An enhancer element in the house-keeping promoter for acetyl-CoA carboxylase gene

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ABSTRACT

The gene for acetyl-CoA carboxylase, the rate-limiting enzyme in the biogenesis of long chain fatty acids, contains two promoter regions which control the generation of different forms of carboxylase mRNA. At least five different forms of carboxylase mRNA are generated by differential splicing of the two transcripts formed under the influence of two promoters. One of the two promoters is mainly responsible for the generation of a class of carboxylase mRNA species, pAU type, induced tissue specifically under lipogenic conditions (8); the other generates ACC mRNAs (FL-type) which are expressed under normal conditions but this expression is also stimulated under lipogenic conditions (8). In the present studies, we have characterized the promoter that is responsible for the FL-type of ACC mRNA. This promoter contains no TATA or CAAT boxes, but five G/C motifs whose sequences are typical of transcriptional factor Sp1 binding sites. However, the presence of these G/C motifs is not sufficient to drive the transcription of the gene under the control of this promoter. Expression of promoter activity requires three copies of 11 to 13mer enhancer elements which are located in the region upstream to the G/C motifs. The presence of such enhancer elements in a house-keeping gene is unusual, and provides a new example where an enhancer element occurs in the CpG island-type promoter of a house-keeping gene.

INTRODUCTION

Long chain fatty acids are basic components of practically all subcellular entities as well as cellular energy reserves. The requirements for fatty acids in different subcellular entities differ, depending on the turnover rate of specific cellular components. Thus, the regulation of acetyl-CoA carboxylase (ACC), the rate-limiting enzyme in the biosynthetic pathway leading to long chain fatty acids, is very complex (1−3). Studies in our laboratory have demonstrated that long term regulation of ACC *in vivo* (4,5) and in various *in vitro* culture systems (6,7) is accomplished by changes in ACC mRNA metabolism. During the conversion of 30A-5 preadipocytes to adipocytes, the amount of ACC mRNA increases markedly as a result of increases in the rate of transcription (7). Furthermore, the repression of ACC by tumor necrosis factor during preadipocyte differentiation is due to the inhibition of the rate of transcription of the ACC gene which, in turn, diminishes the accumulation of ACC mRNA (7). Similar relationships between ACC mRNA metabolism and ACC activity hold in whole animals under different lipogenic conditions (4,5).

Recently, we established the existence of multiple forms of ACC mRNA (8,9). Our characterization of these ACC mRNAs indicated that the 5′ non-translated regions of the mRNA are diverse. The 5′-untranslated region of the ACC gene revealed that different ACC mRNA species are produced by alternative exon splicing and the presence of two spatially distinct promoters (10). We have designated these as promoter 1 (P1) and promoter 2 (P2) (10). Transcription promoted by P1 generates liver and epididymal fat tissue specific pAU-type ACC mRNA species (8), whereas promoter P2 transcripts are found in all the tissues that have been examined so far (FL-type) (8,9).

In the present studies, we have characterized P2. P2 lacks TATA and CAAT boxes, has a high GC content, and has multiple transcription initiation sites (9). These properties are frequently found in 'housekeeping' genes (17−21). The expression of this promoter is under the control of a novel enhancer element which is located about 200 bp upstream from the transcription initiation site. Three symmetrical sequences (11 bp, 13 bp, and 13 bp) comprise the core structure of the enhancer element. Expression of ACC P2 is very low in the absence of the enhancer element. The expression of thymidine kinase (TK) is stimulated about 5-fold when the enhancer element is placed in the TK gene.

MATERIALS AND METHODS

Materials

Enzymes and chemicals were purchased from the following suppliers: Exonuclease III, Klenow fragment, calf intestinal alkaline phosphatase, and dideoxynucleoside triphosphates from Boehringer Mannheim; T4 DNA ligase and restriction endonucleases from Bethesda Research laboratories; S1 nuclease and deoxynucleoside triphosphates from Pharmacia; [γ-^{35}S]-dATP from Amersham; ^{14}C labeled chloramphenicol from New England Nuclear. The chloramphenicol acetyl transferase gene, bearing plasmid pUC-CAT3 (11) was obtained from Dr. J. E. Dixon; pTK-CAT which contains the −109 through +56 sequence of the herpes simplex virus thymidine

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kinase (TK) gene fused to the CAT gene (12) and pRSV-LacZ (13) was obtained from Dr. S. F. Konieczny.

Construction of expression plasmids and generation of 5'-deletion mutants

The construction of plasmid P2-CAT has been described (10). This plasmid contains a HindIII/EspI restriction fragment (1056 bp long) that encompasses the first 62 bases of the first exon of the FL-type of ACC transcript and the corresponding 5' flanking sequences (9). The 1056 bp fragment was inserted in front of the chloramphenicol acetyl transferase gene of pUC-CAT3 (Fig. 1). This construct is designated pP2-CAT O. To create 5'-deletion mutants, pP2-CAT O was linearized by Sma I, and the linearized plasmid (10 μg) was treated with exonuclease III (25 U) in a total volume of 100 μl as follows. Aliquots (12 μl) were withdrawn at 1, 2, 3, 4, 5, and 6 min intervals and frozen on dry ice. Sixty μl of S1 nuclease buffer (14), containing S1 nuclease (15 U), was added to each aliquot. The mixture was incubated at 30°C for 30 minutes and the reaction was stopped by the addition of 5 μl of 1 M Tris HCl (pH 7.5). dNTPs (1 μl, 2.5 mM) and Klenow enzyme (0.5 U) were added to each reaction mixture and incubated for another 15 minutes at room temperature. The mixture was then heated at 68°C for 20 minutes, extracted with phenol/chloroform, and DNA was precipitated with ethanol. The DNA pellets from each sample were resuspended in water (20 μl) and digested with Bam HI (10 U) in a total volume of 30 μl. When digestion was complete, DNA fragments were subjected to fractionation on a 1.8% agarose gel. Inserts of different sizes were recovered from the gel by electrophoresis with the use of an IBI elecroeluter. The recovered inserts were then ligated into pUC-CAT3 which had been double digested with SmaI and BamHI. Exact deletion points were determined by nucleotide sequencing.

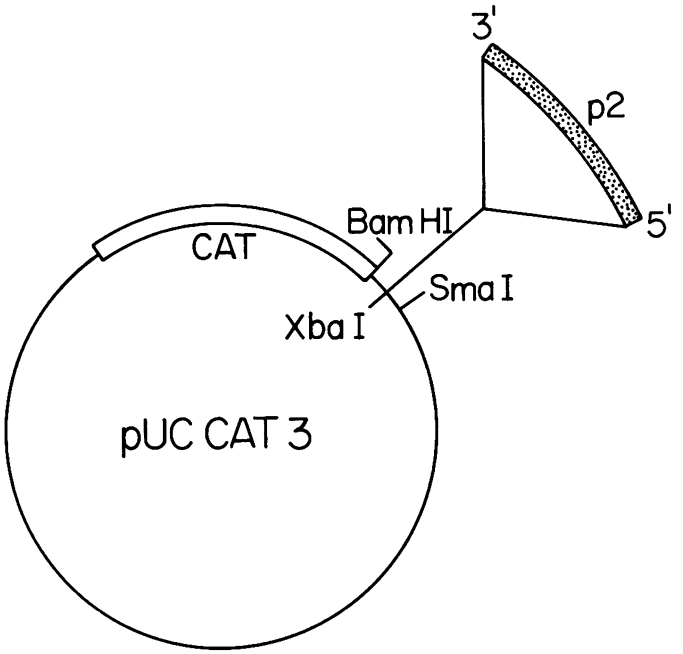

Fig. 1. Construction of P2-CAT. The ACC P2 was inserted in the XbaI site of pUC CAT3. The SmaI site was used to linearize the plasmid, and create the deletion mutants.

Construction of pP2-TK CAT fusion genes

Three P2 fragments of 68 bp (−249 to −181), 86 bp (−249 to −163), and 159 bp (−340 to −181) were inserted in front of the thymidine kinase (TK) promoter of pTK-CAT. The resulting plasmids were designated p68-TK, p86-TK, and p159-TK.

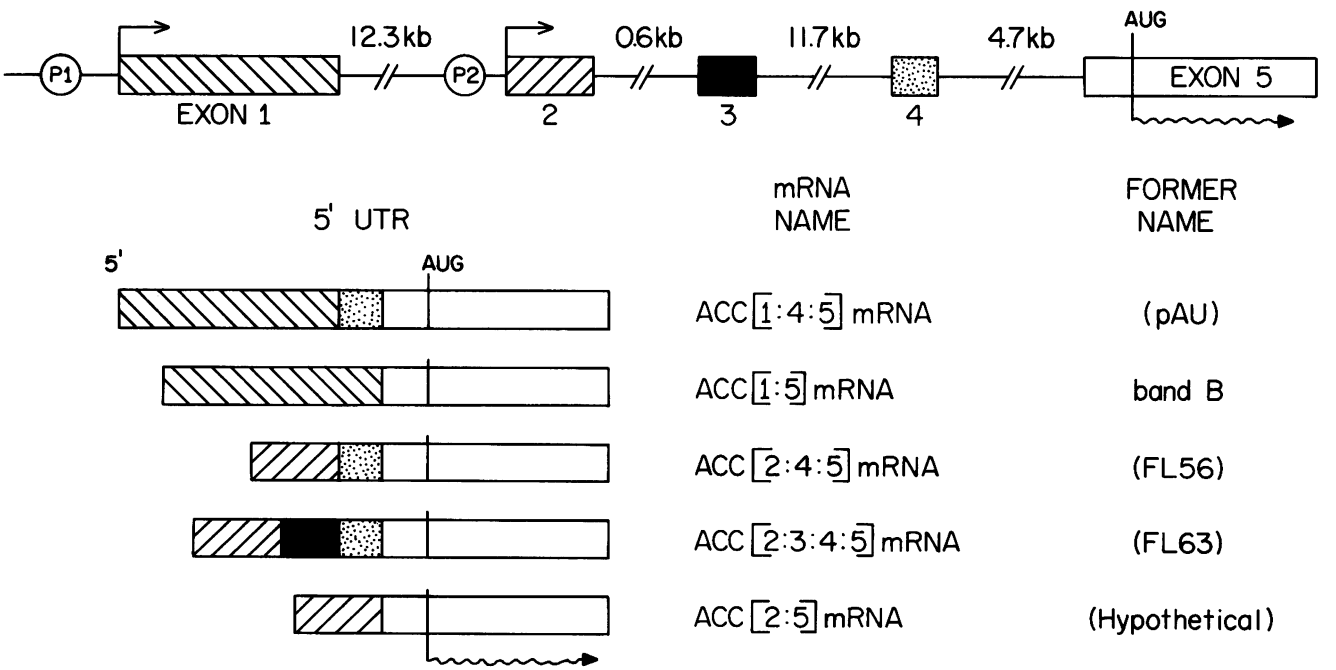

Fig. 2. Genomic structure of the 5'-end of the rat ACC gene. P1 and P2 contain the sequences of the two regions for carboxylase promoters. The five exons and their sizes are indicated. pAU, FL56, and other types of ACC mRNAs with the various exons used to generate these RNAs are also diagrammed. New names for different ACC mRNAs have been proposed to indicate the exons they contain. 5'-UTR, 5' end untranslated region.

DNA-mediated cell transfection and CAT assay

Mouse 30A5 preadipocytes (6,15), 10T1/2 fibroblasts (15) and rat Fao hepatoma cells (16) were cultured in 90 mm petri dishes to about 80% of confluence in Eagle's basal medium supplemented with 10% fetal bovine serum as described previously (6). The cells were transfected with plasmid DNA (15 μg/plate) by the calcium phosphate precipitation method (17). During our studies on transfection, it became apparent that different batches of the same plasmid DNA preparation show different transfection efficiency. However all different DNAs prepared at the same time show the same transfection efficiency. Therefore, all the preparations used in a given experiment were prepared at the same time. Each sample was duplicated and the same experiments were carried out twice to confirm the results. In addition, to examine the transfection efficiency of each construct, the reference pRSV-LacZ plasmid (13) was co-transfected with the test plasmid DNA containing different ACC promoter fragments and β-galactosidase activity was measured (18). After 8 hours of incubation, the DNA-calcium phosphate mixture was removed and cells were shocked for 2 minutes by treatment with 20% glycerol followed by the addition of fresh medium. Following a 60 hour incubation, the transfected cells were washed once with cold PBS buffer and scraped off the culture dishes. The cell pellets were sonicated in 150 μl of 0.25 mM Tris, pH 8.0, for 20 seconds, cell debris was removed by centrifugation for 15 minutes. The supernatants were used for both the β-galactosidase assay, as described by Nielsen et al. (18), and the CAT assay, as described by Gorman et al. (17). The relative CAT activity for each transfection was normalized on the basis of the specific β-galactosidase activity for that transfection (12).

RESULTS

Organization of ACC P2

A schematic representation of the ACC genomic structure containing exons 1−5 is shown in Fig. 2. Based on our present knowledge of promoter function and differential splicing, the FL-type ACC mRNA is a P2 transcript (10). To characterize the nature of P2, a 3 kb genomic fragment containing the first exon for the FL-type ACC mRNA and its upstream region have been sequenced. A portion of this sequence, including a partial sequence of exon 2 (the first exon under the control of promoter 2), and 994 bp of the upstream region are shown in Fig. 3. Analysis of the region flanking the transcription initiation site(s) reveals the typical features of a housekeeping promoter (19−23). The GC content from −1 to −280 is about 80%, but it gradually decreases to about 50% near −500. Five copies of the G/C motif GGGCGG hexanucleotide that forms the core-binding site for transcription factor Sp1 are clustered between positions −14 and −100. In addition, three unusual symmetrical sequences are found between positions −185 and −254, which are underlined with arrows.

Identification of sequences required for promoter activity

Our preliminary experiments indicated that pP2-CAT O was strongly expressed in 30A-5 preadipocytes, suggesting that the pP2-CAT O construct contains nucleotide sequences with strong promoter activity. To identify sequences responsible for promoter activity, portions of the 5′ ends of the P2-CAT O were progressively deleted by exonuclease III treatment. The shortened fragments were inserted in front of the CAT gene in pUC-CAT3. The resulting plasmids were transfected into 30A-5 cells and their transient CAT activities were assayed (Fig. 4).

Several regions within P2 are particularly important for promoter function. Deletion of the region between −994 and −677 reduces CAT activity by about 50% (pP2-CAT1, Fig. 4). However, deletion of sequences between −994 and −340 results in the recovery of CAT activity from 50% to 100% (pP2-CAT2, Fig. 4). CAT activities remain relatively constant until deletion of bases up to −249 (pP2-CAT5, Fig. 4). The dramatic change in promoter activity occurs with deletions between −249 and −135 (pP2-CAT5 and pP2-CA-6, Fig. 3). Deletion of the 115 bp fragment between −249 and −135 leads to about a 94% decrease in CAT activity. Examination of the primary structure in this region reveals the existence of 3 symmetrical sequences: CGCCCCGCCCCGC (−255 TO −243), CGCCCGAGCCCGC (−220 TO −208), AND CCCCCACCCCC (−201 TO −191). This loss of promoter activity occurs in spite of the pP2-CAT 6 still containing 5 G/C rich putative sequences for the Sp1 binding. These may be important elements for transcription of P2. pP2-CAT7 which contains no G/C motif exhibits virtually no CAT activity.

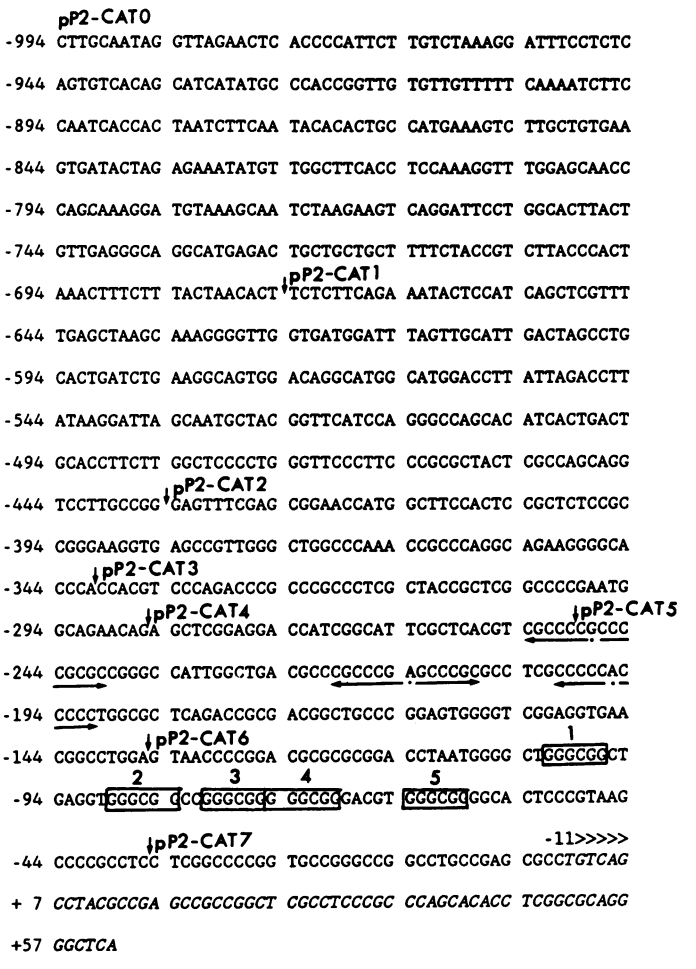

Fig. 3. Nucleotide sequence of 994 bp of the ACC P2. Nucleotides for the coding strand are numbered relative to the starting site of transcription, defined as +1 multiple initiation sites are denoted as >. Boxes denote the five putative Sp1 binding sites. The horizontal arrows indicate the three enhancer sequences and the dots in the middle of the arrows indicate the axis of symmetry of the sequences. Seven deletions were generated by exonuclease III treatment. The end point of each deletion is shown by the vertical arrows.

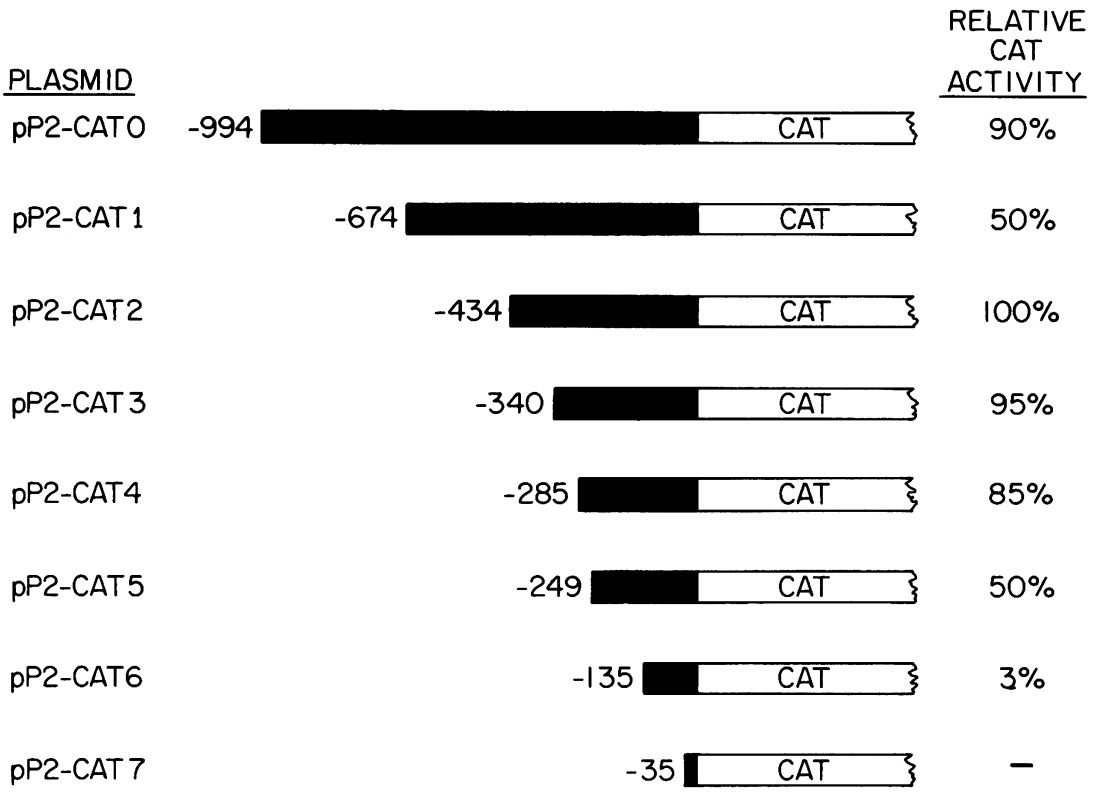

Fig. 4. Structures of ACC P2 deletions and relative promoter activity. The numbers show the length of the P2 fragment upstream of the transcription initiation site. The promoter fragments were inserted upstream of the CAT gene in pUC-CAT3. The resulting plasmids are designated pP2-CAT1 to pP2-CAT 7. Plasmid pP2-CAT0 and its deletion derivatives were transiently transfected into 30A-5 preadipocytes. The relative CAT activity, based on 4 samples, for each deletion is shown.

Identification of positive elements in the upstream region of p2

Basal level activity of P2 was observed as long as the five copies of the Sp1 binding site are kept intact (pP2-CAT6). However, pP2-CAT6 that contains all five G/C motifs for Sp1 binding has only 3% of the transcriptional capacity of the most active construct, pP2-CAT2 (Fig. 4). Since the 115 bp fragment between bases −249 and −135 make a significant contribution to promoter activity, and since this region contains three unusual symmetrical sequences, we examined whether these sequences could act as enhancer-like elements. Three different fragments containing these unusual sequences were inserted upstream of the weak TK promoter in plasmid pTK-CAT. The longest fragment with 159 bp (−340 to −181) contains all three symmetrical sequences and some upstream sequences; the second fragment is 86 bp long (−249 to −163) with two symmetrical core sequences and some downstream sequences; and the shortest fragment is 68 bp long (−249 to −181) and includes two symmetrical core sequences. The resulting plasmids (p159TK-CAT, p86TK-CAT, and p68TK-CAT) were transfected into both 30A-5 preadipocytes and 10T1/2 fibroblasts, and CAT activity was assayed. In 30A-5 cell, the 68 bp and 86 bp fragments stimulate expression of the pTK-CAT about 3-fold, regardless of orientation. The 159 bp fragment can enhance TK promoter activity about 5-fold (Fig. 5). This indicates that the enhancer is not promoter specific. To eliminate the possibility that this region might behave like a promoter itself instead of an enhancer-like element, the 86 bp and the 68 bp fragments were inserted upstream of the CAT gene of pUC-CAT3 and transfected into 30A-5 and 10T1/2 cells. No CAT activity was detected in extracts of cells transfected with these plasmids (data not shown).

Expression of p2 in different cells lines

Since P2 contains many features that are common to the promoters for housekeeping genes, one would expect that this promoter would be active in a variety of cell types. The plasmids pP2-CAT0, pP2-CAT1, pP2-CAT3, and pP2-CAT6 (Fig. 4), were transfected into 30A-5 preadipocytes, 10T1/2 mouse fibroblasts, and Fao rat hepatoma cells. As expected, CAT activity was detected in all three cell lines and the effects of deletions on the expression of CAT activity were similar in different cell lines (Table I). Thus, P2 is active in a variety of cell types, in accordance with the thesis that this promoter is involved in housekeeping functions.

DISCUSSION

ACC catalyzes the rate-limiting step in the biosynthesis of long-chain fatty acids. The importance of lipids for energy storage and membrane biogenesis necessitates a metabolic pathway and enzymes which are always present to meet cellular demands for

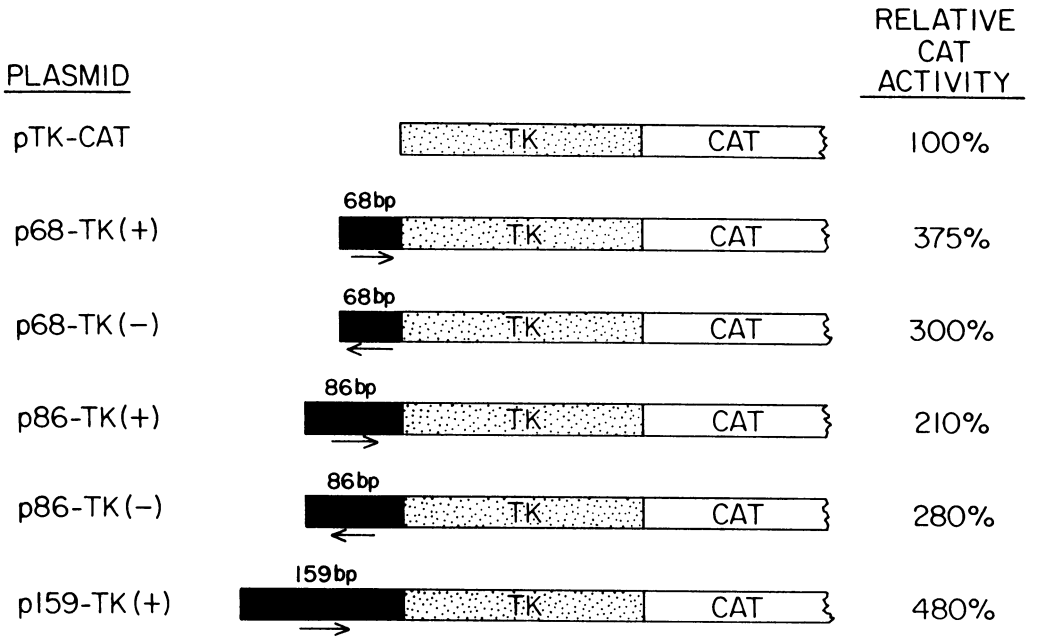

Fig. 5. Plasmids of P2TK-CAT and effects of P2 fragments on TK promoter expression. The 68 bp fragment (−249 to −181) and the 86 bp fragment (−249 to −163) were inserted upstream of the TK promoter of pTK-CAT in both orientations. The 159 bp fragment (−340 to −181) was inserted only in the 5'-3' orientation. The resulting plasmids are designated as p68-TK, p86-TK, and p159-TK. The plasmids were transiently transfected into 30A5 preadipocytes. The relative CAT activities are shown, the calculated values are based on the average of two samples.

fatty acids. However, the role of ACC in meeting the fatty acid requirements for housekeeping or cellular maintenance is different from that of meeting stimulated lipogenesis for energy reserve storage. Under certain physiological conditions stimulated lipogenesis requires induction of ACC. Indeed, conditions leading to stimulated lipogenesis enhance the synthesis of pAU type ACC mRNA which is under the control of P1 (10). Genes coding for enzymes that are constitutively expressed and catalyze basic cellular processes have been termed housekeeping genes. The findings reported in this communication indicate that P2, flanking the 5'end of exon 2, may be responsible for generating the FL type of ACC mRNA to meet this function.

Structural features of ACC P2 reveal marked similarities with other housekeeping genes (19−23). For example, the high GC content, the multiple transcription initiation sites (9), and the lack of TATA and CAAT boxes are all typical of promoters of housekeeping genes. In addition, a functional analysis of ACC P2 indicates that P2 is active in fibroblasts, adipocytes, and hepatoma cells. The present studies suggest, that P2 is the promoter that is constitutively expressed in all cell types examined. The gene for triose phosphate isomerase, a housekeeping enzyme, contains a TATA box in addition to the typical G/C motif (19). In the case of mouse dihydroorotate reductase, a CACAAAAT sequence which may serve as a substitute for the TATA and CAAT boxes is found (20). However, ACC P2 contains neither of these sequences and belongs to the other group of housekeeping genes (21−23). However, the presence of enhancer element in a CpG island-type promoter of a house-keeping gene (24) has never been observed before, as far as we know.

Although we can only speculate about the role that ACC P2 plays in the expression of the ACC gene in different cell types, we were able to analyze sequences important for P2 activity. The

TABLE I. Relative CAT Activity

PLASMID	10T1/2	30A5	Fao
pP2-CAT0	85%	90%	70%
pP2-CAT1	50%	50%	40%
pP2-CAT3	100%	100%	100%
pP2-CAT6	4%	3%	10%

Table I. CAT activity of the CAT plasmids with different promoter fragments. ACC P2 CAT chimeric genes were transiently expressed in different cultured cells. The activity of the chimeric gene, P2-CAT3 was taken as 100%. Since the transfection efficiency for the three cell lines are different, the activities between cell lines are not compared.

data presented here suggest that the regulation of expression of P2 does not rely on one single element, but rather is dependent on the interaction of multiple positive and negative elements.

The single most important sequence in P2 is a positive element located between −249 and −135. When this fragment is deleted promoter activity decreased almost 94%. This 115 bp positive element is composed of three similar 13-mer sequences. When two of these three sequences were inserted upstream of the TK promoter, they caused a 3-fold increase in CAT activity in comparison to the control plasmid. However, if all three of these sequences were inserted upstream of the TK promoter, a greater increase in CAT activity was observed. These data provide direct evidence that the 115 bp fragment does behave like a positive regulatory element for which the three sequences form the core structure. Comparison of the sequence with those of known enhancer regions, such as the SV40 enhancer (25,26), shows no sequence identity. Analysis of these sequences by mutation and DNase I footprinting analysis will allow us to further define the sequences necessary for ACC P2 activity.

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