhib. II, 50 μg of protein kinase inhibitor type II.

phosphorylation of another nuclear protein that interacts with the 422(aP2) promoter.

Graves *et al.* (23) recently identified an upstream enhancer in the 422(aP2) gene that is capable of directing adipose-specific expression in transgenic mice. Moreover, a transgene containing the enhancer, but lacking the segment of the 422(aP2) gene promoter in which the C/EBP binding site and the overlapping negative regulatory element (see Fig. 4) reside, retains its capacity to direct adipose-specific expression. The latter finding is consistent with the evidence (8, 22) that the segment of the gene between nucleotides −122 and −143 in the 422(aP2) promoter (Fig. 4) contains a negative regulatory (repressive) element and that cAMP and possibly C/EBP may act to derepress transcription through this element. We suggest that in the preadipocyte the negative regulatory element may override the effect of the adipose-specific enhancer until differentiation is induced, when the combined actions of cAMP and expression of C/EBP would act to release the promoter from negative control. We cannot rule out the possibility that upon differentiation of 3T3-L1 cells additional factors may be induced that activate the adipose-specific enhancer, although it was shown that proteins binding to the NF-1 site within the enhancer do not change upon differentiation (23).

The cell-free transcription system described in this paper will be useful in assessing these roles of C/EBP and of PKA in the repression and derepression phenomena.

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