Sp1 Mediates Glucose Activation of the Acetyl-CoA Carboxylase Promoter*

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Acetyl-CoA carboxylase (ACC), the rate-limiting enzyme in the biosynthesis of fatty acids, is induced in the presence of high glucose levels. The ACC gene contains two promoters: promoter I (PI) expression is inducible under lipogenic conditions, while promoter II (PII) expression, even though constitutively expressed in all tissues, is also controlled under various physiological conditions. Examination of the expression pattern of a series of deletion constructs of PII showed that the region from −340 to −249 was essential for ACC induction. In addition, by electrophoretic mobility shift assays, supershift assays, and DNase I footprinting studies, we have detected the binding of the transcription factor Sp1 at the two GC-rich sequences located within the −340 to −249 region of promoter II. Mutations at the GC-rich sequences prevented binding of Sp1, and the induction of the PII promoter was no longer observed. Cotransfection studies, in Drosophila Schneider SL2 cells, with the Sp1 expression vector and PII-CAT constructs, have further confirmed the activation of promoter II by Sp1. In addition, we have identified Sp3, another member of the Sp1 family of transcription factors, as a second factor that can bind to the glucose response elements of PII.

Long chain fatty acids are structural components of the cell as well as its energy reserves. In addition, they participate in various cellular and regulatory functions (1-5). Lipogenic tissues of eukaryotes utilize glucose as the primary precursor for the synthesis of these long chain fatty acids. Therefore, a high carbohydrate intake would result in the conversion of excess carbohydrate to triglycerides by the liver. This process is accompanied by the induction of many of the key enzymes of glycolysis, fatty acid synthesis, and those involved in triglyceride synthesis (6, 7). Some of this induction in response to glucose has been found to be at the transcriptional level, for example, the rate-limiting enzyme in the biosynthesis of fatty acids, is induced in the presence of high glucose levels. The ACC gene contains two promoters: promoter I (PI) expression is inducible under lipogenic conditions, while promoter II (PII) expression, even though constitutively expressed in all tissues, is also controlled under various physiological conditions. Examination of the expression pattern of a series of deletion constructs of PII showed that the region from −340 to −249 was essential for ACC induction. In addition, by electrophoretic mobility shift assays, supershift assays, and DNase I footprinting studies, we have detected the binding of the transcription factor Sp1 at the two GC-rich sequences located within the −340 to −249 region of promoter II. Mutations at the GC-rich sequences prevented binding of Sp1, and the induction of the PII promoter was no longer observed. Cotransfection studies, in Drosophila Schneider SL2 cells, with the Sp1 expression vector and PII-CAT constructs, have further confirmed the activation of promoter II by Sp1. In addition, we have identified Sp3, another member of the Sp1 family of transcription factors, as a second factor that can bind to the glucose response elements of PII.

EXPERIMENTAL PROCEDURES

Materials—Commercial products were obtained as follows: Eagle’s basal medium, RPMI 1640 medium, donor calf serum, fetal bovine serum were from Life Technologies, Inc.; dexamethasone and insulin were from Collaborative Research; poly(dI-dC), 3-isobutyl-1-methylxanthine, lactate, and glucose were from Sigma; restriction enzymes and T4 DNA kinase were from New England Biolabs; T4 DNA polymerase was from International Biotechnologies; Klenow fragment of DNA polymerase I, calf intestinal alkaline phosphatase, and DNase I were from Boehringer Mannheim; [14C]chloramphenicol (57 mCi/mmol) was from ICN; [γ-32P]ATP (1000 Ci/mmol) and [γ-32P]ATP (6000 Ci/mmol) were from Amersham; antibodies against c-Myc, Max, Sp1, and Sp3 were from Santa Cruz Biotechnologies. Antibodies against MLTF and LFA1 were generous gifts from Dr. Robert Roeder and Dr. Frances M. Sladek, respectively. The expression plasmid for human Sp1 was a generous gift of Dr. Robert Tjian.

Cell Culture, Transfection, and CAT Assay—Mouse 3T3A5 preadipocytes were cultured in 100-mm Petri dishes to about 90% confluence in Eagle’s basal medium supplemented with 10% donor calf serum. Stable transfectants were obtained by the calcium phosphate precipitation method as described (21). The Schneider line 2 (SL2) cells (22) were grown as described (23), and transfections were carried out by the calcium phosphate method as described (24). For the CAT assay, the cell pellets were sonicated in 150 μl of 0.25 M Tris-HCl (pH 8.0) for 20 s, and cell debris was removed by centrifugation. The supernatant was used for the assay of CAT activity (25). Each value represents the average of at least three determinations.

The glucose treatment was given as follows: day 0 cells, i.e. cells that had just reached confluence, were kept for 48 h in glucose-deficient media.
RPMI 1640 medium and supplemented with 10 mM lactate, 27.5 mM glucose, or 27.5 mM glucose and 10 µg/ml insulin. The day 2 cells were day 0 confluent cells treated with dexamethasone (1.0 µM) and IBMX (0.5 mM) for 48 h and then given 10 mM lactate, 27.5 mM glucose, or 27.5 mM glucose and 10 µg/ml insulin in RPMI 1640 medium for another 48 h. In the case of the day 4 cells, following day 2 an additional treatment of insulin was given for 48 h, and then the cells were kept in 10 mM lactate, 27.5 mM glucose, or 27.5 mM glucose and 10 µg/ml insulin in RPMI 1640 medium for another 48 h. At the end of each of the above three treatments, cells were harvested and assayed for CAT activity. All other experiments were carried out according to the day 2 treatment unless otherwise indicated.

Site-directed Mutagenesis—pPII-CAT2 was digested with EcoRI to remove an 800-bp fragment. This was cloned into the EcoRI site in M13 mp18 vector. The correct orientation was determined by nucleotide sequence analysis (26). Site-directed mutagenesis was carried out by the method described (27). The mutants were then recloned into the pPII-CAT2 in place of the original 800 bp.

Preparation of Nuclear Extracts—Nuclear extracts were prepared according to the method described by Dignan et al. (28). 30A5 cells were given the day 2 treatment, and nuclear extracts were prepared from control cells, i.e. lactate-treated cells, or glucose-treated cells. Extracts were divided into aliquots and stored at −80 °C. 2–3 mg of protein were obtained from 106 cells.

Mobility Shift DNA Binding Assay—Oligonucleotides used for the gel retardation assay are shown in Fig. 5. Double-stranded oligonucleotides were prepared by combining equal amounts of the complementary single-stranded DNA in a solution containing 10 mM MgCl2, heating to 80 °C for 5 min, and then slowly cooling to room temperature. The annealed oligonucleotides were end labeled with [γ-32P]ATP using T4 DNA poly(adenylate) kinase. Binding reactions were carried out in 20 µl containing 10 mM HEPES (pH 7.9), 75 mM KCl, 1 mM EDTA, 5 mM dithiothreitol, 5 mM MgCl2, 10% glycerol, 1 µg of poly(dI-dC). A typical reaction contained 20,000 cpm (0.5–1 ng) of end-labeled DNA with 2–5 µg of nuclear extract. The reaction mixture was incubated on ice for 30 min and then loaded onto a 5% non-denaturing polyacrylamide gel. Electrophoresis was carried out at 100 V for approximately 4 h in 25 mM Tris, 190 mM glycerine, and 1 mM EDTA, pH 8.5. For competition experiments, a molar excess amount of unlabeled DNA fragment was added to the binding mixture. For the supershift assay, 1–2 µl of antiserum was added to the reaction mixture and incubated for at least 1 h at 4 °C prior to adding the labeled probes. The gel was then fixed in 10% methanol, 10% acetic acid, dried, and autoradiographed.

DNase I Footprinting—DNase I footprinting was performed according to the method described by Dynan and Tjian (29) with the following modifications. Binding reactions were carried out as in the gel retardation assay, except the volume varied from 20 to 60 µl. An equal volume of 5 mM MgCl2 and 10 mM CaCl2 was added at the end of the reaction. The reaction mixture was then subjected to DNase I treatment at room temperature for 2 min. After terminating the reaction by the addition of the stop buffer, the samples were extracted and analyzed by an 8% polyacrylamide gel electrophoresis under denaturing conditions, and the gel was exposed to Kodak XAR-5 film for autoradiography.

RESULTS

Mapping of the Glucose-responsive Element in PII—The 1056-base pair fragment of the PII, 5'-flanking region to exon 2, was subcloned into the promoterless pUC-CAT3 plasmid, and progressive deletion mutants were generated as described previously (12). Diagrammatic presentation of these mutants is shown in Fig. 1. Stable clones of 30A5 preadipocytes containing these plasmids were generated, and the ability of PII to respond to glucose was determined by the measurement of CAT activity at three different stages of 30A5 adipocyte differentiation: day 0, day 2, and day 4, as described under “Experimental Procedures.”

As shown in Fig. 2, the expression of promoter II of the ACC gene is activated by glucose at the day 2 stage of differentiation. The response was lower at the day 4 stage, while the day 0 stage cells did not respond to glucose (data not shown). The addition of insulin along with the glucose treatment further increased the maximum response more than 4-fold for the same size deletion clones, although insulin alone had no effect. One possible explanation could be the role played by insulin in the recruitment of glucose transporters to the plasma membrane (30, 31). In a separate experiment, we confirmed that only PII, and not PI, responds to glucose under the same experimental conditions (data not shown).

Progressive deletions of PII, i.e. from pPII-CAT0 to pPII-CAT3 corresponding to −1056 up to −340 bp of promoter II, showed an increase in glucose response of up to 2.7-fold. No glucose effect was observed below pPII-CAT5. These results indicate that some sequence between the pPII-CAT3 and pPII-CAT5 regions of −249 to −340 is needed to respond to glucose. To determine if the presence of these sequences confers glucose responsiveness to a heterologous promoter, the regions between −434 and −285 and between −340 and −181 of promoter II were fused to the thymidine kinase promoter-CAT chimeric gene, and stably transfected 30A5 preadipocytes were generated; they were designated as TK-CAT1 and TK-CAT2, respectively (Fig. 3A). These clones were grown to the day 2 stage and then treated with lactate or glucose and assayed for CAT activity. While the TK-CAT and TK-CAT1 constructs were unable to respond to glucose, TK-CAT2 produced a similar response as seen by pPII-CAT3 (Fig. 3B). This result confirms that the 91-bp sequence between −249 and −340 is sufficient
to confer glucose responsiveness to the TK promoter and the PII promoter of the ACC gene.

Mutagenesis of the Glucose-responsive Region of the ACC Gene—To further define the sequence(s) involved in the glucose responsiveness of the PII promoter, site-directed mutagenesis was performed in the region between −340 and −181 in the PII region of pPII-CAT2. Seven block mutants were constructed as shown in Fig. 4A. Each mutation contained a block of 6–8 bp in which changes have been made from C to T, G to A, A to G, and T to C. The positions of mutations are underlined in Fig. 4A. Stable clones containing these mutant chimeric genes were obtained, and their chimeric gene expression in the presence of either 10 mM lactate or 27.5 mM glucose was determined. Mutants 1, 3, and 4 and double mutant 1,4 resulted in a loss of responsiveness to glucose (Fig. 4B). Mutations in 1 and 4 were at the region of GC-rich sequences in which two Sp1 consensus sequences are found. Thus, both sites appear to be essential for glucose induction of PII expression. On the other hand, mutant 3 did not show any basal promoter activity. These studies suggested the possible involvement of the Sp1 transcription factor in the glucose activation of PII.

Identification of the Nuclear Factor(s) Involved in the Glucose Response—Sequence analysis between −340 and −181 indicated the presence of two GC-rich regions, AGGCGGCGCGGTT (−329 to −317) and CGCGGGGCGGGGC (−254 to −242), the putative binding sites of the transcription factor Sp1 (14), and also two c-Myc-like regions (27, 28) with the imperfect core sequence, CACGTC instead of CACGTG located at −339 to −334 and −260 to −255, for the binding of the MLTF-like transcription factors. This latter sequence has already been implicated in the carbohydrate response of L-type pyruvate kinase (8) and S14 genes (9). To examine whether any of these nuclear factors bind to the sequence between −340 and −181, DNA mobility shift assays, supershift assays, and DNase I footprinting experiments were performed.

Oligonucleotides (Fig. 5) between the regions −341 and −282 (B-50-mer), between −342 and −313 (C-30-mer) and between −282 and −233 (E-50-mer) were synthesized. The B- and E-50-mers were chosen because they both contained one of the two Sp1- and MLTF-like sequences. The C-30-mer, which corresponds to the 5′-end 30 base pairs of the B-50-mer, was used to further define the binding pattern obtained by the B-50-mer. In addition, mutant oligonucleotides, which were either mutated in the Sp1- or MLTF-like sequence, were also used in the binding assays. DNA mobility shift assays were carried out using end-labeled oligonucleotides and nuclear extracts prepared from 30A5 preadipocytes.

The B-50-mer shifted the labeled DNA in three major protein-DNA complexes indicated by arrows as band 1 (B1), band 2 (B2), and band 3 (B3) in lanes 2 and 3 of Fig. 6. Exactly the same pattern was obtained with the E-50-mer (lanes 6 and 7, Fig. 6). When the C-30-mer was used as a probe, only two bands (Band 2 and Band 3) were observed (lanes 4 and 5, Fig. 6). The lower bands shown by an asterisk in Fig. 6 are due to nonspecific bindings. Nuclear extracts from the cells treated with 27.5 mM glucose showed an increase in the formation of the DNA-protein complexes following glucose treatment (lane 2 versus lane 3, lane 4 versus lane 5, and lane 6 versus lane 7, Fig. 6). Since the B- and E-50-mers show the same binding pattern, we
next tested to see if one probe could compete out the other. Labeled B-50-mer is competed out by increasing amounts of itself (lanes 3 and 4, Fig. 7B) and also by increasing amounts of unlabeled E-50-mer (lanes 7 and 8, Fig. 7B). A similar experiment was performed using the E-50-mer as the labeled probe and excesses of unlabeled B- and E-50-mers as competitors. The results were the same as those indicated by Fig. 7B (data not shown). Results based on the competition assays performed between the B- and E-50-mers indicate that not only is the binding pattern identical between the two probes but that the same factors appear to be binding to them. In addition, another oligonucleotide, the D-30-mer, was synthesized to study the binding pattern at the junction of the B- and E-50-mers. This was primarily done to understand why the mutant 3 stable construct did not show even basal CAT activity. This 30-bp probe, which includes the 15-bp sequence between these two 50-mers, did not exhibit the formation of any DNA-protein complex (data not shown).

We next tested mutant oligonucleotides both as probes and

![Image](55x519 to 301x732)

**FIG. 5.** DNA mobility shift competition assays. The 50-bp B- and E-50-mers were end labeled and used as probes in mobility shift assays. Excess of unlabeled B-50-mer, E-50-mer, C-30-mer, mutant C1–30-mer, mutant C2–30-mer, mutant C3–30-mer, and mutant C4–30-mer were used as competitors. Mutants C1 and C4 are mutated at the c-Myc-like sequences, while mutants C2 and C3 are mutated at the Sp1-like sequences of the B- and the E-50-mers. The sequences of the oligonucleotides have been compiled in a table form. The c-Myc-like sequences are double underlined, while the Sp1-like sequences are shadowed. The mutated base pairs in both of these regions are marked by dotted lines. The plus sign represents the oligonucleotides that can compete with the B- and the E-50-mers for binding to the nuclear factors, while the minus sign denotes the non-competitors.

![Image](87x183 to 268x394)

**FIG. 6.** DNA mobility shift assay with 30A5 cell nuclear extract. The B-50-mer (2341 to 2292), the C-30-mer (2342 to 2313), and the E-50-mer (2282 to 2233) of PII were end labeled and incubated with nuclear extracts from 30A5 cells grown in lactate- (lanes 2, 4, and 6) or glucose-containing medium (lanes 3, 5, and 7). Lane 1 shows the position of the labeled DNA alone. B1, B2, and B3 indicate shifted bands with the B- and E-50-mers. The asterisk indicates nonspecific binding.

![Image](321x308 to 548x732)

**FIG. 7.** DNA mobility shift assay with mutant oligonucleotides. A, the B-50-mer, C-30-mer, mutant C1–30-mer (mutated at the c-Myc-like sequence of the B-50-mer), and mutant C2–30-mer (Sp1-like sequence mutant) were end labeled and used as probes. Lanes 1, 2, and 3 correspond to the B-50-mer, lanes 4 and 5 to the C-30-mer, lanes 6 and 7 to mutant C1–30-mer, and lanes 8 and 9 to mutant C2–30-mer. Each probe was incubated with non-treated 30A5 cell nuclear extract (lanes 2, 4, 6, and 8) or with glucose-treated 30A5 cell nuclear extract (lanes 3, 5, 7, and 9). Lane 1 corresponds to the free probe. B, The 50-bp B-50-mer was end labeled and incubated with 30A5 nuclear extract. Lane 1 shows DNA alone and is labeled as F. Lane 2 has no added competitor. A 50- and 150-fold molar excess of unlabeled wild-type DNA (lanes 3 and 4) or mutant C2–30-mer DNA, mutated at the Sp1 site in the B-50-mer (lanes 5 and 6), E-50-mer (lanes 7 and 8), or mutant C3–30-mer, mutated at the Sp1 site in the E-50-mer (lanes 9 and 10), was added to the binding mixture in the mobility shift assay (data not shown). Results based on the competition assays performed between the B- and E-50-mers indicate that not only is the binding pattern identical between the two probes but that the same factors appear to be binding to them. In addition, another oligonucleotide, the D-30-mer, was synthesized to study the binding pattern at the junction of the B- and E-50-mers. This was primarily done to understand why the mutant 3 stable construct did not show even basal CAT activity. This 30-bp probe, which includes the 15-bp sequence between these two 50-mers, did not exhibit the formation of any DNA-protein complex (data not shown).
The nuclear extract was first incubated with labeled and added to the reaction mixture after the antibody reaction. Without the antibody (Fig. 7A), on the other hand, mutations in the c-Myc-like sequence (mutant C1–30-mer) did not have any effect on complex formation (lanes 6 and 7, Fig. 7A) and exhibited the same pattern formation as the C-30-mer (lanes 4 and 5, Fig. 7A). The same results were obtained with oligonucleotides mutated in the Sp1- (mutant C3–30-mer) or the c-Myc-like sequences of the E-50-mer (mutant C4–30-mer) (data not shown). Fig. 7B demonstrates the competition assay of the B-50-mer with the two Sp1 mutant oligonucleotides (mutants C2- and C3-30-mers). When the B-50-mer was end labeled, it was competed off with excess unlabeled B-50-mer (lanes 3 and 4, Fig. 7B) and E-50-mer (lanes 7 and 8, Fig. 7B), while it was not competed off with either mutant C2–30-mer (lanes 5 and 6, Fig. 7B) or mutant C3–30-mer (lanes 9 and 10, Fig. 7B). All the competition results with the wild-type and mutated DNA have been summarized in Fig. 5. Column 1 represents the labeled probes, i.e. the B- and the E-50-mers. The remaining columns from 2 to 7 denote the competitors, i.e. excess of cold B-50-mer, E-50-mer, C-30-mer, and mutants C1-, C2-, C3-, and C4-30-mers. Thus, the similarity of the competition pattern between the B- and the E-50-mers and the inability of the GC-rich region mutants to bind to any factors suggested that the transcription factor Sp1, which is known to bind to GGCGCG-like sequences called GC boxes, could be binding to the GC-rich sequences of promoter II of the ACC gene.

Supershift assays were performed using antibodies against Sp1, MLTF, c-Myc, and also LFA1, which is involved in the glucose response along with MLTF of the L-type pyruvate kinase gene (8). While the antibody against Sp1 was able to shift band 2 in the E-50-mer, the antibodies to c-Myc, MLTF, or LFA1 failed to recognize the factors binding to the E-50-mer (lane 9 versus lanes 3, 5, and 7, Fig. 8A). When the B-50-mer was used as a probe for the supershift assay, the same pattern of DNA band shift was observed (lanes 4 and 5, Fig. 8B). In contrast to the B- or E-50-mers, the Sp1-consensus sequence and C-30-mer generated one band that corresponds to band 2, and most of this band was supershifted by anti-Sp1 as shown in lanes 3 and 7 of Fig. 8B. Purified Sp1 also bound to both the B- and E-50-mers and generated one complex that migrated to the position of band 2 (lane 8, Fig. 8B) and was supershifted by anti-Sp1 (lane 9, Fig. 8B). These results indicate that Sp1 is one of the factors binding to the two GC-rich sequences within −340 to −181 of the PII sequence, which is involved in the glucose activation of the PII promoter. Mutations at either one of the GC boxes eliminated all DNA-protein interactions with the B- and E-50-mers. Hence, it is possible that the band 1 generated by these two oligonucleotides was another member of the Sp1 family of transcription factors. Supershift assays alone and is denoted as F. B, mobility shift assays were performed with (lanes 3, 5, 7, and 9) and without (lanes 1, 2, 4, 6, and 8) anti-Sp1. Labeled B-50-mer (lanes 4, 5, 8, and 9), C-30-mer (lanes 6 and 7), and the consensus Sp1-21-mer (lanes 1–3) were used as probes in the binding assay. The B-50-mer was incubated with 50 ng of purified Sp1 as shown in lane 8 and 9 of the supershift assay. Lane 1 corresponds to DNA alone and is denoted as F, C, the E-50-mer was end labeled and used as a probe in the supershift assay. The probe was added to the reaction mixture after the antibody reaction. The nuclear extract was first incubated with anti-Sp1 (lane 3) or anti-Sp3 (lane 4) antibodies. Lane 1 represents free probe, while lane 2 has only nuclear extract and no antibody.

![Fig. 8. DNA mobility supershift assay. A, the E-50-mer was end labeled and added to the reaction mixture after the antibody reaction. The nuclear extract was first incubated with (lanes 3, 5, 7, and 9) and without (lanes 1, 2, 4, 6, and 8) anti-c-Myc (lane 3), anti-MLTF (lane 5), anti-LFA1 (lane 7), and anti-Sp1 (lane 9) antibodies. Lane 1 has DNA as competitors in DNA mobility shift assays. When the mutant oligonucleotide in the Sp1-like sequence in the B-50-mer (mutant C2-30-mer) was end labeled and incubated with non-treated and glucose-treated nuclear extracts, formation of the three specific complexes, band 1, band 2, and band 3, was eliminated, suggesting that this region is essential for the binding of the nuclear factors to the DNA (lanes 8 and 9, Fig. 7A). On the other hand, mutations in the c-Myc-like sequence (mutant C1–30-mer) did not have any effect on complex formation (lanes 6 and 7, Fig. 7A) and exhibited the same pattern formation as the C-30-mer (lanes 4 and 5, Fig. 7A). The same results were obtained with oligonucleotides mutated in the Sp1- (mutant C3–30-mer) or the c-Myc-like sequences of the E-50-mer (mutant C4–30-mer) (data not shown). Fig. 7B demonstrates the competition assay of the B-50-mer with the two Sp1 mutant oligonucleotides (mutants C2- and C3-30-mers). When the B-50-mer was end labeled, it was competed off with excess unlabeled B-50-mer (lanes 3 and 4, Fig. 7B) and E-50-mer (lanes 7 and 8, Fig. 7B), while it was not competed off with either mutant C2–30-mer (lanes 5 and 6, Fig. 7B) or mutant C3–30-mer (lanes 9 and 10, Fig. 7B). All the competition results with the wild-type and mutated DNA have been summarized in Fig. 5. Column 1 represents the labeled probes, i.e. the B- and the E-50-mers. The remaining columns from 2 to 7 denote the competitors, i.e. excess of cold B-50-mer, E-50-mer, C-30-mer, and mutants C1-, C2-, C3-, and C4-30-mers. Thus, the similarity of the competition pattern between the B- and the E-50-mers and the inability of the GC-rich region mutants to bind to any factors suggested that the transcription factor Sp1, which is known to bind to GGCGCG-like sequences called GC boxes, could be binding to the GC-rich sequences of promoter II of the ACC gene.

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indicated that while band 2 was supershifted by the antibody against Sp1 (lane 3 versus lane 2, Fig. 8C), the antibody against Sp3 was able to bind to the protein in band 1 (lane 4 versus lane 2, Fig. 8C). Hence, Sp3 has now been identified as the other factor that can bind to the glucose-responsive region of PII.

DNase I Footprinting Studies between −340 and −181 of the PII Promoter—To establish if Sp1 binds to both sites in the B- and E-50-mer regions, we carried out DNase I footprinting analysis. The probes (between −340 and −181 of PII) were labeled with 32P at one end of either the coding (probe 1) or non-coding (probe 2) strands. In the presence of Sp1, two regions of probe 2 were protected in a Sp1 concentration-dependent manner, while no such protection occurred in the presence of Bovine serum albumin, which does not bind to the DNA (lanes 7 and 8 versus lanes 9 and 10, Fig. 9). The protected regions of probe 2 included the Sp1 binding sequence in both the B- and E-50-mers. However, with probe 1 we were unable to detect very clear protection with purified Sp1 (lanes 2 and 3, Fig. 9). This is probably because contact between Sp1 and DNA has so far been shown to be at the major groove of only one strand of DNA (32).

By using nuclear extracts from non-treated and glucose-treated cells, we determined that no additional proteins, other than Sp1, bound to the sequence between −340 and −181 of PII. In addition, the protection with the glucose-treated nuclear extract was observed to be stronger than that with the non-treated extract (data not shown). These data agree with the increase in binding observed in mobility shift assays and indicated that glucose treatment increased complex formation between Sp1 and the DNA.

Regulation of the PII Promoter of the ACC Gene by Sp1—The above experimental results show that Sp1 binds to the GC-rich sequences of the B- and E-50-mers of the PII promoter, and such binding activities increased when the cells were treated with glucose. To determine directly if the binding of Sp1 to these sequences is involved in PII activation, Drosophila Schneider SL2 cells were cotransfected with pPII-CAT constructs and the Sp1 expression vector. Drosophila SL2 cells are devoid of endogenous Sp1 (33), and thus allow the effect of varying the amount of Sp1 expression vector to manifest on promoter activity as a function of the intracellular concentration of Sp1. pPII-CAT chimeric constructs were cotransfected with a Drosophila aldehyde dehydrogenase promoter-Sp1 expression vector, and the level of CAT activity was determined (Fig. 10). pSV2CAT, a chimeric construct of the SV40 promoter and the CAT gene, with functional Sp1 binding sites, was used as a positive control in the transfection studies while pX188CAT, which lacks GC-rich Sp1 binding sequences, served as the negative control. The activities of pP1II-CAT2 and pP1II-CAT3, which contain −434 and −340 bp of PII, were induced approximately 3-fold in the presence of the Sp1 expression vector, while pP1II-CAT5, without the two Sp1 sequences between −340 and −249 bp of PII, was not induced at all. This finding suggests that both of the Sp1 consensus sequences located between 340 and 249 of the ACC promoter II can bind Sp1 and that the bound Sp1 activates transcription. Interestingly, mutants at either one of the two Sp1 binding regions were still able to activate the pP1II-CAT2 construct, while the double mutant did not respond to the overexpression of Sp1. This indicates that both the GC-rich sequences between −340 and −249 of PII are functional sites, and overexpression of Sp1 can still induce promoter activity from one site even if the other one is mutated.

**DISCUSSION**

Promoter II of the ACC gene has no CCAAT or TATA box and has the structural features of a housekeeping gene. Even though the ACC gene products of PII are constitutively expressed in all tissues, they increase together with those of promoter I under lipogenic conditions (18, 19).

In 30A5 cells, PII responsiveness to glucose was acquired during the hormone-induced differentiation of the cells as shown in this report. This acquisition of competence for PII to respond to glucose required the participation of Sp1. The region of promoter II responsible for conferring glucose-mediated transcription contains two Sp1 binding sites and also two imperfect CACGTG c-Myc-like binding sequences. In contrast to L-type pyruvate kinase and S14 (15, 16), it was not the c-Myc-

![Fig. 9. DNase I footprinting of PII (−340 to −181) by Sp1. PII fragment −340 to −181 subcloned into pTK-CAT was digested with either EcoRI or HindIII and labeled with 32P to generate either labeled coding or non-coding strands. Probe 1 is the coding strand while probe 2 is the non-coding strand used in the footprinting reactions. The DNA was incubated with 100 ng (lanes 2 and 7) and 200 ng (lanes 3 and 8) of Sp1 or 100 ng (lanes 4 and 9) and 200 ng (lanes 5 and 10) of bovine serum albumin. Lanes 1 and 6 represent the free probe DNA. The mixtures were subjected to 0.00625 units of DNase I for 2 min at room temperature.](https://example.com/figure9.png)
like sequence that is involved in the glucose responsiveness of the ACC gene but the GC-rich consensus sequence for the Sp1 transcription factor.

For this involvement of Sp1, we have presented several lines of evidence. First, we have localized the glucose response element to the region between −340 and −249 by using various deletion mutants of PII. These 91 base pairs are necessary for the glucose-stimulated expression of the ACC gene and are sufficient to confer glucose responsiveness to a heterologous promoter. In addition, by mutational studies along the sequence, we have further narrowed down the elements to the two GC-rich sequences. Mutations at each of these two sequences abolish the stimulation of promoter activity that occurs in cultured 30A5 cells in the presence of high glucose concentrations.

The nature of the glucose response elements was further confirmed by DNase I footprinting analyses, which indicated that only the two regions protected by purified Sp1 were preserved. Each of them is able to recognize the GC box with identical affinities, indicating that their DNA binding domains are highly conserved. Besides, all three proteins contain glutamine and serine/threonine-rich amino terminus domains of which the glutamine-rich sequence has been identified as the transactivation domain. The glutamine-rich domain of Sp1 is necessary for synergistic activation (32). Thus, it is highly possible that another member of the Sp1 family of transcription factors can compete for binding to the two GC-rich sequences in the B- and E-50-mers of the ACC promoter II. This would also explain why only the GC-rich sequences between −340 and −249 of PII are protected in DNase I footprinting assays. Supershift assays have indicated that the other factor involved in the glucose-mediated response of the PII promoter of the ACC gene is Sp3. Further studies need to be carried out to determine how these two members of the Sp1 gene family are involved in mediating glucose action.

Using the Drosophila cell-line SL2, we have shown that Sp1, when cotransfected with p11-CAT2, is able to activate transcription in a concentration-dependent manner. However, it still needs to be seen if Sp3 can function in a similar way.

Competition gel shift assays and supershift assays have confirmed that one of the nuclear factors induced is Sp1. Sp1 has been shown to bind to GGGCGG hexanucleotide GC boxes, and multiple binding sites appear to be a common feature of promoters that are Sp1 responsive (33, 36–39). Purified Sp1 protein bound and comigrated to the position of band 2 in gel shift assays. Introducing a 6-bp mutation in the GC-rich sequence of the oligonucleotide abolished binding almost completely. On the other hand, mutations at either of the two c-Myc-like sequences, i.e., CACGTC, did not have any effect on the binding of factors to the B- or E-50-mers. This would mean that even though these sequences are present in the promoter, the glucose activation of PII is not mediated by a member of the MLTF family of transcription factors (15, 16) but by a member of the Sp1 family of transcription factors.

Two human factors homologous to Sp1 have been cloned and designated Sp3 and Sp4 (38, 39). As members of a gene family, Sp1, Sp3, and Sp4 show similar structural characteristics. Each of them is able to recognize the GC box with identical affinities, indicating that their DNA binding domains are highly conserved. Besides, all three proteins contain glutamine and serine/threonine-rich amino terminus domains of which the glutamine-rich sequence has been identified as the transactivation domain. The glutamine-rich domain of Sp1 is necessary for synergistic activation (32). Thus, it is highly possible that another member of the Sp1 family of transcription factors can compete for binding to the two GC-rich sequences in the B- and E-50-mers of the ACC promoter II. This would also explain why only the GC-rich sequences between −340 and −249 of PII are protected in DNase I footprinting assays. Supershift assays have indicated that the other factor involved in the glucose-mediated response of the PII promoter of the ACC gene is Sp3. Further studies need to be carried out to determine how these two members of the Sp1 gene family are involved in mediating glucose action.

Since Sp1 is an ubiquitously expressed transcription factor, an immediate question arises as to if all the genes that contain Sp1 binding site(s) are responsive to glucose. The following examples illustrate the complexity of Sp1 function; however, its responsiveness to glucose may be gene specific. It has been observed that Sp1 does not bind to all the consensus sequences in a given promoter, but the binding is selective in the activation of the gene. A good example of this is the TK-CAT fusion construct that we used to study glucose responsiveness in a heterologous promoter. This construct was unaffected by glucose despite the two Sp1 binding sites present in the sequence. In addition, it is known that different activation domains of Sp1 molecules are responsible for different functions (41). Both the interactions between Sp1 molecules at different sites on genes as well as interactions of Sp1 with other nuclear factors (42, 43) indicate that Sp1 functions differently, i.e. in a gene-specific manner. Glucose activation of PII involves increased

Fig. 10. The effect of overexpression of Sp1 on pII-CAT constructs and mutant constructs in Schneider cells. Different pII-CAT constructs were cotransfected with the Sp1 expression vector (pADHSp1) or the empty vector (pADHR1). pSV2CAT was used as the positive control and pX188CAT as the negative control for responsiveness to Sp1. The cells were harvested after 24 h and assayed for CAT activity. Each value represents the mean ± standard deviation calculated from three experiments.

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binding of Sp1 to the promoter, which itself is a novel mechanism of Sp1 function. Recently, v-Raf was reported to activate the murine rep-3b promoter through the modification of the bound Sp1 without affecting Sp1 binding to the promoter (43). These latter examples show that modification of Sp1 may lead to specific gene activation without involving the interaction of Sp1 with other proteins. The presence of a modifying factor such as v-Raf in the specific nucleus and its responsiveness to such agents as glucose would determine, for example, whether or not a particular gene is going to respond to glucose.

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