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Characterization of the transcriptional regulatory region of the human dihydrolipoamide dehydrogenase gene

(gene expression/chloramphenicol acetyltransferase assays/housekeeping genes)

GARY L. JOHANNING, JONATHAN I. MORRIS, K. T. MADHUSUDHAN, DAVID SAMOLS, AND MULCHAND S. PATEL*

Department of Biochemistry and Pew Center for Molecular Nutrition, Case Western Reserve University School of Medicine, Cleveland, OH 44106

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ABSTRACT **Dihydrolipoamide dehydrogenase (E_3; EC 1.8.1.4) is the common component of the three mammalian α-ketoacid dehydrogenase complexes and the glycine cleavage system. To study regulation of E_3 gene expression, a 12-kilobase clone from a human leukocyte genomic library was isolated, and a 1.8-kilobase fragment containing part of the first intron, the first exon, and 1.5 kilobases of the 5′ flanking region of the E_3 gene was sequenced. The nucleotide sequence of the E_3 promoter region revealed consensus sequences for several DNA binding proteins but no apparent TATA box or Sp1 sites. Although the 1.6-kilobase 5′ flanking region has a low percentage of G+C (44%), the nucleotide sequence between +1 and −150 base pairs has a G+C content of 67%. Primer extension analysis showed a major transcriptional start site located 95 nucleotides upstream from the translation initiation codon. A series of 5′ deletions from the E_3 promoter-regulatory region were ligated to the bacterial chloramphenicol acetyltransferase (CAT) gene, and the resulting constructs were transfected into HepG2 cells. The longest E_3 promoter-CAT construct had a relatively high level of CAT enzyme activity, and deletion of a promoter element between −769 and −1223 base pairs resulted in a 3-fold increase in reporter gene expression. These results suggest that the human E_3 promoter has characteristics of housekeeping and facultative promoters and that a negative regulatory element is present between 769 and 1223 base pairs upstream from the transcription start site.**

Dihydrolipoamide dehydrogenase (E_3; EC 1.8.1.4) is the common flavoprotein component of the three mammalian α-ketoacid dehydrogenase complexes—namely, the pyruvate dehydrogenase complex (PDC), the α-ketoglutarate dehydrogenase complex, and the branched-chain α-ketoacid dehydrogenase complex. The E_3 component is also present in the glycine cleavage system. E_3 functions to reoxidize dihydrolipoamide that is covalently attached to lysine residue(s) of the dihydrolipoamide acyltransferase component of these complexes, using NAD^+ as the electron acceptor. Human E_3 cDNA clones have recently been isolated (1, 2), and the gene has been mapped to chromosome 7 (3).

E_3 is regulated by the NAD^+/NADH ratio (4). In some circumstances E_3 may be regulated at the level of enzyme synthesis. E_3 is present in a fixed ratio relative to the other components of the three mammalian α-ketoacid dehydrogenase complexes, and it is possible that its rate of synthesis and/or degradation is finely regulated to maintain constant relative ratios in these complexes. During differentiation of 3T3-L1 preadipocytes into 3T3-L1 adipocytes, the activities of pyruvate and branched-chain α-ketoacid dehydrogenase complexes are increased by severalfold (5, 6). The increase in PDC activity was paralleled by increases in its component proteins $E_1\alpha$, $E_1\beta$, and E_3 as well as increases in their relative rates of synthesis in 3T3-L1 adipocytes (5, 7).

The promoter-regulatory regions of several components from these three complexes have recently been characterized. The sequences of the 5′ flanking regions of the α (8) and β (9) genes of the E_1 component of the human PDC and the $E_1\beta$ gene of the human branched-chain α-ketoacid dehydrogenase (10) have been reported, but the promoter of the E_3 component has not been characterized. E_3 catalyzes a reaction required in most cell types and thus would be expressed in most tissues. As such, the E_3 gene could be classified as a housekeeping gene, or one that carries out essential metabolic functions and has a wide tissue distribution (11). Although PDC has variable tissue activities, it is present in all human tissues assayed (12). In this paper, the DNA sequence of the proximal promoter of the human E_3 gene and the effect of deletions of cis-acting regulatory elements on reporter gene expression in a transient assay are reported.†

MATERIALS AND METHODS

Genomic Library Screening. A human leukocyte genomic library in λ EMBL 3 (Clontech) was screened with a 375-nucleotide 5′ terminal *Xba* I fragment of the human E_3 cDNA (nucleotides +1 to +375; ref. 2). The genomic library was plated at a density of $\approx 1 \times 10^5$ plaques per 150-mm plate. Nylon (Nytran; Schleicher & Schuell) filter lifts were prepared from the plates; the filters were alternatively denatured and neutralized in 0.5 M NaOH/1.5 M NaCl and 0.5 M Tris·HCl, pH 8/1.5 M NaCl, respectively. The filters were rinsed in 2× SSC (1× SSC = 0.15 M NaCl/0.015 M sodium citrate, pH 7.0), air-dried, and heated at 80°C in a vacuum oven for 30 min. The filters were prehybridized for 4 hr in a solution containing 50% deionized formamide, 5× SSC, 3× Denhardt's solution (1× Denhardt's solution = 0.02% Ficoll/0.02% polyvinylpyrrolidone/0.02% bovine serum albumin), 0.5% sodium dodecyl sulfate (SDS), and 200 μg of denatured, fragmented salmon sperm DNA per ml. The 375-nucleotide *Xba* I probe prepared by the random priming method (13), using [α-^{32}P]dCTP, was then added and hybridization was continued for 16–20 hr at 42°C. The filters were rinsed with 2× SSC/0.2% SDS, washed twice with 2× SSC/0.2% SDS at 65°C for 30 min, washed with 1× SSC/0.1% SDS at 65°C for 30 min, and subjected to autoradiography. The positive clones were plaque purified and a large-scale preparation of

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Abbreviations: E_3, dihydrolipoamide dehydrogenase; PDC, pyruvate dehydrogenase complex; CAT, chloramphenicol acetyltransferase.

*To whom reprint requests should be addressed at: Department of Biochemistry, Case Western Reserve University School of Medicine, 10900 Euclid Avenue, Cleveland, OH 44106-4935.

†The sequence reported in this paper has been deposited in the GenBank data base (accession no. M99384).

the phage was made and purified using a glycerol step gradient (14).

Characterization of Genomic Clones. The phage insert was digested with various combinations of restriction enzymes and the resulting restriction fragments were separated by agarose gel electrophoresis. These fragments were transferred to nylon filters (colony/plaque screen; New England Nuclear) that were hybridized with the 5′ terminal *Xba* I probe described above. The filters were prehybridized in a solution containing 50% formamide, 7% SDS, 0.1% nonfat dried milk, 0.25 M Na_2HPO_4/NaH_2PO_4 (pH 7.2), 0.25 M NaCl, 1 mM EDTA, and 250 μg of denatured, fragmented salmon sperm DNA per ml for 4 hr at 42°C, after which time the ^{32}P-labeled probe was added. The filters were hybridized for 16–20 hr at 42°C and washed with 0.1× SSC/1% SDS at 55°C for 15–20 min. Fresh buffer was added and washing was repeated as above until the background radioactivity was low, and the filters were then autoradiographed. Positive fragments were subcloned into the plasmid vector pBluescript (KS; Stratagene) and the DNA sequence of both strands was determined by the dideoxy chain-termination method (15), using Sequenase (United States Biochemical) and alkali-denatured double-stranded templates. Exonuclease III digestion was used to generate sets of ordered deletions for sequencing (Erase-a-Base; Promega). The reaction products were separated on 8 M urea/6% acrylamide sequencing gels. Sequencing reaction primers were the universal M13 forward and reverse primers or custom-synthesized 18-base-pair (bp) oligonucleotides.

Primer Extension and S1 Nuclease Mapping. A synthetic 30-bp oligonucleotide primer, 5′-TCCTTTCCGCCAATACTTTCACCTCCGCTG-3′, beginning 16 nucleotides upstream from the E_3 translation initiation codon and complementary to the mRNA, was labeled at the 5′ end using [γ-^{32}P]ATP and T4 polynucleotide kinase (14). The primer (5×10^5 cpm) was combined with 40 μg of tRNA, 40 μg of total RNA from HepG2 cells, or 2 μg of poly(A)$^+$ RNA from human heart (16). Samples were resuspended in 30 μl of aqueous hybridization buffer (17), heated to 60°C for 10 min, and incubated overnight at 42°C. The procedure for primer extension was as described (17), using 40 units of avian myeloblastosis virus reverse transcriptase (Boehringer Mannheim) and a 90-min incubation at 42°C. Primer extension products were analyzed on an 8 M urea/6% acrylamide sequencing gel, along with a dideoxy sequencing ladder for precise sizing.

The probe for S1 mapping was prepared by digesting a 2.5-kilobase (kb) double-strand fragment, which contained the E_3 promoter and a portion of the E_3 coding region with *Msc* I, an enzyme that digests this fragment uniquely at a position 34 nucleotides downstream from the translation initiation codon. The end-labeled (14) probe (50,000 cpm) was hybridized at 52°C with 70 μg of fresh human placental RNA, and the mixture was subjected to S1 nuclease digestion as described (18).

Reporter Gene Constructs Used for Transfections. A portion of the E_3 promoter beginning at a *Nar* I site within the putative 5′ untranslated region and extending to −1853 bp was cloned into a vector (pBSKCAT) containing the coding region of the *Escherichia coli* chloramphenicol acetyltransferase (CAT) gene and the simian virus 40 (SV40) splicing and poly(A) signals (19). The pBR322 sequence in this construct was replaced with the pBluescript (SK) vector by subcloning the *Eco*RI/*Bam*HI fragment of CAT [including the SV40 small tumor intron and poly(A) site] into the *Sma* I site of pBluescript. This construct was designated pE3CAT1.8. A set of nested deletions from the E_3 promoter region was generated by removing segments of the promoter at convenient restriction sites. The resultant constructs, pE3CAT1.8 to pE3CAT0.02, were transfected into HepG2 cells. A second plasmid, pRSVβgal, which contained the β-galactosidase gene driven by the Rous sarcoma virus promoter/enhancer subcloned into the *Hind* III site of the pBluescript (SK) vector, was cotransfected with the pE3CAT constructs as an internal control to assess transfection efficiency. An additional vector (pSV2CAT) (19) was used as a positive control. The pSV2CAT vector contained the SV40 early promoter/enhancer driving CAT expression.

Cell Culture and Transfection Conditions. Human hepatoma HepG2 cells were grown to 70% confluence in modified Eagle's medium (GIBCO) supplemented with 10% fetal calf serum, penicillin (100 units/ml), streptomycin (100 μg/ml), and 2 mM glutamine at 37°C under 5% CO_2. Cells from a single plate ($\approx 3 \times 10^7$ cells) were washed with sterile phosphate-buffered saline, trypsinized, and cotransfected with 10 μg of the test plasmid and 5 μg of pRSVβgal control plasmid. Transfection was carried out by the calcium phosphate coprecipitation procedure (37). Cells were harvested after 48 hr by first rinsing twice with cold phosphate-buffered saline and then collecting from the plate by scraping with a rubber spatula. The cells were then pelleted in a Microfuge, resuspended in 0.25 M Tris·chloride (pH 7.5), and lysed by three freeze-thaw cycles using a dry ice/ethanol bath and 37°C water bath. After centrifuging the resultant lysate to remove particulate material, aliquots of the supernatant were assayed for β-galactosidase activity (14) and protein concentration (20). The remainder of the supernatant was heated to 65°C for 5 min to inactivate deacetylase, and the soluble lysate was stored at −20°C prior to determination of CAT activity.

CAT Activity. CAT assays were by the phase-extraction method (38) using [^3H]chloramphenicol as substrate. The reaction conditions were chosen so that substrate in the presence of butyryl-CoA was converted into product within the linear range of the assay (0.01–50%). After the incubation butyrylated chloramphenicol was extracted with xylenes, the xylenes were back-extracted twice with water to decrease the background, and ^3H radioactivity was determined by scintillation spectroscopy. The CAT activity was normalized to the β-galactosidase activity and expressed as fold increase in activity over that of the full-length (1.8-kb) promoter.

Statistical Analysis. Results are presented as means ± SD for the number of determinations indicated (Fig. 3). The significance of differences between groups was determined by analysis of variance (ANOVA one-way).

RESULTS AND DISCUSSION

Isolation, Structure, and Sequencing of Genomic DNA. A 375-nucleotide 5′ terminal *Xba* I fragment of the human E_3 cDNA was used to screen a human placental DNA genomic library. One positive clone was isolated from a total of 1.5×10^6 plaque-forming units of recombinant phage. The human genomic DNA insert released from the phage arms by *Xho* I digestion was about 12 kb in length. The insert was partially mapped with restriction enzymes and subjected to Southern blot hybridization analysis. A 5-kb *Eco*RI/*Eco*RI fragment was found to hybridize strongly with the 5′ E_3 cDNA probe. This fragment, upon further digestion with *Pst* I, yielded a 2.5-kb fragment that also hybridized. These *Eco*RI and *Pst* I fragments were subcloned into the plasmid vector pBluescript, and both strands of the *Pst* I fragment were sequenced.

Nucleotide Sequence of the 5′ Flanking Region of the Human E_3 Gene. In the present work, we have characterized the proximal promoter region of the E_3 gene. The nucleotide sequence of a portion of the first intron, the first exon, and 1.5 kb of the proximal 5′ flanking region of the gene is shown in Fig. 1. The sequence from the ATG translation initiation site to the 3′ end of the first exon in Fig. 1 (39 nucleotides) matches exactly the corresponding human E_3 cDNA sequence previously reported (1, 2). Primer extension was used

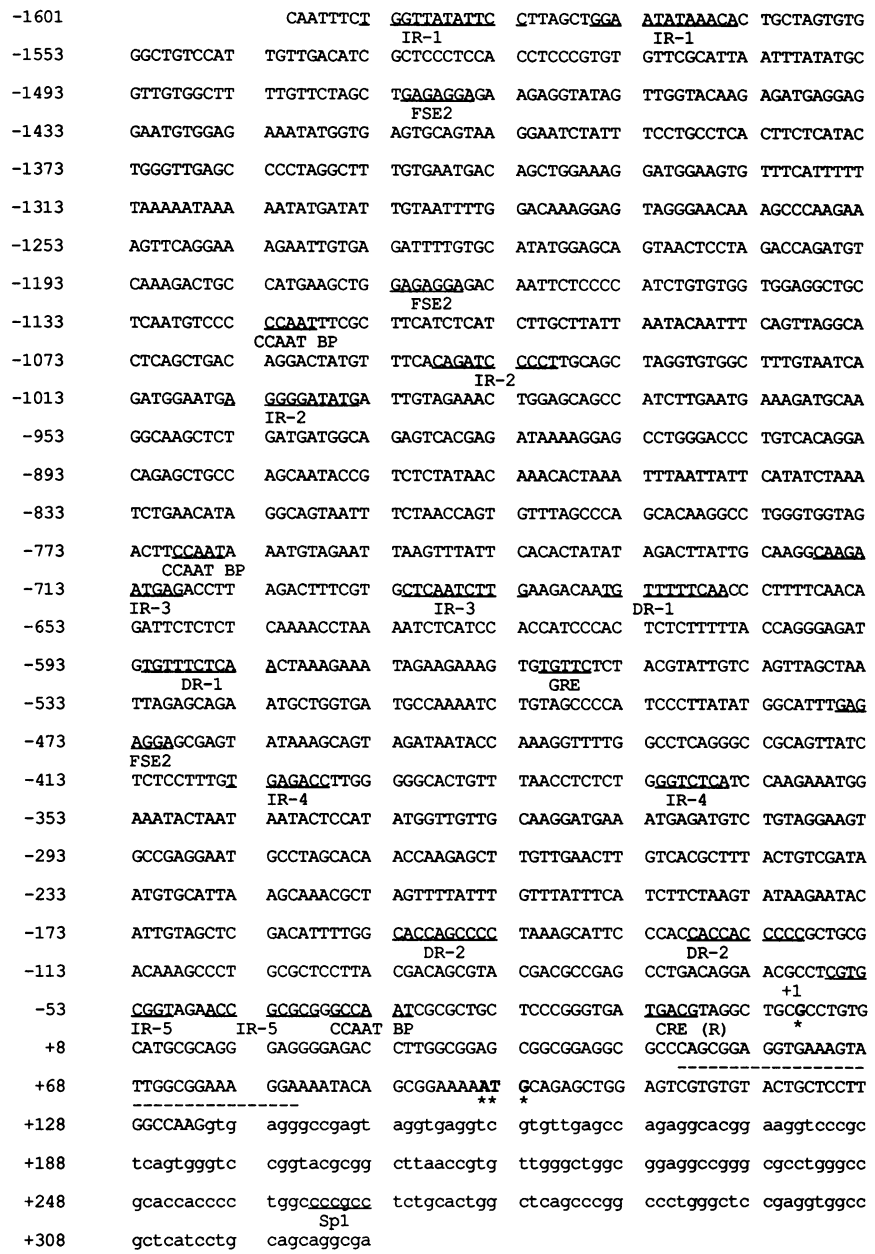

FIG. 1. Nucleotide sequence of the transcriptional regulatory region of the human E_3 gene. Nucleotides are numbered relative to the deduced major transcription start site, designated +1, as determined by primer extension. Uppercase letters denote exon and 5′ flanking sequences; lowercase letters denote intron sequences. Direct repeats (DR), inverted repeats (IR), and potential consensus sequences for regulatory elements and transcription factor binding sites are underlined. The initiator ATG is indicated by three asterisks and the transcription start site (+1) is indicated by an asterisk. The region corresponding to the primer used for primer extension is underlined with a dashed line. CCAAT, CCAAT-binding protein; CRE, cyclic AMP response element; FSE2, fat-specific element 2; GRE, glucocorticoid response element; R, sequence in reverse (antisense) orientation.

to determine the transcription start site of the E_3 mRNA. An antisense 30-bp oligonucleotide starting 16 bp 5′ from the initiator methionine was end-labeled with [γ-^{32}P]ATP, hybridized to human HepG2 total RNA, and extended with reverse transcriptase. The longest and most abundant reaction product identified (designated +1 in Fig. 1) was 95 nucleotides upstream of the ATG initiation codon (Fig. 2). The same nucleotide was identified in a separate experiment using human heart poly(A)$^+$ RNA (results not shown). S1 nuclease mapping revealed a protected fragment of 88 bp (results not shown), identifying a site in the close vicinity (7-bp difference) of the transcription start site determined by primer extension.

Several discrepancies exist between the sequence of Fig. 1 and previously reported sequences of the 5′ untranslated region of cDNA clones of human E_3. These include a single base change (from T to G) at position +48, compared to the human E_3 cDNA sequence of Pons *et al.* (2), and insertions of G residues at positions +37, +46, and +48, compared to the human E_3 cDNA sequence of Otulakowski and Robinson (1).

The promoter-regulatory region of the E_3 gene has characteristics in common with the 5′ flanking regions of other constitutively expressed genes. First, it lacks appropriately positioned TATA and CCAAT consensus sequences found approximately 30 and 80 bp, respectively, 5′ of the transcriptional start site in many facultative genes but in some cases absent in housekeeping genes. Initiator elements present at the transcription start site of several housekeeping genes have recently been described (21, 22). There is no homology

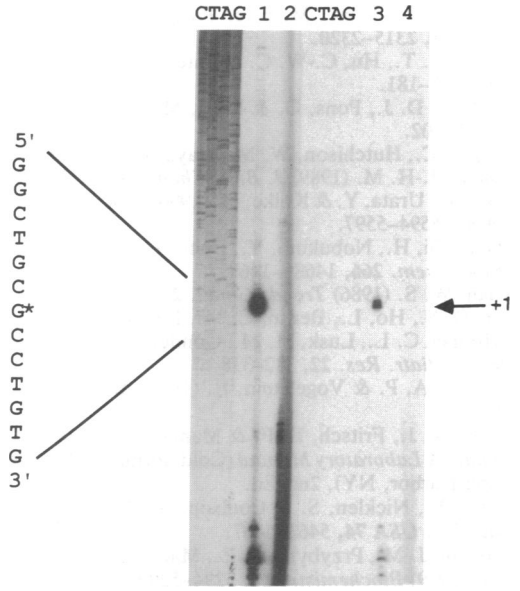

FIG. 2. Transcription start site identification by primer extension. A sample of total RNA from human HepG2 cells was hybridized with a synthetic 5′ end-labeled 30-bp oligonucleotide probe and extended with reverse transcriptase. Lanes CTAG, dideoxy sequencing ladder obtained by extending the same primer using cloned human E_3 genomic DNA as a template; lane 1, HepG2 total RNA, 40 μg; lane 2, tRNA, 40 μg. Lanes CTAG (right side) and lanes 3 and 4 represent a shorter exposure (≈4 hr) of lanes CTAG and lanes 1 and 2, respectively, shown on the left-hand side.

to the consensus sequences for these elements at the transcription start site of the E_3 gene, and in this respect the promoter resembles that of the mouse DNA methyltransferase gene, which also lacks TATA and initiator elements (23).

The human E_3 promoter has structural characteristics similar to the human thymidylate synthase gene in that it has a high GC content within the first 150 nucleotides of the transcription start site, and its 5′ flanking region lacks TATA and Sp1 binding consensus sequences (24). The first intron in the thymidylate synthase and E_3 genes is (G+C)-rich and contains at least one Sp1 site. If a single Sp1 consensus sequence mismatch is allowed, four Sp1 sites (base pairs −150 to −145, −127 to −122, −45 to −40, and −43 to −38) can be identified within the first 175 bp upstream of the transcription start site. The promoter sequence from −1601 to +1 shown in Fig. 1 has a low GC content (44% G+C for 1601 nucleotides upstream from the transcription initiation site), but the sequence from −100 to +327 nucleotides has 67% G+C. The CpG/GpC ratio from base pairs −100 to +327 is 0.78, compared with 0.40 for the sequence from −1601 to +1. These data indicate that the region surrounding the transcription start site has the characteristics of a CpG island (25) often found in housekeeping genes. These CpG islands typically extend from just upstream of the transcription start site through the first one or two exons and are characterized by an abundance of the sequence CpG and a lack of methylation. The extent of methylation of the E_3 promoter has not been determined.

The human E_3 promoter contains sequence motifs related to elements known to be important in modulating transcription. Potential binding sites for the glucocorticoid receptor (26), fat-specific element 2 (27), a cAMP response element (28), and CCAAT-binding proteins (29) are present in the promoter (Fig. 1). If a single mismatch from the consensus sequence is allowed, potential sites for AP-1 (30), AP-2 (31), and Oct-1 (32) are present. In addition, an Sp1 site (33) is found in the first intron. The extent to which these transcription factors may mediate the response of E_3 to hormonal or other regulatory stimuli *in vivo* is unknown. There are also several repeated sequences (direct and inverted repeats) in the 5′ flanking region of the gene (Fig. 1). These repeats may regulate gene expression by altering the secondary structure of the promoter, as has been suggested for the $E_1\alpha$ promoter of the human pyruvate dehydrogenase gene (8).

Reporter Gene Analysis of the Human E_3 Promoter. The pE3CAT deletion clones were used to assess the influence of deleted regions from the E_3 promoter on gene expression of CAT in HepG2 cells. It was observed that the entire 1853 bp of the E_3 promoter region resulted in a relatively high level of CAT expression (≈4-fold higher) compared to the pSV2CAT vector (results not shown). Expression of CAT activity was dependent on the presence [see pBSKCAT(-E3)] as well as orientation [see pE3CAT(rev)] of the E_3 promoter sequence (Fig. 3).

As shorter segments of the E_3 promoter were used for transient transfection analyses, changes in CAT expression were noted. A marked increase (about 3-fold; $P < 0.05$) in CAT activity was observed with deletion of the 5′ flanking region to −700 (pE3CAT0.7) over the activity expressed by the construct with 1200 bp upstream of +1 (pE3CAT1.2) (Fig. 3). A small but consistently observed decrease ($P < 0.05$) in CAT expression relative to pE3CAT0.7 was seen when a deletion was made to −551 (pE3CAT0.5), suggesting removal of a positive-acting sequence element. An increase ($P < 0.05$) in CAT activity was observed for pE3CAT0.4 compared to pE3CAT0.5, whereas a large reduction ($P < 0.05$) in CAT expression was noted when the E_3 promoter segment was reduced from base pair −332 to base pair −19. The increase in CAT activity upon deletion of a segment of a promoter-regulatory region suggests that a negative element has been removed. If positive regulatory elements are not found further upstream (in regions not yet assayed for promoter activity), then the region between bases −1223 and −769 and between bases −551 and −488 may play important roles in

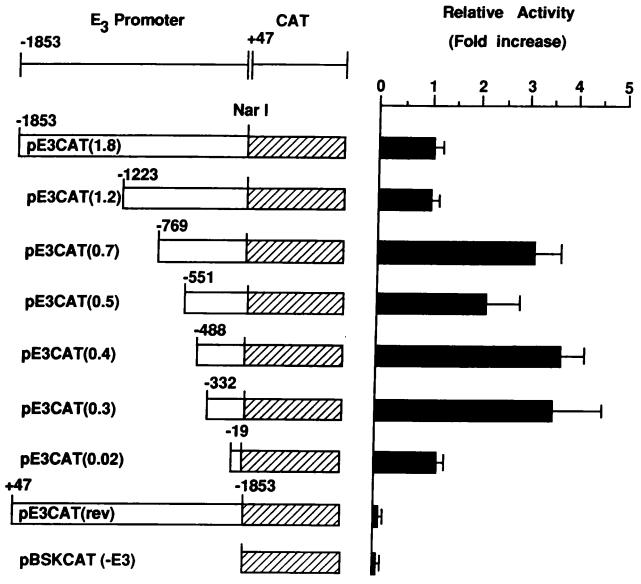

FIG. 3. Human E_3 promoter activity in HepG2 cells. The coding region of the CAT gene was fused to the E_3 genomic sequence downstream of the major transcription start site, but upstream of the ATG codon, to generate a series of pE3CAT constructs. The E_3 promoter sequences are represented by open boxes and the CAT coding region is represented by hatched boxes. Each pE3CAT construct (10 μg) was cotransfected with pRSVβgal (5 μg) into HepG2 cells, and 48 hr later the activities of CAT were measured. Transfection efficiency for CAT constructs was normalized to the β-galactosidase activity and the CAT activity is expressed as fold increase over that of the pE3CAT1.8 construct. The results are the means ± SD of six independent transfections.

regulation of transcription. The repressor element may contain regulatory site(s) for binding of factors unique to E_3 gene expression or could be an as yet uncharacterized element.

Sequences in these two regions were inspected for homology with other negative regulatory elements. The E_3 promoter sequence from −1187 to −1180 (CTGCCATG) resembles the sequence (GCTGCCATG) of an element that negatively regulates human interleukin 3 expression (34). In addition, the sequence from −877 to −869 (ACCGTCTCT) is similar to the sequence of a silencer element (ACCCTCTCT) found to regulate the tissue-specific expression of the human collagen II gene (35). The sequence from −531 to −522 (AGAGCAGAAT) also shares some homology to a negative regulatory site (GGAGAGCACATTTG) in the human insulin gene (36). Site-directed or block mutations in restricted areas of this element should more precisely define the nucleotide sequence responsible for reduction in transcription.

The shortest CAT construct studied, which contained E_3 promoter sequences from base pair −19 to base pair +47, had greater activity than a construct containing no E_3 insert (Fig. 3), suggesting that this short promoter region contained sequences adequate for expression of the E_3 gene. Another CAT construct containing E_3 promoter sequences from base pair −1853 to base pair −19 was cotransfected with pRSVβgal in HepG2 cells. CAT expression by this construct [pE_3(−1853 to −19)CAT], lacking the sequence (−18 to +47) in the immediate vicinity of the putative transcription start site, had <3% of the CAT activity expressed by the pE3CAT1.8 construct (−1853 to +47) (results not shown). This large decrease in CAT activity upon deletion of the −19 to +47 region of the E_3 promoter is consistent with primer extension data indicating the location of the transcription start site in this region.

Although E_3 is a component of enzyme complexes considered to be housekeeping enzymes, the level of enzyme activity of these complexes varies with tissue type. The activity of the PDC, for example, is higher in kidney and heart compared to the activity in liver (12). It would be of interest to study expression of the E_3 gene in cells derived from different tissues, to assess whether there is an altered level of expression in these cells and whether regulation of gene expression is also observed in cell types other than those derived from liver. In addition, treatment of HepG2 cells containing the pE3-CAT construct with a battery of hormones, and transfection with various transcription factor expression vectors, should provide more detailed information on the mechanism of *in situ* regulation of the E_3 gene expression.

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