A region in the first exon/intron of rat carnitine palmitoyltransferase Iβ is involved in enhancement of basal transcription

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Carnitine palmitoyltransferase-Iβ (CPT-Iβ) catalyses the transfer of long-chain fatty acids to the enzymes of β-oxidation of muscle and heart. Transcriptional control of this regulatory protein is relevant to disorders of fatty acid oxidation and the switch to glucose metabolism that occurs in cardiac pathology. The presence of a transcriptional enhancer sequence in the first untranslated exon and first intron of the CPT-Iβ gene was identified using deletional and mutational analysis, and by ligation of an oleate responsive element (fatty acid response element) to a minimal promoter. The enhancer sequences are contained in the first 40 bases downstream of the transcription start site and increase CPT-Iβ reporter gene expression independent of any 5′ *cis*-acting elements. Deletion of the first 40 bases of the 3′-untranslated region does not affect the up-regulation of transcription by 10 μM phenylephrine. However, mutation and/or deletion of bases between +11 and +30 dramatically decreases reporter gene expression. Electrophoretic mobility-shift assays reveal two DNA (+11 to +36)–protein complexes that appear cardiac specific. The exon/intron element enhances activation of the heterologous thymidine kinase promoter in a position- and orientation-dependent manner. Therefore we have identified a novel region in the first exon/intron of the CPT-Iβ gene that acts as a non-classical transcriptional enhancer downstream of regulatory elements characterized previously in the 5′-flanking region of the minimal promoter.

Key words: EMSA, phenylephrine, untranslated exon.

INTRODUCTION

Cellular factors that target expression of proteins in cardiac muscle have been well characterized with respect to the components of excitation–contraction coupling. There have also been significant reports, although less in number, concerning cardiac-specific expression of mitochondrial proteins involved in electron transport [1,2]. Only recently has attention focused on genes responsible for mitochondrial transformation of long-chain fatty acids to produce energy as the cardiac myocyte hypertrophies and contractile function increases [3–5]. One of these proteins is carnitine palmitoyltransferase Iβ (CPT-Iβ), the muscle isoform of carnitine palmitoyltransferase I (also known as M-CPT-I), which is the predominant CPT-I isoform in the heart and is the sole CPT-I enzyme expressed in skeletal muscle.

The first studies carried out on regulation of the human CPT-Iβ gene suggested that exposure of serum-deprived cardiac myocytes to long-chain fatty acids activated human CPT-Iβ gene expression via a fatty-acid-response element (FARE). This element was shown to be activated by peroxisomal-proliferator-activated receptor α (PPARα) both in CV-1 (monkey kidney fibroblasts) cells and in neonatal cardiac myocytes [3,4]. In our studies using the rat CPT-Iβ gene, the FARE site was localized to −303 to −289. We observed a 2–3-fold induction of CPT-Iβ reporter gene expression at high physiological concentrations of oleate, i.e. 2:1 molar ratios of fatty acid to BSA [5]. In contrast with the robust response of human CPT-Iβ to long-chain fatty acids, the rat CPT-Iβ gene is strongly GATA-4-dependent and is co-activated by serum response factor (SRF). This latter regulatory paradigm is characteristic of cardiac-specific genes [6]. We proposed that the combinatorial interaction between GATA-4 and SRF is an important mechanism by which the expression of cardiac-specific genes involved in energy expenditure and ATP production is synchronized between the cytosolic compartment and the mitochondria.

CPT-Iβ is similar to the liver isoform, CPT-Iα (or L-CPT-I), in that the promoter is TATA-less and basal expression is driven by specificity protein 1 (Sp1) [5]. However, CPT-Iα is known to be sensitive to hormonal and nutritional factors [7], whereas transcriptional activation of the muscle isoform appears to be independent of diet and diabetes, conditions which alter hormonal and metabolic levels of circulating fatty acids [8]. CPT-Iα requires elements in the first intron for transcriptional responsiveness to thyroid hormone [9]. We have demonstrated that CPT-Iβ mRNA is up-regulated with prolonged electrical stimulation of neonatal cardiac myocytes in culture, co-incident with the development of a hypertrophic response [10]. One or more enhancer regions in the first intron of other cardiac genes are implicated in the response to hypertrophic stimuli, e.g. the Na^+/Ca^{2+} exchanger [11]. Since it is increasingly evident that introns may play additional regulatory roles in gene function, we tested the hypothesis that elements in the untranslated first exon

Abbreviations used: BCS, bovine-calf serum; CPT-I, carnitine palmitoyltransferase I; CPT-Iα, liver isoform of CPT-I (L-CPT-I); CPT-Iβ, muscle isoform of CPT-I (M-CPT-I); EMSA, electrophoretic mobility-shift assay; DMEM, Dulbecco's modified Eagle's medium; DPE, downstream promoter element; FARE, fatty acid response element; hsv, herpes simplex virus; MED, multiple start site element downstream; MEF, myocyte enhancer factor; MZF, myeloid zinc finger; NCX, Na^+/Ca^{2+} exchanger; Nkx, muscle homologue of the *Drosophila* gene *tinman*; NKE, Nkx-binding element; PPARα, peroxisomal-proliferator-activated receptor α; Sp1, specificity protein 1; SRE, serum response element; SRF, serum response factor; TFIID, transcription factor IID; TK, thymidine kinase.

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or the first intron of CPT-Iβ may influence the responsiveness of this protein to a hypertrophic stimulus. Alternatively, it may serve a regulatory function by modulating transcription. Our results suggest that a *cis*-element in the first intron enhances CPT-Iβ (p$-$361/$+$80) transcriptional activity by 2–3-fold.

EXPERIMENTAL

Cell culture and transfections

Primary cultures of neonatal rat cardiac myocytes were prepared as described previously [12]. Cells were plated in 6-well plates (Primeria; Fisher, Pittsburgh, PA, U.S.A.) at a density of 6×10^5 cells/well. The cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Fisher) with 1% penicillin/streptomycin (Life Technologies, Grand Island, NY, U.S.A.) and 10% (v/v) bovine-calf serum (BCS; Hyclone Laboratories, Logan, UT, U.S.A.). The cells were incubated at 37 °C in the presence of 95% air and 5% CO_2 for 24–36 h before transfection. HEK-293 cells were cultured under identical conditions. Myocytes were transfected using calcium phosphate precipitation in the presence of serum as previously described [5,13]. The calcium phosphate transfection contained 2.0 μg CPT-Iβ firefly luciferase vector. Control transfections for transfection efficiency contained 0.25 μg of pRL-CMV *Renilla* luciferase expression construct (Promega, Madison, WI, U.S.A.). At 6 h following transfection, the medium was removed and the transfected myocytes maintained in DMEM containing 10% (v/v) BCS for 36 h. The cells were lysed and the lysate was used to measure luciferase expression with the dual luciferase reporter assay (Promega). Transfections in HEK-293 cells were also carried out in the presence of calcium phosphate as described for cardiac myocytes. Thymidine kinase (TK)/luciferase constructs were transfected into cardiac myocytes, CV-1 and HEK-293 cells with LIPOFECTAMINE™ Plus reagent (Life Technologies) as described previously [5].

For treatment with oleate, the transfected cells were kept in DMEM containing 10% (v/v) BCS for 12 h, and then washed twice with PBS. The myocytes were incubated for 24–30 h in either serum-free DMEM or serum-free DMEM containing 0.5 mM oleate, complexed in a 2:1 molar ratio with BSA (fatty acid free; Sigma, St Louis, MO, U.S.A.). In studies of the responsiveness of the CPT-Iβ reporter gene constructs to phenylephrine in neonatal cardiac myocytes, 1.0 μg of CPT-Iβ firefly luciferase vector and 0.2 μg of pRL-CMV construct were transfected into the myocytes with LIPOFECTAMINE™ Plus reagent in the absence of serum. After 3 h, the transfection medium was replaced with DMEM containing 10% (v/v) BCS overnight. The cells were then maintained in serum-free medium for 36 h, after which the serum-free medium was changed and the myocytes cultured for 48 h in the absence or presence of 10 μM phenylephrine. The firefly luciferase expression activity in the CPT-Iβ construct was normalized against *Renilla* luciferase expression (pRL-CMV) for each separate experiment after correcting for protein.

Generation of luciferase reporter constructs

A series of CPT-Iβ promoter constructs were created by PCR amplification from the plasmid template p$-$391/$+$80 [13]. In a series of deletion constructs, the 5′-end was shortened from the longest construct, p$-$361/$+$80, to the shortest construct at p$-$31. Similarly, a range of 3′ sequences was ligated on to the 5′ constructs starting with the longest 3′ sequence, $+$80 (containing the first untranslated exon and 60 bp of intron 1), to $-$1 (minus the first exon and intron sequences). Specific primers were designed with an artificial restriction site at the 5′-end of the desired region of the CPT-Iβ promoter sequence. In some experiments, primers were constructed that contained the CPT-Iβ promoter FARE site sequences (ggtgacctttccct, $-$303 to $-$289) at the 5′-end of p$-$31 artificial primers. The PCR products were restricted and cloned into the multiple cloning site of the promoterless firefly luciferase vector pGL3-Basic (Promega). The QuickChange™ site-directed mutagenesis kit (Stratagene, La Jolla, CA, U.S.A.) was used for making the point mutation and short deletion constructs. Briefly, HPLC-purified primers containing the desired site mutant (Table 1) were used in concert with the PfuTurbo DNA polymerase for PCR amplification. After PCR, the wild-type parental pGL3-CPT-Iβ DNA template was digested by *Dpn*I, and the remaining mutated plasmid DNA was transformed into *Escherichia coli*.

Table 1 Oligonucleotides for gel-shift assays and site-mutation constructs

The upper strand sequences are shown. (**a**) Oligonucleotides for making site-mutation or short deletion constructs. (**b**) Oligonucleotides used in gel-shift assays. The wild-type sequences of the CPT-Iβ promoter are in capitals and the mutated nucleotides are shown in lower case letters. The consensus sequences for factor binding and mutations are underlined. Δ indicates the position of deleted bases, with the removed bases shown on the right (delete). The italic letters indicate sequences of pGL3 vector (**a**) or *Nhe*I enzyme linker (**b**).

(**a**)

Element (location)	Oligonucleotide sequences (5′ to 3′)	Wild type
p$-$159($-$70mt)/$+$80 ($-$73 to $-$66)	GTGAGGACAGaaCaGaGtCGGGCAGGGGT	GGCGGGGC
p$-$361/$+$40mt1 ($+$34 to $+$39)	GAGTTGGGGACgAtaAgA*agatctgcga*	CAGCAC
p$-$361/$+$40mt2 ($+$28 to $+$32)	TGAGCTGTGAGTTcatctCCAGCACAaga	GGGGA
p$-$361/$+$40mt3 ($+$22 to $+$27)	AACCTGAGCTGcaAGTcGGGGACCAG	TGAGTT
p$-$361/$+$40d ($+$21 to $+$26)	AACCTGAGCTΔTGGGGACCAGCACA	delete: GTGAGT
p$-$361/$+$40d ($+$11 to $+$30)	GACAGAAGCAΔACCAGCACA	delete: AACCTGAGCTGTGAGTTGGG

(**b**)

Element (location)	Oligonucleotide sequences (5′ to 3′)
FARE ($-$303 to $-$289)	*ctagc*GACTCCTGGTGACCTTTTCCCTACATTCAGC*g*
Exo-Int ($+$11 to $+$36)	*ctagc*AACCTGAGCTGTGAGTTGGGGACCAG*g*
Exo-Intmt ($+$22 to $+$27)	*ctagc*AACCTGAGCTGcaAGTcGGGGACCAG*g*
Nkx ($-$94 to $-$88)/GATA ($-$96 to $-$93)	*ctagc*AGTGGGTATCAAGTTCCCAGTGA*g*
Nkxmt ($-$94 to $-$88)	*ctagc*AGTGGGTAaCAcagTCCCAGTGA*g*

The correct insertion of the CPT-Iβ fragment in every construct was confirmed by DNA sequencing.

To investigate the function of the first untranslated exon/first intron on the transcriptional activity of the heterologous promoter herpes simplex virus (hsv) TK, the following plasmid constructs were employed. Synthesized oligonucleotides to the exon/intron fragment, +11 to +36, inserted in both forward and reverse directions at the *Bam*H1 site upstream from the hsv TK promoter, were isolated from the cloned plasmids. The hsv TK promoter was contained in the pUC plasmid, where the chloramphenicol acetyltransferase ('CAT') open reading frame had been replaced by firefly luciferase (kindly provided by Dr Jon Brandt, Department of Pediatrics, Washington University School of Medicine, St Louis, MO, U.S.A. and Dr Daniel Kelly, Department of Pharmacology and Molecular Biology, Washington University School of Medicine, St Louis, MO, U.S.A.). Clones containing the synthesized oligonucleotides to the first exon/intron, +1 to +40, were also isolated in forward and reverse directions after insertion into the *Hin*dIII site, downstream of the hsv TK promoter constructed in the pGL3-Basic luciferase vector. The hsv TK promoter open reading frame from the pRL-TK vector was inserted into pGL3-Basic luciferase vector between the *Bag*lII/*Hin*dIII restriction sites.

Nuclear extract preparation and electrophoretic mobility-shift assays (EMSA)

Nuclear extracts were prepared from cultured primary rat neonatal cardiomyocytes, neonatal liver cells and HEK-293 cells using the method of Muller et al. [14]. To maintain the phosphorylation state of the nuclear protein extracts, phosphatase inhibitors (50 mM NaF and 1 mM Na_3VO_4) were present in all extraction buffers. The wild-type and mutant oligonucleotides of identified enhancer regions [5] were designed from the rat CPT-Iβ promoter. The sequences of the upper strand of these oligonucleotides with 5′-*Nhe*I ends are shown in Table 1. Double-stranded oligonucleotides were synthesized (Operon Technologies, Alameda, CA, U.S.A.) and 5′ end-labelled with [γ-^{32}P]ATP by T4 polynucleotide kinase. Myocyte nuclear extract (20 μg), neonatal liver (20 μg) or nuclear extracts from HEK-293 cells (5 μg) were incubated for 20 min at 25 °C in a 20 μl reaction mixture containing 25 mM Hepes (pH 7.8), 100 mM KCl, 2 mM dithiothreitol, 0.5 % (v/v) Nonidet P40, 5 % (v/v) glycerol, 0.5 mM PMSF, 0.2 ng/μl each of aprotinin and leupeptin and 2.5 ng/μl dIdC. For competition and supershift experiments, a 20–50-fold excess of unlabelled competitor oligonucleotide or 2 μl of antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.) was preincubated with nuclear extract during the first incubation. Protein–DNA complexes were resolved on 4 % (w/v) polyacrylamide gels under non-denaturing conditions in 0.5 × TBE [45 mM Tris/borate (pH 8.0) and 1 mM EDTA] at 10V/cm for 2 h. The gel was dried and exposed overnight at −70 °C. Immunoblotting was carried out using enhanced chemiluminescence according to the manufacturer's instructions (Perkin-Elmer Life Sciences, Boston, MA, U.S.A.).

Statistics

The reported values represent the means ± S.D. or S.E.M. of three separate cultures, as indicated. Each experiment was performed in triplicate. Each separate neonatal rat myocyte culture was taken from individual biweekly preparations of hearts from 30–50 rat pups. Statistical significance was determined using Student's *t* test for non-paired and paired variates (Sigma Plot Statistics software; SPSS Science, Chicago, IL, U.S.A.).

RESULTS

The first intron contributes to the basal expression of CPT-Iα [7], and thyroid induction of CPT-Iα expression depends on sequences in this region [9]. To examine whether positive transcriptional elements might reside within the first untranslated exon/intron of the CPT-Iβ gene, a series of deletion mutations were designed, each with and without the first exon (+1 to +20) and intron fragment (+21 to +80). Sequential deletion of the minimal CPT-Iβ promoter gradually decreased reporter gene expression in neonatal rat cardiac myocytes (Figure 1), as previously shown [5]. Strong repression of luciferase expression occurred both in the absence and presence of the +80 fragment when a CA box at −117 to −112 was deleted (Figure 1, construct 6) or when the Sp1 site at −74 to −68 was mutated (Figure 1, construct 7). The approx. 50 % decrease in activity with the deletion of +80 in the p−111 and p−159(−70mt) clones was only slightly less than the decrease seen in the full-length p−361/−1 (70 %). These results suggest that there is no preferential interaction between the exon/intron and the vicinal CA box and Sp1 of the CPT-Iβ promoter. Therefore regardless of the specific enhancer region(s) within each fragment, the presence of the first exon/intron consistently and significantly increased reporter gene expression by 2–3-fold (Figure 1).

The results suggest that an independent *cis*-transcriptional element resides within the +1 to +80 region of the CPT-Iβ gene. Therefore we tested whether the positive transcriptional effect would be effective in the context of a small stretch of CPT-Iβ DNA, which contains the transcriptional start site and FARE. In this model construct, the intervening enhancer [GATA and serum response element (SRE)] and initiator (Sp1 and the CA box) elements were deleted and activation of gene expression was physiologically attained in the transfected cells by addition of oleate to the culture medium. FARE (−303 to −289) was ligated to p−31 constructs of the CPT-Iβ reporter gene. Oleate (0.5 mM; 2:1 molar ratio with BSA) was added to activate reporter gene expression. In the absence of FARE, the −31/+80 construct did not respond to oleate (Figure 2, construct 1), and luciferase activity was 12 % of that seen in the p−318/+80 construct (Figure 3). The presence of the FARE element (pFARE−31/+80) restored responsiveness to fatty acid and a 2-fold induction by oleate was observed (Figure 2, construct 2). Similarly, when the FARE site was ligated to −31/+40 (pFARE−31/+40), a 2-fold induction of luciferase expression was observed. Although the presence of FARE also permitted a 2-fold induction of luciferase gene expression in the pFARE−31/−1 construct, the relative magnitude of the induction was reduced by 70 % in the absence of the first exon and intron (Figure 2, construct 4). These data reinforce the concept that an independent positive transcriptional element is contained within the CPT-Iβ exon 1 and first intron. The data suggest further that the sequences are located between +1 and +40, since deletion of +40 to +80 had no effect on the magnitude either of basal transcription or transcription in the presence of oleate (Figure 2, construct 3).

The potential requirement for enhancer regions responsive to the presence of fatty acids, but separate from the peroxisome-proliferator-responsive element ('PPRE') consensus sequence, has been suggested in recent studies on the liver isoform CPT-Iα [15]. The full-length exon/intron (+80) was ligated into deletion constructs of CPT-Iβ and transfected into neonatal cardiac myocytes in the absence and presence of oleate. Constructs

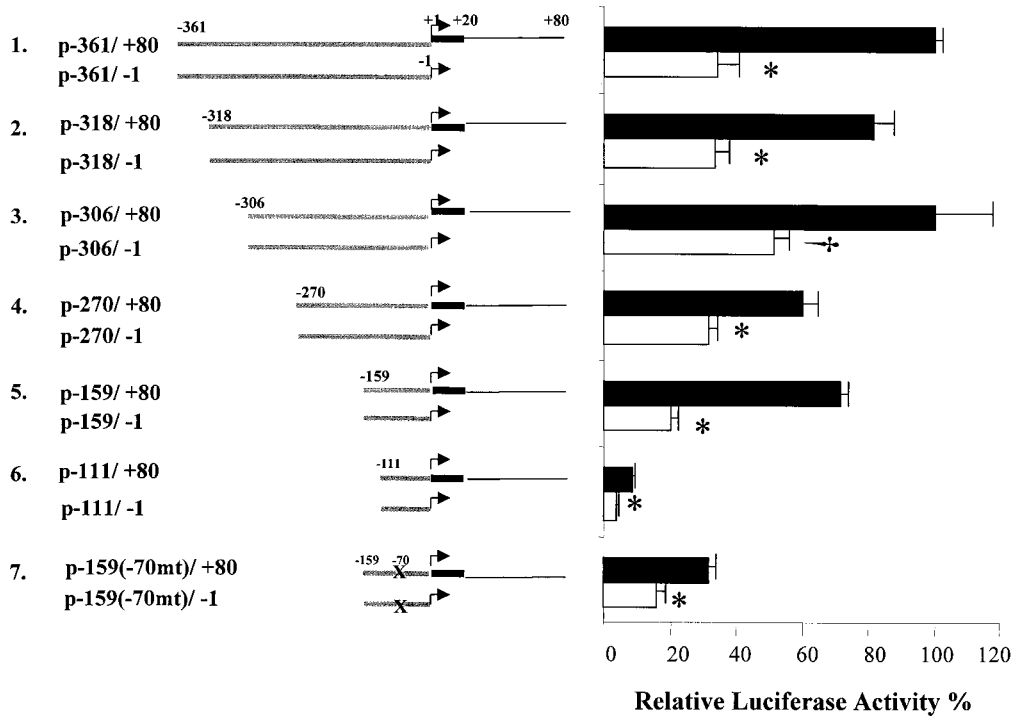

Figure 1 Identification of the active *cis*-transcriptional element(s) within the first exon (+1 to +20) and partial first intron (+21 to +80)

Sequential deletion constructs of the minimal CPT-Iβ promoter with or without the first exon and partial first intron were transfected into neonatal cardiac myocytes. The firefly luciferase expression activity in each CPT-Iβ construct was compared with p−361/+80 (100%) after correction against *Renilla* expression from the co-transfected pRL-CMV plasmid. Solid bars are the CPT-Iβ construct containing the untranslated first exon (+1 to +20) and intron (+21 to +80) sequences. The open bars are values in the absence of the same 3′ sequences. Results are the means ± S.D. of three or more separate experiments, each performed in triplicate. $^*P < 0.01$ and $\dagger P < 0.05$ indicate significant changes in reporter gene expression of each deletion or mutation construct compared with p−361/+80.

containing the FARE site (p−318/+80, p−306/+80 and p−318[dMEF]/+80; where MEF is myocyte enhancer factor) increased luciferase expression by 2.92±0.1-fold (Figure 3). However, constructs lacking the FARE sequence with sequential deletions between p−318 and p−159 also demonstrated enhanced transcription in response to oleate (1.54±0.09-fold increase, Figure 3). Therefore at least 50% of the enhancement in reporter gene expression results from oleate stimulation at sites other than FARE. With the control plasmid (p−31/+80), in the absence of identifiable enhancer sequences, the oleate effect was abolished (Figure 3).

The presence of oleate in the culture medium could exert positive effects on CPT-Iβ transcription as an activator of PPARα as well as by its known role as a major energy substrate of the heart. To test the effect of oleate on myocyte growth, the protein levels of myocytes both in serum-free and serum-containing medium were measured in the presence and absence of oleate. These data suggest that oleate increased the general protein content of the cells over a range from 20% (serum alone, 19.6±0.6 μg per well; serum+oleate, 23.6±1.6 μg per well, $P < 0.05$) to 30% (serum-free, 13.7±0.19 μg per well; serum-free+oleate, 18.2±0.41 μg per well, $P < 0.05$). Thus the stimulatory effect of oleate, independently of FARE, may be to provide the energetic support to synthesize cytosolic and nuclear proteins and cofactors in the myocytes.

The addition of phenylephrine (10 μM) to cultured neonatal cardiac myocytes produced a small, but significant, augmentation in CPT-Iβ gene expression in the full p−361/+80 clone (Table 2). An exon/intron segment confers sensitivity to the cardiac Na^+/Ca^{2+} exchanger in neonatal heart cells [11]. Deletion of the +41 to +80 (p−361/+40) and +1 to +80 (p−361/−1) segment of the untranslated exon and first intron had no effect on the magnitude of the phenylephrine-induced luciferase expression (Table 2).

The data using FARE as an enhancer for CPT-Iβ gene expression (Figure 2) suggested that the positive sequences in the exon/intron are located between nucleotides +1 and +40. To delineate the region that contributes to increased gene transcription, deletion constructs of the exon/intron were prepared within the minimal CPT-Iβ promoter (p−361/+80). The relative luciferase activity of the full clone (p−361/+80) was unchanged in magnitude when compared with the clone where the +41 to +80 region was deleted (Figure 4, compare constructs 1 and 2). These results confirm the absence of any positive effectors within the terminal +41 to +80 bp of the fragment. This conclusion is supported further by a dramatic reduction (3-fold) of reporter gene activity when the p−361 was ligated to +41 to +80 (deletion of +1 to +40; Figure 4, construct 10). Consistent with this finding, the luciferase reporter gene expression of the p−361/+41 to +80 construct (Figure 4, construct 10) was not significantly different from p−361/−1, where the entire exon/intron fragment was deleted (Figure 4, construct 11).

We have identified potential consensus sequences within the +21 to +40 bp of the first intron. These consisted of a downstream promoter element (DPE; +34 to +39; Table 1), myeloid zinc finger 1 (MZF1; +28 to +32; Table 1) and an 'Nkx-like' site (+22 to +27; Table 1; where Nkx is the muscle homologue of the *Drosophila* gene *tinman*). Each sequence was

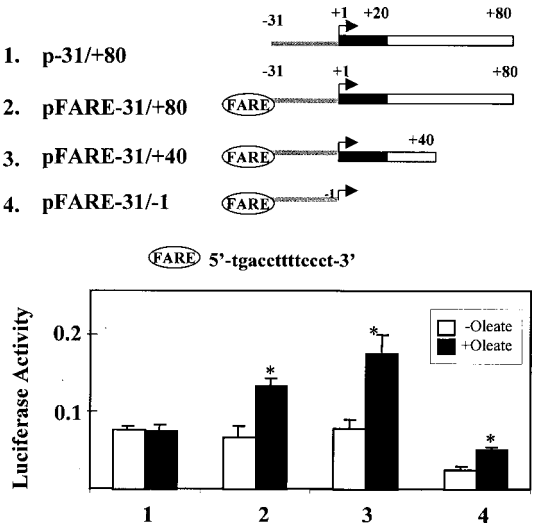

Figure 2 Fatty acid responsiveness of the basal promoter is mediated by the FARE element independently of the first exon (+1 to +20) and partial first intron (+21 to +80)

5′ FARE sequences (−303 to −289) were ligated to CPT-Iβ promoter constructs with or without the first exon and partial first intron, and transfected into neonatal cardiac myocytes (upper panel). The cells were cultured in serum-free medium in the absence (open bars) or presence (solid bars) of 0.5 mM oleate (in a 2:1 molar ratio to BSA) (lower panel), as described in the Experimental section. Firefly luciferase activity in each CPT-Iβ construct was compared with p−31/+80 after normalization to protein content and *Renilla* expression. Results are means ± S.D. of three different experiments, each performed in triplicate. $^*P < 0.05$ compared with treatment without oleate.

Table 2 Effect of first exon/intron (+1 to +40) on phenylephrine up-regulation of CPT-Iβ gene expression

Three 3′ end deletion constructs, p−361/+80 (exon 1+60 bp of intron 1), p−361/+40 (exon 1+20 bp of intron 1) and p−361/−1 (without exon 1 and intron 1) were transfected into myocytes and subsequently treated with 10 μM phenylephrine (PE) as described in the Experimental section. Firefly luciferase expression activity in each CPT-Iβ construct was compared with the p−361/+80 construct after correction for protein content and *Renilla* luciferase expression. Fold induction was calculated by dividing the activity in the presence of PE (+PE) with that in the absence of PE (−PE). Results are means ± S.D. ($n = 3$ different culture preparations). $^*P < 0.05$ when PE-treated cells (+PE) were compared with cells in the absence of PE (−PE).

	Relative luciferase activity		
Plasmid	−PE	+PE	Fold Induction
p−361/+80	1.00 ± 0.11	1.57 ± 0.10*	1.57 ± 0.10*
p−361/+40	0.96 ± 0.11	1.47 ± 0.06*	1.53 ± 0.11*
p−361/−1	0.40 ± 0.05	0.65 ± 0.13*	1.61 ± 0.13*

individually mutated as indicated in Table 1 within the p−361/+40 construct. Neither mutation of the DPE (mt1; Figure 4, construct 3) nor MZF1 [16] (mt2; Figure 4, construct 4) affected reporter gene expression. In contrast with the above mutations, the p−361/+40mt3 (deletion of +22 to +27; Figure 4, construct 5) decreased reporter gene activity by 50%. Furthermore, deletion of that region [d(+21 to +26); Figure 4, construct 6] within +1 to +40, reduced reporter gene expression to 39% of that for p−361/+40. Deletion of +1 to +20 and ligation of p−361 to +21 to +40 [p−361/(+21 to +40); Figure 4, construct 7] restored reporter gene activity to 73% of the control

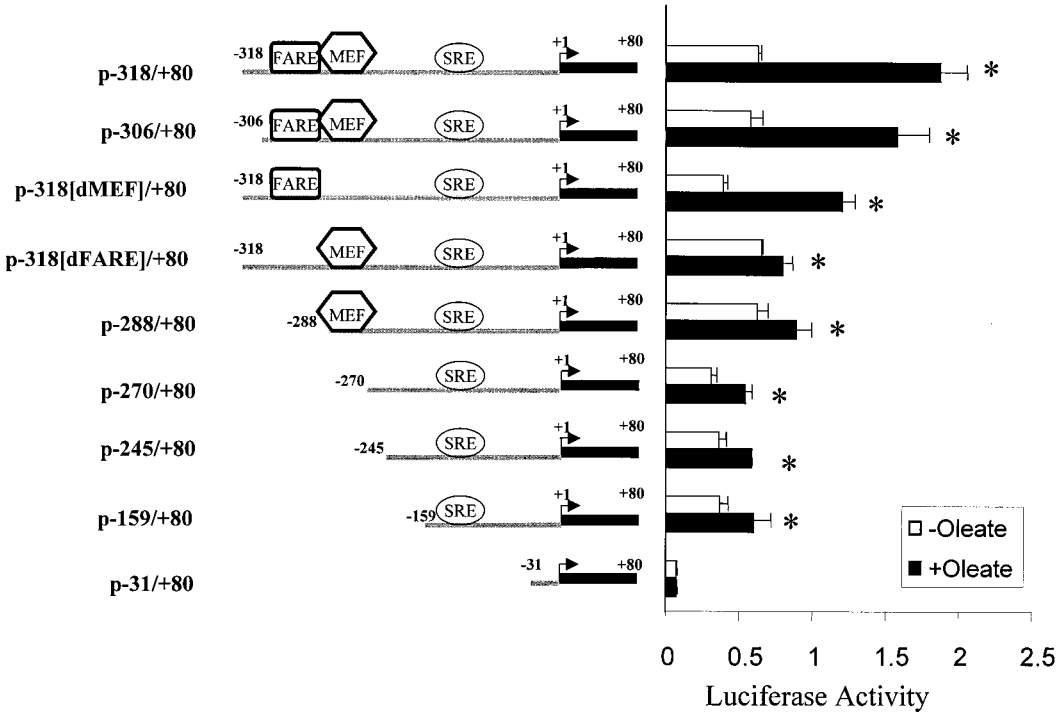

Figure 3 Fatty acid activation of the CPT-Iβ promoter in the presence or absence of FARE

Serial truncated and/or deleted constructs on the basis of p−318/+80 were transfected into neonatal cardiac myocytes as described in the Experimental section. The cells were then cultured in serum-free medium in the absence (open bars) or presence (solid bars) of 0.5 mM oleate (in a 2:1 molar ratio to BSA) for 30 h. Firefly luciferase expression in each CPT-Iβ construct was corrected for protein content and *Renilla* expression. Results are means ± S.D. of three different experiments, each performed in triplicate. $^*P < 0.05$ compared with treatment without oleate. The location of FARE, MEF and SRE sites are indicated.

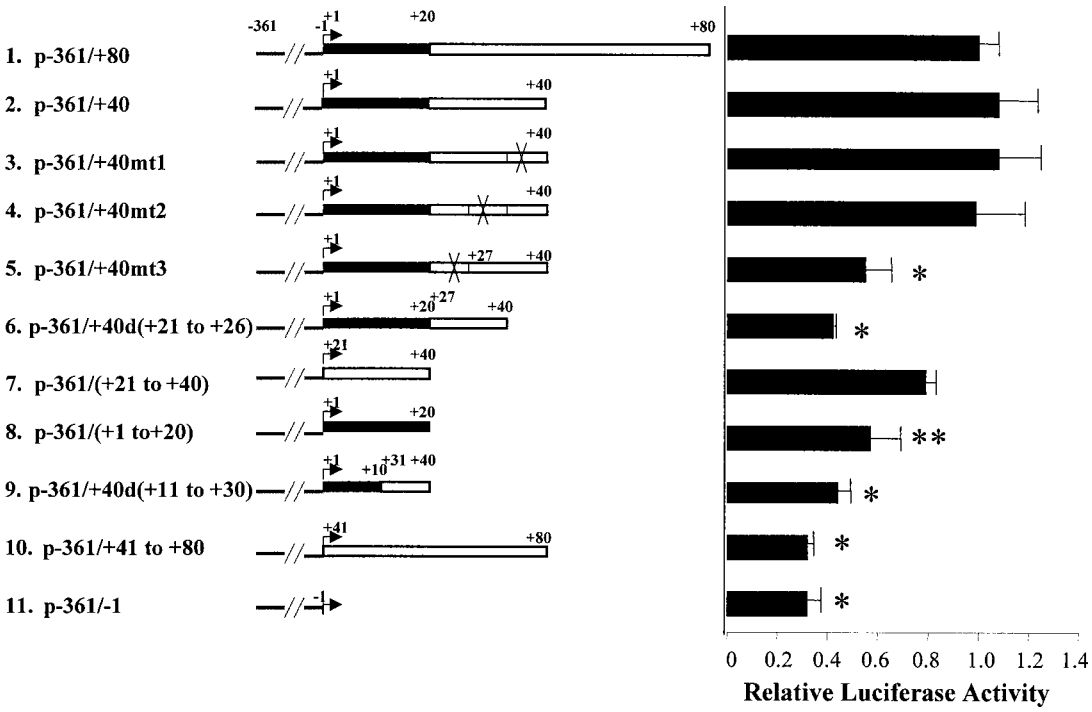

Figure 4 Delineation of the *cis*-transcriptional element(s) in the first exon and partial intron

Serial 3′-end deletions, short deletions and point mutations on the basis of p−361/+80 were transfected into neonatal cardiac myocytes. 'X' represents the point mutation region in the construct. The firefly luciferase expression activity in each CPT-Iβ construct was compared with p−361/+80 (1.0) after normalization to *Renilla* expression. Results are means ± S.D. from three independent experiments, each performed in triplicate. $^{*}P < 0.01$ and $^{**}P < 0.05$ compared with control (p−361/+80).

value. The reporter gene activity of p−361/(+1 to +20) was identical with the activity of the p−361/+40mt3, i.e. 0.51±0.12 compared with 0.53±0.1 (means±S.D.) respectively (Figure 4, compare constructs 5 and 8). Deletion of sequences +11 to +30 in the p−361/+40d(+11 to +30) construct (Figure 4, construct 9) decreased luciferase activity by 60%, confirming that the region encompassing bp +21 to +26 (Figure 4, construct 6) was essential for increased expression of CPT-Iβ.

The seven bases from +21 to +26 (GTGAGTT) have similarity (86%) to the consensus Nkx-binding element (NKE) sequence (TNAAGTG). Previous findings showing NKE in untranslated regions of genes suggest that they might be regulated during development in a modular fashion [17,18]. Co-expression of Nkx2.5 and the CPT-Iβ reporter gene (p−391/+80) elevated luciferase activity by 6.64±0.58-fold in CV-1 cells. The reporter construct, p−361/+80mt3, contains the identical mutations in +22 to +27 as described for the p−361/+40mt3 (see Table 1). The former construct was co-transfected with the Nkx2.5 expression vector into CV-1 cells. The reporter gene activity was again enhanced 6.867±0.365-fold when the reporter gene was mutated in the first intron (mt3). Double mutations in the putative Nkx2.5 site in the 5′-flanking region at −96 to −88 and in the first intron at +22 to +27 decreased reporter gene expression to 4.23±0.716-fold, a 38% decrease in CPT-Iβ gene expression. We have shown previously that the point mutation in the Nkx site (−96 to −88) is adjacent to, and overlaps, the GATA site [5]. This mutation was proposed to interrupt GATA-4 binding at −96 to −93. Nonetheless, no change in the magnitude of the inductive effect of Nkx overexpression on reporter gene activity in the presence or absence of mt3 (+22 to +27) suggested that it is Nkx interaction at −94 to −88 (but not +22 to +27) that enhances CPT-Iβ gene expression.

EMSAs were used to examine the protein–DNA interactions between nucleotides spanning the first exon and the first intron (+11 to +36). Nuclear extracts were prepared from neonatal cardiac myocytes, neonatal rat liver and HEK-293 cells. The latter cell line was included, since it was used for transfection studies using a heterologous promoter containing the exon/intron sequences (see below). As shown in Figure 5, two complexes were retained on the gel by the myocyte nuclear proteins, as indicated by arrows marked 1 and 2 (left). These shifts were completely inhibited by unlabelled oligonucleotides to the exon/intron (+11 to +36) and intron (+21 to +40) regions (Figure 5). Partial competition of the higher-molecular-mass-band shift occurred in the presence of unlabelled exon sequences (+1 to +20). These results suggest that the lower-molecular-mass complex (band 2) is derived from within the intron, whereas the higher-molecular-mass complex (band 1) results from protein interaction with nucleotides that may span the exon/intron boundary.

Nuclear extracts from neonatal liver have a very low abundance of discreet proteins specific for this region (results not shown). However, nuclear extracts from HEK-293 cells produced two band shifts (Figure 5). These band shifts (arrows 3 and 4) ran at different mobilities compared with the cardiac extracts. Whereas the upper band was inhibited effectively by all three unlabelled probes, the higher molecular-mass band (band 4) was partly inhibited only by the exon/intron oligonucleotide. Differences in the protein binding patterns between the cell types suggested tissue differences in nuclear–protein interaction with the untranslated regions from the muscle-specific CPT-Iβ (Figure 5).

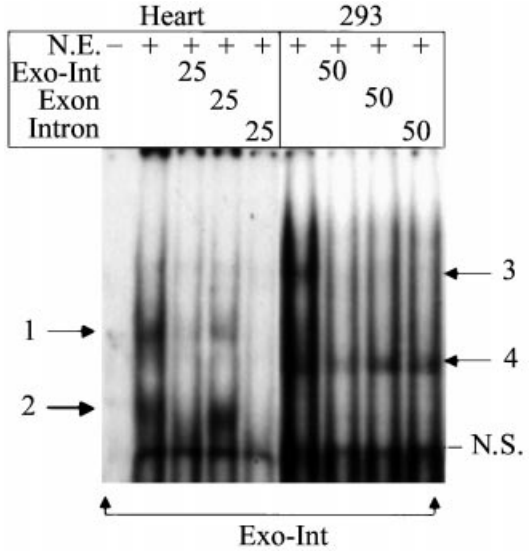

Figure 5 Identification of protein binding to the region of the active *cis*-transcriptional element

Oligomers of the exon/intron sequence +11 to +36 (Exo-Int) were 5′-end-labelled with [γ-^{32}P]ATP and incubated with nuclear protein extracts (N.E.) from neonatal cardiac myocytes (heart) or HEK-293 (293) cells. Unlabelled competitor oligonucleotides +11 to +36 (Exo-Int), +1 to +20 (Exon) and +21 to +40 (Intron) were included as indicated. Protein–DNA complexes were resolved on 4% (w/v) polyacrylamide gels under non-denaturing conditions. Arrows indicate the positions of the major specific protein–DNA complexes in myocytes (numbered 1 and 2) and HEK-293 cells (numbered 3 and 4). N.S. indicates the position of non-specific protein–DNA interaction.

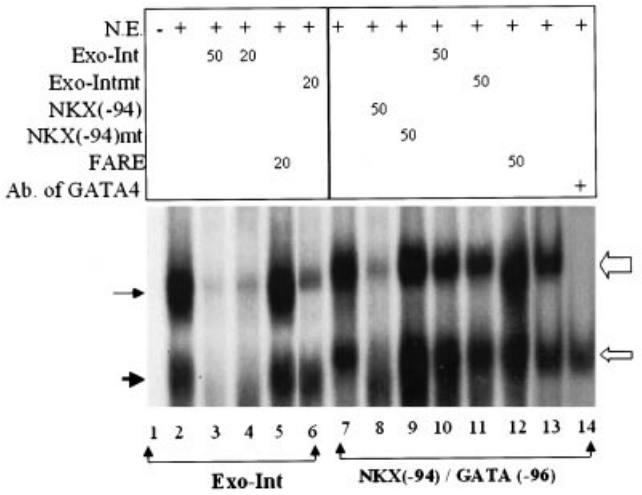

Figure 6 Band-shift analysis of the first exon/intron region (+11 to +36) compared with Nkx/GATA-binding sites (−96 to −88) in neonatal cardiac myocytes

Oligonucleotides representing the exon/intron region +11 to +36 (Exo-Int) and Nkx/GATA sites −96 to −88 [Nkx (−94)/GATA (−96), Nkx (−94 to −88)/GATA (−96 to −93)] were 5′ end-labelled with [γ-^{32}P]ATP and incubated with nuclear protein extracts (N.E.) of neonatal myocytes. Unlabelled competitor oligonucleotides of wild-type [Exo-Int; and NKX(−94), Nkx (−94 to −88)] or mutant [Exo-Intmt, Exo-Intmt (+22 to +27); and NKX(−94)mt, Nkxmt (−94 to −88)] and non-consensus sequences [FARE, FARE (−303 to −289)], or GATA-4 antibody for supershift experiments, were included as indicated. Protein–DNA complexes were resolved on 4% (w/v) polyacrylamide gels under non-denaturing conditions. The positions of the major specific protein–DNA complexes bound to Exo-Int (+11 to +36) and on Nkx (−94 to −88)/GATA (−96 to −93) region (−102 to −80) are indicated by solid and open arrows respectively. The absence of a band at the upper open arrow in lane 14 is due to cropping of the supershifted GATA-4–antibody complex [5].

Protein–DNA complex formation was assessed in nuclear extracts from neonatal cardiac myocytes using the exon/intron (+11 to +36) and putative Nkx/GATA-binding site (−96 to −88) oligonucleotide probes (Figure 6). In the left portion of Figure 6 (labelled Exo-Int), the +11 to +36 double-stranded oligonucleotide was incubated with nuclear extracts from neonatal cardiac myocytes. Again, two prominent bands were present (lane 2, left arrows). These bands were competed by 50- and 20-fold of unlabelled +11 to +36 (lanes 3 and 4), but not by FARE (−303 to −289) (lane 5) or the exon/intron mutation oligonucleotide (Exo-Intmt; lane 6), demonstrating specificity for protein binding to +11 to +36. The Nkx/GATA-site EMSA, corresponding to the region −96 to −88 (containing overlapping Nkx/GATA sites), was run simultaneously with the exon/intron EMSA, and also revealed two major protein bands. However, these latter protein–DNA complexes were present at slightly higher molecular masses (Figure 6, compare lane 7 with lanes 5 and 6). Moreover, neither the mutated −94 to −88 (Nkxmt; Figure 6, lane 9) nor a 50-fold excess of unlabelled Exo-Int or Exo-Intmt oligonucleotides (Figure 6, lanes 10 and 11) competed with either of the −96 to −88 oligonucleotide-shifted bands. Similar results were seen with the FARE (−303 to −289) probe (Figure 6, lane 12). Finally, only one of the Nkx/GATA-4 shifts was competed and supershifted by the GATA-4 antibody (Figure 6, lane 14, upper right arrow). The lower band in the Nkx/GATA oligonucleotide gel shift was not competed by the GATA-4 antibody. Its role as a potential Nkx-binding complex has not been established. Nevertheless, this band is unlikely to have bear any relationship with the second lower-molecular-mass species in the exon/intron gel shift (lower left arrow). This conclusion is reached on the basis of the differences in complex mobility. The experiments demonstrating reporter gene induction by Nkx overexpression in the presence of the +22 to +27 mutation (mt3; see above) reinforce the likelihood that the exon/intron does not contain Nkx-binding sites.

Three different CPT-Iβ constructs, including the control reporter construct missing the exon/intron (p−361/−1), and the constructs either containing the entire first 40 bases, or only the +21 to +40 sequences of the untranslated region, were transfected into cardiac myocytes and HEK-293 cells (Figure 7A). Myocyte activity of the p361/+40 plasmid was 14-fold higher than reporter gene expression in the HEK-293 cells. Truncation of both the exon and intron regions (p−361/−1) decreased CPT-Iβ reporter gene expression by 4-fold, but had no effect on the low activity present in the HEK-293 cells (Figure 7A). When the partial first intron (+21 to +40) was ligated to p−361, the reporter gene activity in the HEK-293 cells was unaffected. In cardiac myocytes, transfection of p−361/(+21 to +40) restored luciferase expression to 73% of control (p−361/+40). These results in myocytes are identical with those seen in Figure 4 (construct 7) for the p−361/(+21 to +40) clone, and suggest that sequences in both the first exon and first intron are necessary for cardiac-specific enhancement of CPT-Iβ gene expression.

The ability of the exon/intron region to enhance reporter gene expression was examined outside the context of the CPT-Iβ gene, and in myocytes versus HEK-293 cells. Either the +1 to +40 or the +11 to +36 sequence was ligated to the hsv TK/luciferase reporter gene in sense and antisense directions. When the exon/intron region was placed following transcription start of the hsv TK/luciferase promoter, i.e. in its natural position in

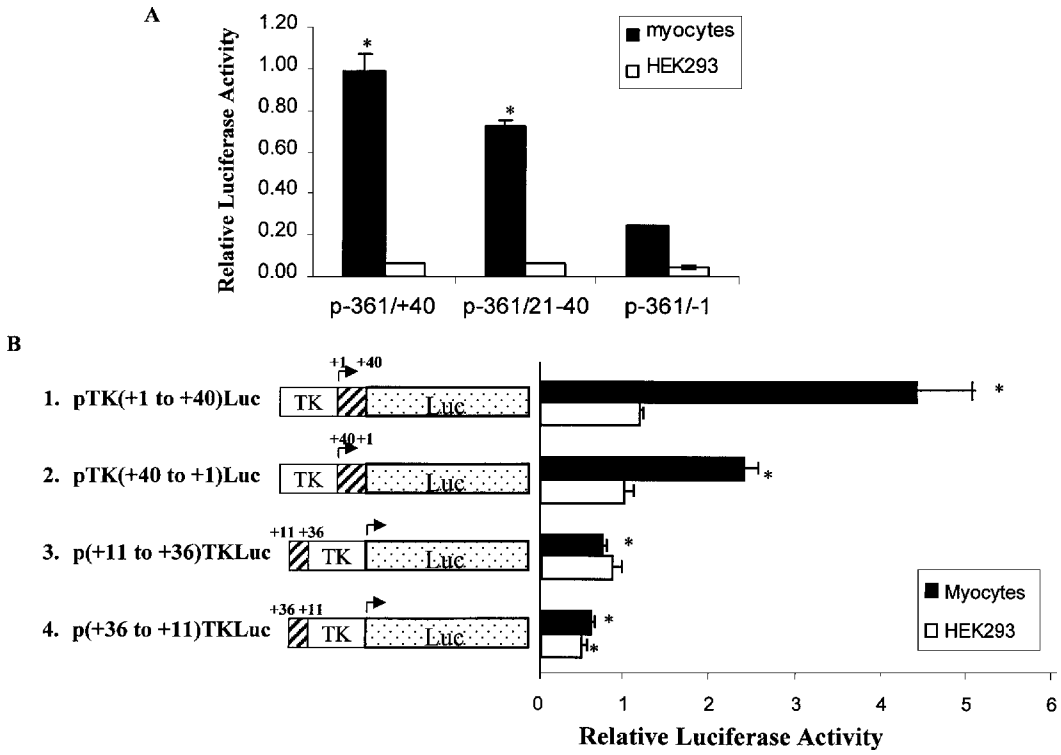

Figure 7 Cardiac preference, positional and orientation effects of the exon/intron-enhancer element of CPT-Iβ

(**A**) Three CPT-Iβ promoter constructs, with (p$-$361/$+$40) and without the 3′ untranslated region (p$-$361/$-$1), or with only the partial first intron ($+$21 to $+$40), were transfected into neonatal cardiac myocytes (solid bars) and HEK-293 cells (open bars). Firefly luciferase expression activity in each construct was normalized to *Renilla* expression. The luciferase activity was then normalized to p$-$361/$+$40. Results are means$\pm$S.D. of three separate experiments from different cell cultures, each performed in triplicate. *$P < 0.05$ compared with p$-$361/$-$1. (**B**) Four CPT-Iβ constructs of the exon/intron elements were ligated into the heterologous hsv TK promoter, and transfected into neonatal cardiac myocytes (solid bars) and HEK-293 cells (open bars) as described in the Experimental section. In constructs 1 and 2, the $+$1 to $+$40 element was inserted 3′ to the TK transcription start in the 5′ to 3′ (construct 1) and 3′ to 5′ (construct 2) orientations respectively. In constructs 3 and 4, both position and orientation were changed by ligating the $+$11 to $+$36 bases at the 5′-end of the hsv TK promoter in the 5′ to 3′ (construct 3) and in the 3′ to 5′ (construct 4) orientation. The firefly luciferase expression activity was normalized to the respective empty TK/luciferase constructs after correcting for protein concentration and *Renilla* expression. Results are means$\pm$S.E.M. of three independent experiments, each performed in triplicate. *$P < 0.05$ when compared with the respective TK/luciferase control vector. Luc, luciferase.

the CPT-Iβ gene, transfection into cardiac myocytes produced a 4-fold enhancement of luciferase expression (Figure 7B). No enhancement was seen in transfected HEK-293 cells, so that luciferase activity was no different when normalized to expression from the empty vector (i.e. 1; Figure 7B, construct 1). Reversing the orientation of the inserted DNA still produced a doubling of TK/luciferase expression, suggesting that the inverse orientation still allowed transcriptional activation of luciferase expression (Figure 7B, construct 2). Interestingly, moving the exon/intron element to the 5′-end of the hsv TK promoter dramatically diminished TK/luciferase expression to levels seen with the empty vector (Figure 7B, constructs 3 and 4). In all cases the expression of the TK reporter gene in HEK-293 cells was not significantly different from the empty vector or slightly inhibitory in both cell types when added before the hsv TK promoter.

DISCUSSION

Intron transcriptional enhancers have been identified in a variety of genes. Their function is described as directing tissue-specific expression [18,19], developmental regulation of gene demethylation [20], complex modular organization to accommodate anatomical evolution of an organ [17], alternative promoter utilization [21], and protein concentration dependent-up-regulation of transcriptional activity, important in cell transformation [22]. Expression of CPT-Iα has been shown to increase 25-fold in hyperthyroid rats compared with hypothyroid rats [23]. Several protein-binding domains in the first intron of the CPT-Iα gene are required for the 3,3′,5-tri-iodothyronine induction in the absence of any intronic thyroid-response element ('TRE') sequences [9]. Similar to the liver isoform of CPT-I, CPT-Iα, there exists at least one upstream enhancer region in CPT-Iβ that augments reporter gene activity in cardiac myocytes. This region has been localized to nucleotides $+$11 to $+$36, which span the latter part of the first exon and the initial segment of the first intron. In contrast with the CPT-Iα intronic element, which is specifically required for the 3,3′,5-tri-iodothyronine induction of the liver isoform gene expression, the nucleotides that up-regulate CPT-Iβ expression in cardiac myocytes appear independent of any specific enhancer element in the 5′-flanking sequences [9]. The exon/intron nucleotides impose a general effect on gene expression. The proteins that bind to the region ($+$11 to $+$36) exhibit unique mobilities compared with protein complexes from HEK-293 cells. Furthermore, we could not detect any significant level of liver nuclear protein binding to these sequences (results not shown). The cardiac binding proteins may play a more important role in fine-tuning the levels of this enzyme to the energetic needs of the postnatal heart, since GATA-4 and SRF are clearly required for transcriptional activation of CPT-Iβ [5].

In addition to intron regulation of CPT-Iα and CPT-Iβ, other cardiac genes are regulated by enhancer elements present in the first intron. The human cardiac homeobox gene CSX1, containing the 3′ half of the first intron, is transactivated by overexpression of CSX1, suggesting lineage-restricted autoactivation of this gene [18]. The cardiac Na^+/Ca^{2+} exchanger (NCX) gene also contains an 32 bp intronic region that appears requisite for α-adrenergic stimulation [11]. This region may contain more than one element, and its sequences bear no consensus with any known transcription factors. Its activity also spans the first exon/intron boundary, as reported in the present study for the CPT-Iβ gene. However, the sequence information for the intronic enhancer region in the NCX gene does not resemble our putative enhancer, and in contrast with the NCX gene, no role in α-adrenergic regulation of CPT-Iβ was demonstrated. It is also important to note that the presence of the first exon/intron in the minimal promoter of the CPT-Iβ gene ($-31/+40$) demonstrates no up-regulation of gene expression by long-chain fatty acids independent of the FARE site. These results are in contrast to recent findings on the first intron of the liver CPT-Iα gene [15]. However, more extensive analysis of the entire CPT-Iβ first intron (469 bp, [13]) is required.

Enhancers and promoters have been identified in *cis*-elements located downstream from transcription initiation sites in several genes [20,21,24]. Within the context of the $+40$ downstream stimulatory region, we identified a consensus sequence for a conserved DPE. This sequence is present in many TATA-box-deficient promoters and is conserved from *Drosophila* to humans [25,26]. It is believed that the DPE acts in conjunction with the initiation site sequence to provide a binding sequence for transcription factor IID (TFIID) in the absence of a TATA box [25]. However, in the context of the CPT-Iβ gene, mutation of the DPE consensus sequence (p$-361/+40$mt1) had no effect on the extent of reporter gene enhancement imparted by the p$-361/+40$ construct.

Binding of Sp1 to regulatory sequences within the CPT-Iβ promoter region is important for transcriptional up-regulation and also may be important for start-site selection [27]. For the first time, recent reports suggest that Sp1-like proteins bind to *cis*-elements located downstream from initiation start sites to impart tissue-specific expression [24]. Another element, termed multiple start site element downstream (MED) 1 may be important for start-site selection in some TATA-less promoters [28]. It is not clear whether MED-1 and its cognate binding proteins act as selectors to activate multiple start sites. Instead, MED-1 may support the use of the $+1$ site in many cell types and, together with other *trans*-acting factors, act to impose a higher-order of regulation on the recognition of the start site element. Although these particular sequences are not present in the active region of our exon/intron boundary, there is 83% similarity of $+21$ to $+26$ bases in the CPT-Iβ first intron to a sequence 35 nucleotides downstream of *Drosophila* Class II genes, i.e. (G/A/T)-(C/T)-A-(T/G)-T-G [29]. This sequence contributes to the interaction of TFIID on a wide spectrum of promoters. In our gene, however, this intron span is effective, but not completely sufficient, since first exon sequences also are required to restore completely the enhancement in reporter gene activity.

Finally, we report that the CPT-Iβ-enhancer element when placed in the context of the heterologous hsv TK promoter significantly increases reporter gene expression in a position-dependent fashion. Transcriptional activation is abolished when the $+11$ to $+36$ exon/intronic sequences are placed 5′ to the hsv TK promoter. When placed 3′ to the TK transcription start site, the reversed orientation of the enhancer still stimulates transcription of the luciferase reporter, indicating appropriate mRNA splicing. However, the extent of transcriptional activation by the reverse construct is diminished by 50%, suggesting that the orientation of the enhancing sequence imparts regulatory significance to transcriptional enhancement. Finally, the exon/intron sequences are silent or inhibitory when placed 5′ to the hsv TK promoter, i.e. the element can not act distally to the transcription start site. Thus the features of this sequence are not consistent with the characteristics of a 'classical' enhancer in that it exhibits both position and orientation dependence. These results are reminiscent of the recent report on the human norepinephrine transporter gene where a *cis*-element at an exon/intron junction exhibits promoter-enhancing effects in a position and orientation-dependent manner [30]. Inhibitory effects on transcription, which occur upon inversion of this element in the correct downstream position, suggest that the proteins bound to the DNA may be sufficiently asymmetrical, so that the resulting configuration is less efficient functionally.

In summary, we report in the present study the enhancing effect of untranslated sequences downstream from the initiation start site of CPT-Iβ. The region responsible for up-regulation of reporter gene expression appears to span the first exon/intron boundary, and mutation in the first intron decreases reporter gene expression by 70%. However, the full magnitude of the enhancement of gene expression also requires sequences present in the first, untranslated exon. Using nuclear extracts from cardiac myocytes, the proteins that bind to these regions migrate with distinct mobilities compared with DNA–protein complexes where nuclear protein was derived from HEK-293 cells. Since the increase in gene expression appears to be independent of any specific upstream enhancer element, our data suggest that there is a binding sequence spanning the exon/intron boundary, possessing both positional and orientation dependence, which acts to regulate transcriptional activity and increase utilization of the CPT-Iβ initiation start site. The magnitude of gene regulation of the muscle-specific mitochondrial protein may depend not only on the energy needs of the cardiac myocyte, but also on the availability of binding factors and cofactors that interact in a complex transcriptional code.

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