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Synergistic role of specificity proteins and upstream stimulatory factor 1 in transactivation of the mouse carboxylesterase 2/microsomal acylcarnitine hydrolase gene promoter

Tomomi FURIHATA, Masakiyo HOSOKAWA[1], Tetsuo SATOH and Kan CHIBA

Laboratory of Pharmacology and Toxicology, Graduate School of Pharmaceutical Sciences, Chiba University, Chiba 260-8675, Japan

Mouse carboxylesterase 2 (mCES2), a microsomal acylcarnitine hydrolase, is thought to play some important roles in fatty acid (ester) metabolism, and it is therefore thought that the level of transcription of the *mCES2* gene is under tight control. Examination of the tissue expression profiles revealed that mCES2 is expressed in the liver, kidney, small intestine, brain, thymus, lung, adipose tissue and testis. When the *mCES2* promoter was cloned and characterized, it was revealed that Sp1 (specificity protein 1) and Sp3 could bind to a GC box, that USF (upstream stimulatory factor) 1 could bind to an E (enhancer) box, and that Sp1 could bind to an NFκB (nuclear factor κB) element in the *mCES2* promoter. Co-transfection assays showed that all of these transcription factors contributed synergistically to transactivation of the *mCES2* promoter. Taken together, our results indicate that Sp1, Sp3 and USF1 are indispensable factors for transactivation of the *mCES2* gene promoter. To our knowledge, this is the first study in which transcription factors that interact with a *CES2* family gene have been identified. The results of the present study have provided some clues for understanding the molecular mechanisms regulating *mCES2* gene expression, and should be useful for studies aimed at elucidation of physiological functions of mCES2.

Key words: acylcarnitine hydrolase, carboxylesterase, promoter characterization.

INTRODUCTION

Recent studies have suggested that carboxylesterases (CESs; EC 3.1.1.1) are involved in fatty acid (ester) metabolism, because it has been shown that acyl-CoA [1,2], acylcarnitine [3,4], acylglycerol [5] and phospholipid [6] can be substrates for CESs. CESs belong to a serine hydrolase superfamily, and are expressed ubiquitously in various mammals [7], with high levels of CES activity often being detected in the liver, small intestine and kidney. CESs comprise a multigene family, and tissue distribution profiles and preferential substrates of fatty acid esters are clearly different among CES isoenzymes, suggesting that each CES isoenzyme plays a distinct role in fatty acid (ester) metabolism.

We have recently reported that an inducible acylcarnitine hydrolase in mouse liver microsomes, termed mouse carboxylesterase 2 (mCES2), belongs to the CES2 family [3]. mCES2 showed a significant contribution to acylcarnitine hydrolysis in di-(2-ethylhexyl)phthalate-treated mouse liver microsomes, and is therefore thought to play some important roles in fatty acid (ester) metabolism in the ER (endoplasmic reticulum). It has been reported that, as observed in mitochondria or peroxisomes, the carnitine/acylcarnitine system exists in microsomes to supply the intraluminal space with acyl-CoA, which is widely used in the formation of various fatty acid esters. Abo-Hashema et al. [8] presented evidence of microsomal TAG (triacylglycerol) synthesis via a lipolysis/esterification pathway, the critical point in very-low-density lipoprotein assembly [9], involving CATs (carnitine acyltransferases). In their studies, microsomal TAG synthesis was found to be dependent on the presence of both extraluminal carnitine and intraluminal CoASH, and glibenclamide, an inhibitor of liver microsomal CAT [10], was found to greatly reduce the amount of luminal synthesis of TAG. Results of subsequent studies supporting the results of their study have recently been reported by Washington et al. [11]. They showed that the carnitine/acylcarnitine system exists in the ER of the rat small intestine, and that its inhibition by etomoxir, a specific carnitine palmitoyltransferase-1α inhibitor [12], causes significant decrease in lymphatic output of chylomicron TAG. Since mCES2 can preferentially hydrolyse medium- and long-chain acylcarnitines, the expression level of mCES2 might significantly affect the rate of TAG synthesis in the ER by modulating the amount of acyl-CoA generated from acylcarnitine by intraluminal CAT. Therefore determination of the mechanisms by which *mCES2* gene expression is regulated would be an important step towards elucidation of how mCES2 is involved in TAG synthesis, as well as other fatty acid ester metabolisms within the ER.

Experimental data to elucidate molecular mechanisms controlling *CES* gene expression are limited. The mouse CES1 isoenzyme showed a developmental expression pattern in the liver, and it has been suggested that Sp1 (specificity protein 1) and Sp3 interacting with the mouse *CES1* promoter region could be involved in the ontogenic profile of the mouse *CES1* gene expression [13]. Other investigators showed that SREBP2 (sterol regulatory element-binding protein 2) and Sp1 could bind to

Abbreviations used: CAT, carnitine acyltransferase; C/EBP, CCAAT/enhancer binding protein; (m)CES, (mouse) carboxylesterase; DMEM, Dulbecco's modified Eagle's medium; dsDNA, double-stranded DNA; E box, enhancer box; EMSA, electrophoretic mobility-shift assay; ER, endoplasmic reticulum; FAS, fatty acid synthase; GAPDH, glyceraldehyde-3-phosphatase dehydrogenase; NFκB, nuclear factor κB; PLB, passive lysis buffer; RLM 5′-RACE, RNA ligase-mediated and oligo-capping rapid amplification of 5′-cDNA ends; RT-PCR, reverse transcriptase-PCR; Sp1, specificity protein 1; SL2, Schneider's *Drosophila* line 2; SREBP2, sterol regulatory element-binding protein 2; TAG, triacylglycerol; USF, upstream stimulatory factor.

[1] To whom correspondence should be addressed (email masakiyo@p.chiba-u.ac.jp).

The nucleotide sequence data reported will appear in DDBJ, EMBL, GenBank® and GSDB Nucleotide Sequence Databases under the accession number AB110074.

the promoter regions of the rat and human CES1 isoenzymes respectively [14,15]. With regard to the CES2 family, it has recently been reported that an approx.-1.6-kbp fragment of the 5′-flanking region of the human *CES2* gene was isolated and found to contain three functional promoters [16]. However, transcriptional factors interacting with the promoters have not been identified. No other information about the mechanisms regulating *CES2* gene transcription is currently available.

The aim of the present study was to determine the molecular mechanisms controlling *mCES2* gene expression. First, we obtained the tissue expression profiles of mCES2 mRNA and protein. Given the fact that mCES2 is ubiquitously expressed, we characterized the promoter region of the *mCES2* gene and analysed the mechanisms regulating the basal promoter activity. The results showed that three transcriptional factors contribute synergistically to the promoter activity of the *mCES2* gene. This study provides useful information for further studies aimed at elucidation of the molecular mechanisms controlling *mCES2* gene expression.

EXPERIMENTAL

Preparation of mouse tissue homogenates and esterase activity staining after non-denaturing PAGE

Three 8-week-old C57BL/6 male mice (Japan SLC, Inc., Shizuoka, Japan) were killed, and nine tissues (brain, thymus, lung, heart, liver, kidney, small intestine, epididymal adipose tissue and testis) were removed from each mouse. Tissue samples of equal weights were pooled, and the homogenate was prepared using a homogenizer. The protein concentration was determined by using a Bio-Rad *Dc* Protein Assay Kit (Bio-Rad Laboratories, Hercules, CA, U.S.A.). Esterase activity staining after non-denaturing PAGE was performed according to the method of Mentlein et al. [17]. Different amounts of homogenate were used in this method to adjust band intensity: 20 μg of brain, 15 μg of thymus, 7 μg of lung, 10 μg of heart, 1 μg of liver, 5 μg of kidney, 5 μg of small intestine, 2.5 μg of adipose tissue and 10 μg of testis. The homogenate of Sf9 cells expressing mCES2 was used as a positive control [3].

Total RNA preparation and RT-PCR (reverse transcriptase-PCR)

Three 8-week-old C57BL/6 male mice (Japan SLC) were killed, and removed tissue samples of equal weights were pooled. Total RNA from each tissue (brain, thymus, lung, heart, liver, kidney, small intestine, epididymal adipose tissue and testis) was extracted using TRIzol® reagent (Invitrogen, Carlsbad, CA, U.S.A.). To prevent contamination with genomic DNA, the extracts were treated with DNase I (Takara Shuzo, Shiga, Japan). Subsequently, first-strand cDNA was synthesized from 2 μg of each tissue RNA by Ready-To-Go RT-PCR Beads with an oligo(dT) primer (Amersham Biosciences, Piscataway, NJ, U.S.A.). For verification of synthesis of equal amounts of cDNA, PCR was performed (94 °C for 15 s, 49 °C for 20 s, 68 °C for 40 s; 35 cycles) using a set of primers for detection of mouse *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase) gene expression: sense, 5′-TGCACCACCAACTGCTTA-3′; and antisense, 5′-GGATGCAGGGATGATGTTC-3′. Another set of primers (sense, 5′-TTCAAGGATGTCAGACCACC-3′; and antisense, 5′-AACACATTTTTTTTGATACAGGGTA-3′) was used for PCR (94 °C for 15 s, 55 °C for 20 s, 68 °C for 40 s; 35 cycles) to detect *mCES2* gene expression. PCR conditions are shown in the parentheses. A plasmid vector containing mCES2 cDNA [3] was used as a template for a positive control. The PCR product was purified by a Wizard SV Gel and PCR Clean-up System (Promega, Madison, WI, U.S.A.), and was confirmed to be a fragment of mCES2 cDNA by DNA sequencing. The DNA sequence was determined using a Dye Terminator Cycle Sequencing-Quick Start Kit (Beckman Coulter, Fullerton, CA, U.S.A.) and a CEQ 2000 DNA Analysis System (Beckman Coulter). The results were confirmed by sequencing at least twice in each direction.

Mouse USF1 (upstream stimulatory factor 1) cDNA was obtained from the liver cDNA by PCR using the following primers: sense, 5′-CGCCTCGGATGAGCCCC-3′; and antisense, 5′-ATGGGGTTAGCCTGTTGCTC-3′. The isolated cDNA was inserted into the pCR-Blunt II TOPO vector (Invitrogen), and the nucleotide sequence was determined as described above.

Isolation of the 5′-flanking region of the *mCES2* gene

Mouse genomic DNA was extracted from the liver of a 5-week-old C57BL/6 male mouse (Japan SLC) by DNAzol Reagent (Invitrogen). The 5′-flanking region of the *mCES2* gene was isolated by PCR with the sense primer 5′-CCCATTGTGTGTCCAGTATCAC-3′ (− 1385 nt) and the antisense primer 5′-GCTCTCAGTCCTAAGTCTGTGC-3′ (+ 60 nt) using the genomic DNA as a template. The relative 5′ positions of primers with respect to the transcription start site determined in the present study are shown in parentheses. The primers were designed on the basis of results of the Mouse Genome Project (http://www.sanger.ac.uk). The amplicon was subcloned into the pCR-Blunt II TOPO vector, and the nucleotide sequence was determined as described above.

Determination of the transcription start site of the *mCES2* gene

The method of RLM 5′-RACE (RNA ligase-mediated and oligo-capping rapid amplification of 5′-cDNA ends) was used to determine the transcription start site of the *mCES2* gene (GeneRacer kit; Invitrogen). This method ensures the capture of only full-length 5′ ends of specific transcripts via elimination of truncated messages from the amplification process. Total RNA from mouse liver was treated with calf intestinal phosphatase and tobacco acid pyrophosphatase according to the manufacturer's instructions. After selective ligation of an RNA oligonucleotide to the 5′-ends of decapped mRNA, first-strand cDNA was synthesized. Using the supplied sense primer and the *mCES2* gene-specific primer designed, 5′-AGCAGCAGCCCGAAGAACCC-3′ (+ 121 nt), touchdown PCR amplification was performed (94 °C for 2 min, followed by 5 cycles of 94 °C for 30 s, 66 °C for 90 s, 5 cycles of 94 °C for 30 s, 64 °C for 90 s, and 25 cycles of 94 °C for 30 s, 63 °C for 30 s and 68 °C for 90 s) by using high-fidelity DNA polymerase (KOD plus; Toyobo, Tokyo, Japan). The product was subcloned into the pCR-Blunt II TOPO vector, and sequenced as described above.

Plasmid construction

The *mCES2* gene promoter region was removed from the cloning vector and inserted into the pGL3-Basic vector (Promega) by *Xho*I and *Kpn*I digestion. The plasmid is referred to as p − 1385/+ 60. Seven deletion constructs were generated by 'nested' PCR of the primary clone using the following sense primers: 5′-TGTGACCTCGCATAACTATCAT-3′ (p − 966/+ 60), 5′-CAAAGCACAGGAGTCTTGAAGC-3′ (p − 363/+ 60), 5′-AGAGAGAACAGAAGCAGCAGAG-3′ (p − 163/+ 60), 5′-GCCATTGGCTCCTATCACT-3′ (p − 120 + 60), 5′-TTCACCCGTCACACAATG-3′ (p − 69/+ 60) and 5′-TTAAATAGCTGCCTGTGC-3′

(p − 29/+ 60). The antisense primer used was the same as that used in the genome cloning. All constructs are named and are shown in parentheses. The obtained 5′-deletion fragments were transferred into pGL3-Basic vectors, as described above.

To introduce mutations into the reporter plasmids, complementary primers harbouring a few mutations were designed for each target site as follows: 5′-GTCACACAATGTTTCCACCTACACCCT-3′ and 5′-AGGGGTGTAGGTGGAAACATTGTGTGAC-3′ for the GC box; 5′-TCCTATGACTAGTGACTAATTACTGTTCC-3′ and 5′-GGAACAGTAATTAGTCACTAGTCATAGGA-3′ for the E box (enhancer box); and 5′-GCAGCAGAGGCTCCTTTCCCCTACCTCC-3′ and 5′-GGAGGTAGGGGAAAGGAGCCTCTGCTGC-3′ for the NFκB (nuclear factor κB) element. The mutagenic sites are underlined. Inverse-PCR amplification was performed by using KOD PLUS and using p − 163/+ 60 as a template. PCR products were treated with *Dpn*I to digest the residual template plasmid, and were applied to the transformation. The mutations introduced were confirmed by DNA sequencing as described above. All mutational constructs are named as follows: pGCmt for the GC box mutant; pEmt for the E box mutant; pNFmt for the NFκB element mutant; pGCEmt for the GC box and E box mutant; pGCNFmt for the GC box and NFκB element mutant; pENFmt for the E box and NFκB element mutant; and pAllmt for the GC box, E box and NFκB element mutant.

An Sp1 mammalian expression vector, pCMVSp1, and *Drosophila* Sp3 expression vector, pPacUSp3, were generously given by Dr Robert Tjian (Howard Hughes Medical Institute, Department of Molecular and Cell Biology, University of California, Berkeley, CA, U.S.A.) and Dr Guntram Suske (Philipps-Universitat, Marburg, Germany) respectively. To construct an Sp1 *Drosophila* expression vector, the coding region of Sp1 [18,19] was amplified by PCR and subcloned into the pCR-Blunt II TOPO vector. The sense primer was 5′-CACCATGGATCCCTCGA-3′, and the antisense primer was 5′-TGCCTGATCTCAGAAGCC-3′. Then, the coding region of Sp1 was inserted into pAc5.1/V5-His (Invitrogen) (pAcSp1). Likewise, mouse (m)USF1 cDNA was subcloned into pAc5.1/V5-His (pAcUSF1) by *Eco*RI digestion coupled with alkaline phosphatase treatment to enable it to be expressed in *Drosophila* cells. DNA sequencing as described above confirmed the orientations and the nucleotide sequences of these constructs.

Cell cultures

Hepa1-6 cells and COS-7 cells were obtained from Riken Cell Bank (Tsukuba, Japan). Both cell lines were maintained at 37 °C with 5 % CO_2 in DMEM (Dulbecco's modified Eagle's medium) (Invitrogen) supplemented with 10 % (v/v) heat-inactivated fetal bovine serum, and 50 units/ml penicillin and 50 μg/ml streptomycin. *Drosophila* SL2 cells, purchased from A.T.C.C. (Rockville, MD, U.S.A.), were grown at 24 °C without CO_2 in *Drosophila* Schneider's medium (Invitrogen) supplemented with 10 % fetal bovine serum, and 50 units/ml penicillin and 50 μg/ml streptomycin.

Transient transfections and dual luciferase assays

Hepa1-6 cells were plated at a density of 0.8×10^5 cells/well in a 24-well dish 1 day before transfection. When the cells had reached 60–80 % confluence, they were transiently transfected with 200 ng of the reporter plasmids and 4 ng of phRL-TK vector (Promega) using LIPOFECTAMINE™ (Invitrogen) and PLUS Reagent (Invitrogen), according to the manufacturer's protocol. At 3 h after transfection, the medium was removed and the cells were cultured for a further 24 h in complete supplemental DMEM. Then, the cells were washed with PBS before harvesting. The cells were lysed with passive lysis buffer (PLB; Promega), and luminescence from transfected cell lysates was determined using a Dual-Luciferase Reporter Assay System (Promega) and a TD-20/20 luminometer (Promega) according to the manufacturer's directions. Results were expressed as a relative ratio (firefly luciferase activity:*Renilla* luciferase activity). Experiments were performed in triplicate, and each value presented is the mean (± S.D.) from three independent assays.

COS-7 cells were plated at a density of 1.3×10^6 cells/dish in a 100 mm dish 1 day before transfection. When the cells had reached 60–80 % confluence, they were transiently transfected with 5 μg of pCMVSp1 using the same reagents as those used for Hepa1-6 cells. The medium was removed 3 h after transfection and the cells were cultured for a further 72 h in complete supplemental DMEM. The cells were harvested, and the nuclear extracts were prepared as described below.

SL2 cells were plated at a density of 5×10^5 cells/well in a 24-well plate. At 18 h after plating, the cells were transfected with 150 ng of the reporter plasmid and various amounts of a mixture of expression plasmid (pAcSp1, pPacUSp3 and/or pAcUSF1) using Cellfectin reagent (Invitrogen), according to the provided protocol. The total amount of DNA in the mixture was adjusted to 300 ng by adding an empty pAc5.1/V5-His vector. Complete growth medium was added to each well 5 h after transfection. The cells were harvested 48 h after addition of the medium. After being washed with PBS, the cells were lysed with PLB, and single luciferase activity was determined using the same system as that described above. The luciferase activity was normalized by total protein amount determined by using a Bio-Rad *Dc* Protein Assay Kit. Experiments were performed in duplicate, and each value presented is the mean(± S.D.) from three independent assays.

A Student's *t* test was performed to determine significance of difference between the groups in mutation analysis. Similarly, ANOVA was performed to determine significance of synergism between the groups in co-transfection assays. *P* values less than 0.05 were taken to be significant.

Nuclear extraction, EMSA (electrophoretic mobility-shift assay) and supershift assays

Nuclear extracts were prepared from the cultured cells by using a CelLytic Nuclear Extraction Kit (Sigma-Aldrich Co., St Louis, MO., U.S.A.) according to the manufacturer's protocol. Hepa1-6 cells or COS-7 cells were washed twice with ice-cold PBS and collected. After extracting nuclear contents, the protein concentrations were determined as described in the previous section. The nuclear extracts were stored at − 80 °C until use.

Probe dsDNA (double-stranded DNA) was generated by annealing the complementary oligonucleotides listed in Supplementary Table s1 (http://www.BiochemJ.org/bj/384/bj3840101add.htm). Consensus and mutated consensus oligonucleotides for the Sp family, USFs, C/EBPs (CCAAT/enhancer binding proteins) and NFκB were designed following the instructions of Santa Cruz Biotechnology (Santa Cruz, CA., U.S.A.). dsDNA was end-labelled with [γ-^{32}P]dATP (Amersham Biosciences) using T4 polynucleotide kinase (Promega). Each binding reaction mixture contained 10 mM Tris/HCl, pH 7.5, 50 mM NaCl, 1 mM $MgCl_2$, 0.5 mM EDTA, 0.5 mM dithiothreitol, 4 % (v/v) glycerol, 0.5 μg of poly(dI-dC)/poly(dI-dC) and 1–2 μg each of the nuclear extracts. The reaction mixtures were pre-incubated on ice for 10 min before the addition of a [γ-^{32}P]dATP-labelled probe. Samples were kept for a further 20 min at room temperature with the probe, and the mixtures were electrophoresed on a 6 % (w/v)

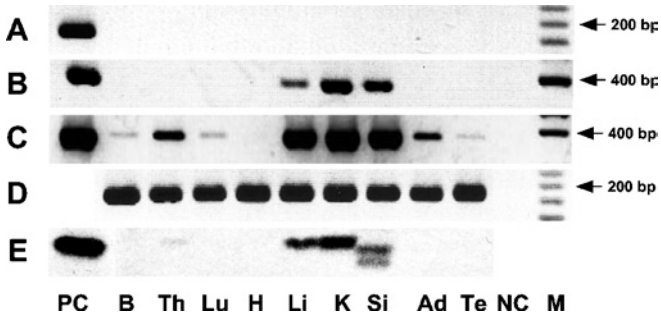

Figure 1 Tissue expression profiles of mCES2 mRNA and protein

(**A**–**D**) The results of RT-PCR are shown; (**E**) shows the results of esterase activity staining after non-denaturing PAGE. PC, positive control; B, brain; Th, thymus; Lu, lung; H, heart; Li, liver; K, kidney; Si, small intestine; Ad, adipose tissue; Te, testis; NC, non-template control; M, DNA size markers. In (**A**), mouse *GAPDH* gene-specific primers were used, and mouse tissue RNA was used as a template. Mouse liver cDNA prepared in our previous study [3] was used as a positive control. In (**B**) and (**C**), mCES2 mRNA expression was detected. Mouse tissue cDNA was used as a template. mCES2 cDNA in the pFAST BAC1 vector [3] was used as a positive control. In (**C**), template tissue cDNA was increased to a 12-fold excess amount of that used in (**B**). In (**D**), mouse GAPDH mRNA expression was detected. Mouse tissue cDNA was used as a template. In (**E**), mCES2 protein activity was detected. Various amounts of mouse tissue homogenates were used to adjust band intensity: 20 μg of brain, 15 μg of thymus, 7 μg of lung, 10 μg of heart, 1 μg of liver, 5 μg of kidney, 5 μg of small intestine, 2.5 μg of adipose tissue and 10 μg of testis homogenates.

polyacrylamide gel in 0.5 × 45 mM Tris/borate buffer with 1 mM EDTA at 4 °C. Gels were dried and autoradiographed by BASS 2000 II (Fuji Film, Tokyo, Japan).

Competition experiments were performed as described above, except that unlabelled competitive dsDNA (Supplementary Table s1; http://www.BiochemJ.org/bj/384/bj3840101add.htm) was added to the binding reaction mixture at a 50-fold excess of the probe amount before addition of the probe. For supershift assays, 2 μg of antibodies against Sp1 (sc-59x), Sp3 (sc-644x), USF1 (sc-229x), NFκB p65 (sc-109x) or AP1 (sc-45x; all antibodies listed from Santa Cruz Biotechnology) was added to the binding reaction mixture at room temperature for 30 min before addition of the probe.

All results are representative of two or three independent experiments.

Chemicals used in this study were of reagent grade and obtained from commercial sources.

RESULTS

Tissue expression profiles of mCES2 mRNA and protein

At the start of the present study, we obtained tissue expression profiles of mCES2 mRNA and protein by performing RT-PCR, and esterase activity staining after non-denaturing PAGE, respectively. The results of PCR using mouse *GAPDH* gene-specific primers showed that mouse tissue cDNAs were free of contamination with genomic DNA (Figure 1A) and were synthesized in equal amounts (Figure 1D). PCR using *mCES2* gene-specific primers revealed limited expression of mCES2 mRNA in the liver, kidney and small intestine (Figure 1B). When template cDNAs were increased to 12-fold excess amounts of those used in the experiment, for which the results are shown in Figure 1(B), mCES2 mRNA expression was also detected in the thymus, adipose tissue, brain, lung and testis (Figure 1C). There was no band observed in the heart. Nucleotide sequences of all of the PCR products showed 100 % identity with that of mCES2 cDNA. Consistent with the results of RT-PCR, a strong activity band was detected in the liver, kidney and small intestine, and a weak activity band was detected in the thymus by esterase activity staining after non-denaturing PAGE (Figure 1E). The band detected on the lane of the small intestine was a doublet with a slightly different migration pattern.

Isolation of the mCES2 5′-flanking region

To understand the mechanism by which *mCES2* gene transcription is regulated, the 5′-flanking region of the *mCES2* gene was isolated from the C57BL/6 mouse genomic DNA by PCR amplification. The obtained nucleotide sequence is shown in Supplementary Figure s1 (http://www.BiochemJ.org/bj/384/bj3840101add.htm), together with the sequence around the translation start site obtained from the results of RLM 5′-RACE and cDNA cloning of mCES2 [3]. When the nucleotide sequence of the clone was compared with the results of the genome project, a thymine nucleotide deletion was found in the clone at position − 978 with respect to the transcription start site (+ 1), which was determined in this study. Regions homologous with known *cis* elements were identified using searching programs for transcription factor-binding sites (http://www.gene-regulation.com/ and http://www.drkazu.com/KENKYUU.htm/). The results showed that there were a GC box, an E box and an NFκB element which preceded a translation start codon, in addition to other elements scattered throughout the sequence, such as binding sites for hepatocyte nuclear factors or C/EBPs. A TATA box, however, was not found.

Determination of the transcription start site of the *mCES2* gene

The RLM 5′-RACE method was used to determine the transcription start site of the *mCES2* gene. DNA sequencing analysis of a PCR product showed that it contained a partial nucleotide sequence of the *mCES2* gene ligating to the adapter sequence. The partial sequence started at 65 bp upstream from the translation start codon (Supplementary Figure s2; http://www.BiochemJ.org/bj/384/bj3840101add.htm). The data showed that this relative position was the transcription start site of the *mCES2* gene, and this position is hereafter referred to as + 1 (indicated by the arrow in Supplementary Figure s1; http://www.BiochemJ.org/bj/384/bj3840101add.htm).

Identification of the major *cis* elements responsible for transcriptional activity of the 5′-flanking region of the *mCES2* gene

Deletion analysis and mutation analysis were performed to examine the transcriptional activity of the 5′-flanking region of the *mCES2* gene, and to identify the elements that contribute to its transcriptional activity. Seven deletion constructs were transiently transfected into mouse hepatoma cells (Hepa1-6 cells). Deletion analysis showed that the highest level of transcriptional activity was in p − 163/+ 60 (Figure 2), and that the level of activity was gradually reduced as the fragment became longer or shorter. Deletion of the region from − 163 to − 121 nt, which contained the NFκB element (p − 120/+ 60), resulted in a decrease in the level of transcriptional activity by 50 %. Further deletion of the region from − 120 to − 70 nt, which contained the E box (p − 69/+ 60), resulted in a decrease in the level of activity to one-fourth of the highest level observed in p − 163/+ 60. The activity was almost abolished by deletion of the region from − 69 to − 30 nt, in which the GC box was located (p − 29 + 60).

The results of mutation analysis were similar to those obtained from deletion analysis of the 5′-flanking region of the *mCES2* gene (Figure 3). All of the mutational constructs except for pNFmt

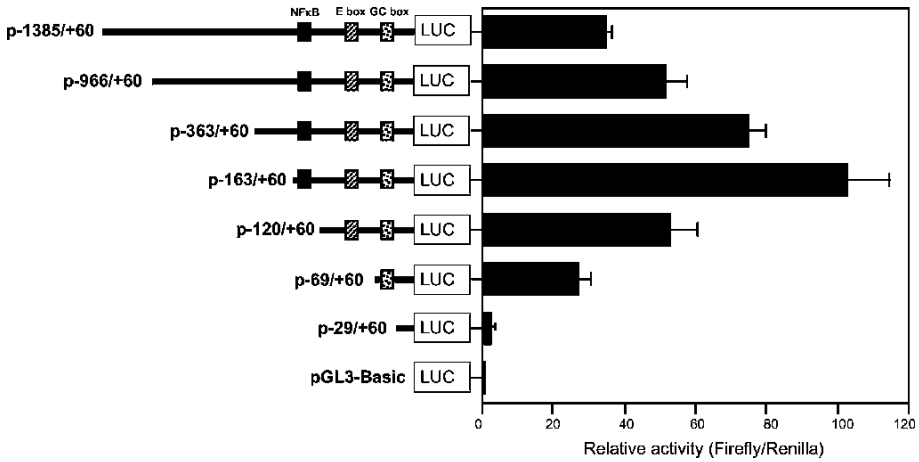

Figure 2 Deletion analysis of the 5′-flanking region of the *mCES2* gene

Luciferase (LUC) genes regulated by various lengths of the *mCES2* 5′ deletion fragments (200 ng) were transiently transfected into Hepa1–6 cells. A GC box, an E box and an NFκB element are shown as differently patterned boxes. Names of constructs are shown on the left side. Details of the deletion constructs are provided in the Experimental section. Each value is the mean $\pm$ S.D. of relative activity (Firefly/*Renilla*) for three separate experiments, each performed in triplicate.

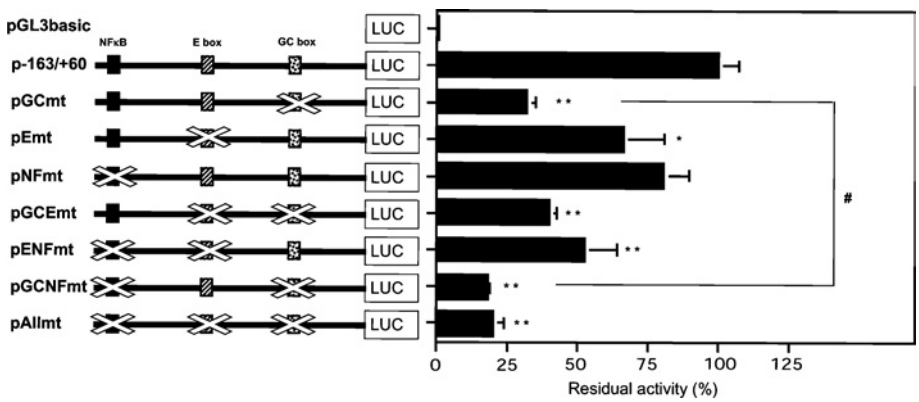

Figure 3 Mutation analysis of the 5′-flanking region of the *mCES2* gene

Luciferase genes regulated by the *mCES2* promoter constructs harbouring various mutations (200 ng) were transiently transfected into Hepa1–6 cells. A GC box, an E box and an NFκB element are shown as differently patterned boxes, and mutations are indicated by crosses. Details for the mutation constructs are provided in the Experimental section. Luciferase activity was determined as relative activity (Firefly/*Renilla*), and each value is the mean $\pm$ S.D. of residual activity (%) of the control (p $-$ 163/+ 60) for three separate experiments, each performed in triplicate. * and ** indicate statistically significant differences compared with the control (p $-$ 163/+ 60; $P < 0.05$ and $P < 0.005$ respectively). # indicates a statistically significant difference between pGCmt and pGCNFmt ($P < 0.05$).

showed significant decrease in transcriptional activity compared with that of the intact p $-$ 163/+ 60. The introduction of mutation into the GC box (pGCmt) and the E box (pEmt) caused 68 % ($P < 0.005$) and 33 % ($P < 0.05$) decreases in the activity level, respectively, relative to the activity level of p $-$ 163/+ 60. The effects of double mutations on transcriptional activity differed among constructs. Additional mutations in the NFκB element to pGCmt (pGCNFmt) resulted in a decrease in the transcriptional activity level (decrease of a further 14 %; $P < 0.05$). However, the activity level of the double GC box and E box mutant (pGCEmt) was comparable with that of pGCmt. The activity level of the double E box and NFκB element mutant (pENFm) was 53 % that of the control (p $-$ 163/+ 60), and was slightly lower than that of pEmt. The activity level of the constructs harbouring GC box, E box and NFκB element mutations (pAllmt) was significantly low (20 % of the control level; $P < 0.005$), but this activity level was almost the same as that of pGCNFmt.

Identification of nuclear proteins interacting with the GC box of the *mCES2* gene promoter

The results of deletion and mutation analyses showed that the 5′-flanking region of -163 to $+60$ nt consists of a minimal promoter of the *mCES2* gene, and that three *cis* elements located within the region, GC box, E box and NFκB element, were important for transactivation of the promoter. EMSAs were performed to determine which nuclear proteins could interact with these elements. The probes used in EMSAs are listed in Supplementary Table s1 (http://www.BiochemJ.org/bj/384/bj3840101add.htm).

Figure 4 shows the results of EMSAs and supershift assays for identification of proteins interacting with the GC box. The probe, $-36/-62$WT, was incubated with Hepa1-6 nuclear extracts, resulting in the formation of three DNA–protein complexes, termed complex 1, complex 2 and complex 3 (lane 3). There was an unknown factor in the nuclear extracts, indicated in

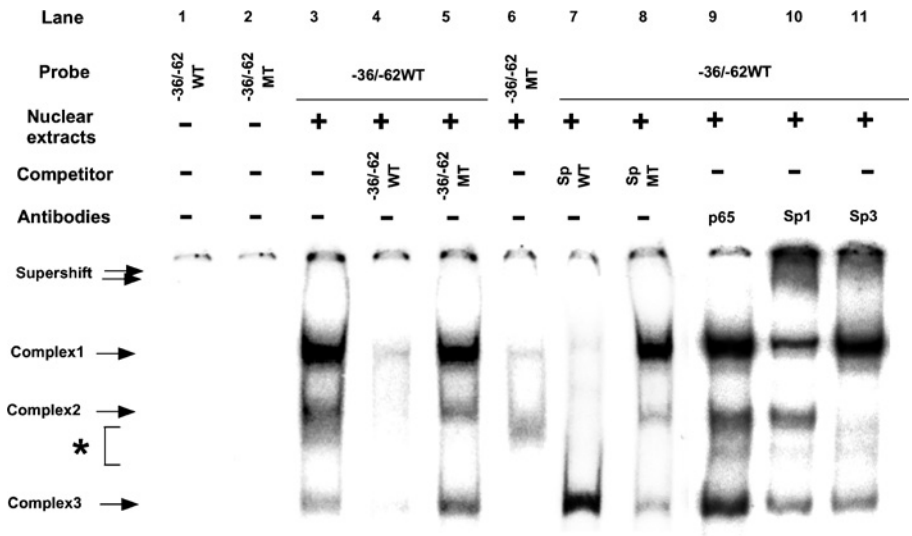

Figure 4 Identification of nuclear proteins interacting with the GC box in the *mCES2* gene promoter

The probes and competitors are listed in Supplementary Table s1 (http://www.BiochemJ.org/bj/384/bj3840101add.htm). Symbols (−) and (+) indicate the absence and presence of nuclear extracts or competitor, respectively. DNA–protein complexes are indicated by arrows and are named. Asterisk indicates unknown complexes. EMSAs were performed using the following probes: −36/−62WT in lanes 1, 3–5 and 7–11 and −36/−62MT in lanes 2 and 6. Oligonucleotide competitors were added with 50-fold excess amounts of the following probes: −36/−62WT in lane 4, −36/−62MT in lane 5, SpWT in lane 7, and SpMT in lane 8. Supershift assays were performed using antibodies specific for Sp1 or Sp3. Anti-Sp1 antibodies (2 μg) were added to the reaction mixtures in lane 10, and anti-Sp3 antibodies (2 μg) were added to the reaction mixtures in lane 11. Anti-p65 antibodies (2 μg) were used as a negative control (lane 9). The arrows indicate shifted bands. Other procedures are described in the Experimental section.

Figure 4 by an asterisk. All three complexes disappeared when a 50-fold excess amount of −36/−62WT was added as a competitor (lane 4), whereas a competitor containing a degenerate GC box of the *mCES2* gene, −36/−62MT, did not affect the formation of any of the three complexes (lane 5). The labelled probe −36/−62MT could hardly produce all three complexes (lane 6). Complex 1 and complex 2 were completely competed-out by the addition of SpWT, an Sp family consensus oligonucleotide (lane 7), but not by the addition of SpMT, a mutated Sp family consensus oligonucleotide (lane 8). Neither SpWT nor SpMT could prevent the formation of complex 3 (lanes 7 and 8). To identify the proteins comprising the three complexes, supershift assays were performed using anti-Sp1 and anti-Sp3 antibodies. The addition of anti-Sp1 antibodies generated a supershifted band together with a decrease in the amount of complex 1 formed (lane 10), and, similarly, the addition of anti-Sp3 antibodies completely disrupted the formation of complex 2 accompanied by the generation of a supershifted band (lane 11). Anti-p65 antibodies, used as a negative control, did not influence the formation of any complexes (lane 9). These data show that Sp1 and Sp3 proteins are bound to the GC box of the *mCES2* gene promoter. The formation of complex 3 is not dependent on the canonical GC box, but is dependent on the GC box of the *mCES2* promoter. Nuclear proteins in complex 3 remain unknown.

Identification of nuclear proteins interacting with the E box of the *mCES2* gene promoter

Next, we examined nuclear proteins that interacted with the E box of the *mCES2* promoter (Figure 5). Incubation of the labelled probe −92/−123WT with nuclear extracts resulted in the formation of complex 4 (lane 4).

Formation of complex 4 was not observed in the presence of −92/−123WT as a competitor (lane 5), and this complex was detected in the presence of a competitor containing a mutated E box, −92/−123MT (lane 6). In addition, −92/−123MT could not bind to the nuclear protein forming complex 4 (lane 7). This complex was eliminated by the addition of the competitor USFWT, a USF consensus oligonucleotide (lane 8), whereas it was resistant to the competitor USFMT, a mutated USF consensus oligonucleotide (lane 9). A protein–DNA complex migrating at the same position as that of complex 4 was detected in lane 12, where USFWT was used as a probe. To determine the proteins forming complex 4, EMSAs were performed using antibodies against USF1. Anti-USF1 antibodies prevented the formation of complex 4 (lane 10), whereas non-related antibodies (anti-AP1 antibodies) did not (lane 11). These results demonstrate that USF1 proteins exist in complex 4.

Identification of nuclear proteins interacting with the NFκB element of the *mCES2* gene promoter

Results of deletion and mutation analyses showed that, even under normal cell culture conditions, the NFκB element is important for the *mCES2* gene promoter to exert its maximum level of transcriptional activity. To identify the constitutive factor(s) bound to the NFκB element of the *mCES2* gene promoter, EMSAs were performed using consensus or mutational probes for Sp family and C/EBPs (Figure 6A). This is because it has been reported that the upstream regions of P-selectin [20], angiotensinogen [21], MHC H2K [22], and cytochrome P450 *3A7* [23] genes possess NFκB elements that can bind to a constitutive factor in a sequence-dependent manner (Supplementary Table s2; http://www.BiochemJ.org/bj/384/bj3840101add.htm). The probe −123/−149WT could bind to nuclear proteins, resulting in the formation of complex 5 and complex 6 (lane 3). These complexes were not observed in the presence of the competitor −123/−149WT (lane 4), but were found in the presence of the mutational competitor −123/−149MT (lane 5). The formation of complex 5 was competed away by addition of the competitor SpWT (lane 7), but was not affected by the competitor SpMT (lane 8). The competitor NFκBWT slightly diminished the rate

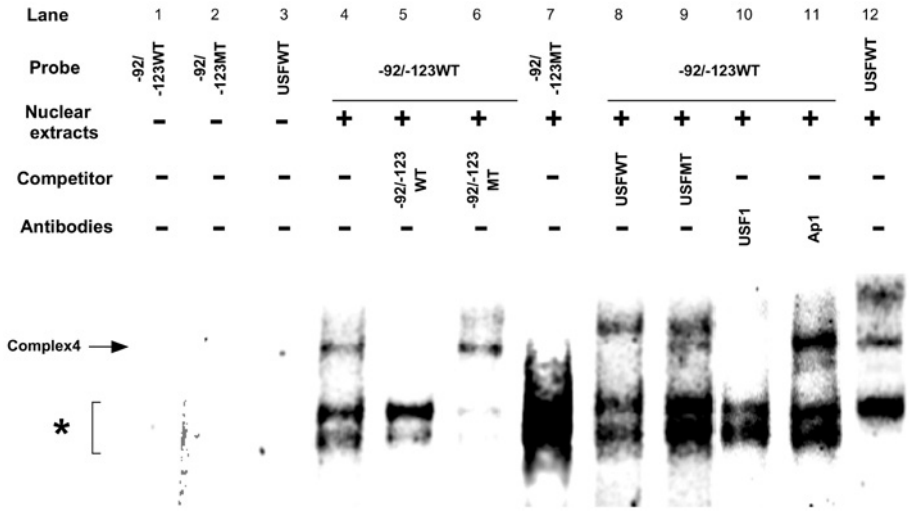

Figure 5 Identification of nuclear proteins interacting with the E box in the *mCES2* gene promoter

The probes and competitors are listed in Table s1 (http://www.BiochemJ.org/bj/384/bj3840101add.htm). Symbols are explained in the legend for Figure 4. EMSAs were performed using the following probes: −92/−123WT in lanes 1, 4–6 and 8–11, −92/−123MT in lanes 2 and 7, and USFWT in lanes 3 and 12. Oligonucleotide competitors were added with 50-fold excess amounts of the following probes: −92/−123WT in lane 5, −92/−123MT in lane 6, USFWT in lane 8 and USFMT in lane 9. EMSAs were also performed using antibodies specific for USF1. Anti-USF1 antibodies (2 μg) were added to the reaction mixtures in lane 10. Anti-AP1 (activator protein 1) antibodies (2 μg) were used in lane 11 as a negative control. Other procedures are described in the Experimental section.

of formation of complex 5, and simultaneously abolished the formation of complex 6 (lane 9). No effect of the competitor NFκBMT on the formation of complexes was observed (lane 10). Neither the C/EBP consensus competitor nor its mutant affected the formation of complexes (lanes 11 and 12). When antibodies were added to the reaction mixture, the formation of complex 5 was reduced in the presence of anti-Sp1 antibodies, but not in the presence of anti-p65 or anti-Sp3 antibodies (results not shown). To verify the binding of Sp1 to the *mCES2* NFκB element, EMSAs were performed using nuclear extracts from COS7 cells in which Sp1 was overexpressed (Figure 6B). The probe −123/−149WT could produce complex 7 with nuclear extracts (lane 2), and this complex was eliminated by the addition of either a self-competitor or SpWT (lanes 3 and 4 respectively). The formation of complex 7 was not disturbed by SpMT (lane 5). Furthermore, complex 7 disappeared in the presence of anti-Sp1 antibodies (lane 6), whereas anti-p65 antibodies did not disturb the formation of complex 7 (lane 7). Together with the results shown in Figure 6(A), our results show that Sp1 is involved in the formation of these complexes.

Synergistic transactivation of the *mCES2* promoter by Sp1, Sp3 and USF1 in *Drosophila* SL2 cells

We finally analysed the functional capabilities of Sp1, Sp3 and USF1 for transactivation of the *mCES2* gene promoter. p −163/+60 was co-transfected with pAcSp1, pPacUSp3 and/or pAcUSF1 in *Drosophila* SL2 cells, which possess a null background for these transcription factors [24], and luciferase activities were compared (Figure 7). When transfected alone, Sp1 was shown to be a strong transactivator of the promoter (2.4-fold increase in promoter activity), whereas USF1 induced only a weak transactivation (1.4-fold increase in promoter activity) and Sp3 was almost inert. However, in the case of a combination of Sp1 with Sp3 or USF1, the promoter activity level was increased 3.7-fold ($P < 0.01$) and 4.2-fold ($P < 0.05$) respectively. The combination of Sp3 and USF1 also showed a synergistic transactivation (3.5-fold increase; $P < 0.005$). Moreover, potent synergistic transactivation (8.7-fold increase) was observed in the case of a combination of all three factors, and this induction level of the promoter activity was significantly higher than those obtained with the Sp1/Sp3 combination or the Sp1/USF1 combination ($P < 0.005$). These results indicate co-operative transactivation of the *mCES2* promoter by Sp1, Sp3 and USF1.

DISCUSSION

We have presented here some experimental data showing tissue expression profiles of mCES2, which indicate that Sp1, Sp3 and USF1 contribute to synergistic transactivation of the *mCES2* promoter.

As for the examination of tissue expression profiles of mCES2, RT-PCR is not a completely quantitative method for evaluation of mRNA expression; nonetheless, the amplification levels of specific bands in tissue cDNA were apparently different. Relatively high expression levels of mCES2 in the liver and small intestine are reasonable, since it has been demonstrated that the carnitine/acylcarnitine system does work in the ER in these tissues [8,11]. The reason why mCES2 is expressed in the kidney, thymus, adipose tissue, brain, lung and testis, but is not expressed in the heart, is not known at present. It has been reported that human CES2 is expressed at high levels in the kidney and heart, and at low levels in the brain and lung, but is not expressed in the thymus [16]. Although physiological functions of these CES2 isoenzymes remain obscure, the similarities and differences between expression profiles of these CES2 isoenzymes may mirror functional similarities and species differences of the CES2 family.

The possibility that mCES2 is post-translationally modified, or forms a complex with another protein or other proteins in a small-intestine-specific manner, should be pointed out. Results of esterase activity staining after non-denaturing PAGE showed that the bands observed in the small intestine were doublet bands with slightly different rates of migration, although all tissue samples

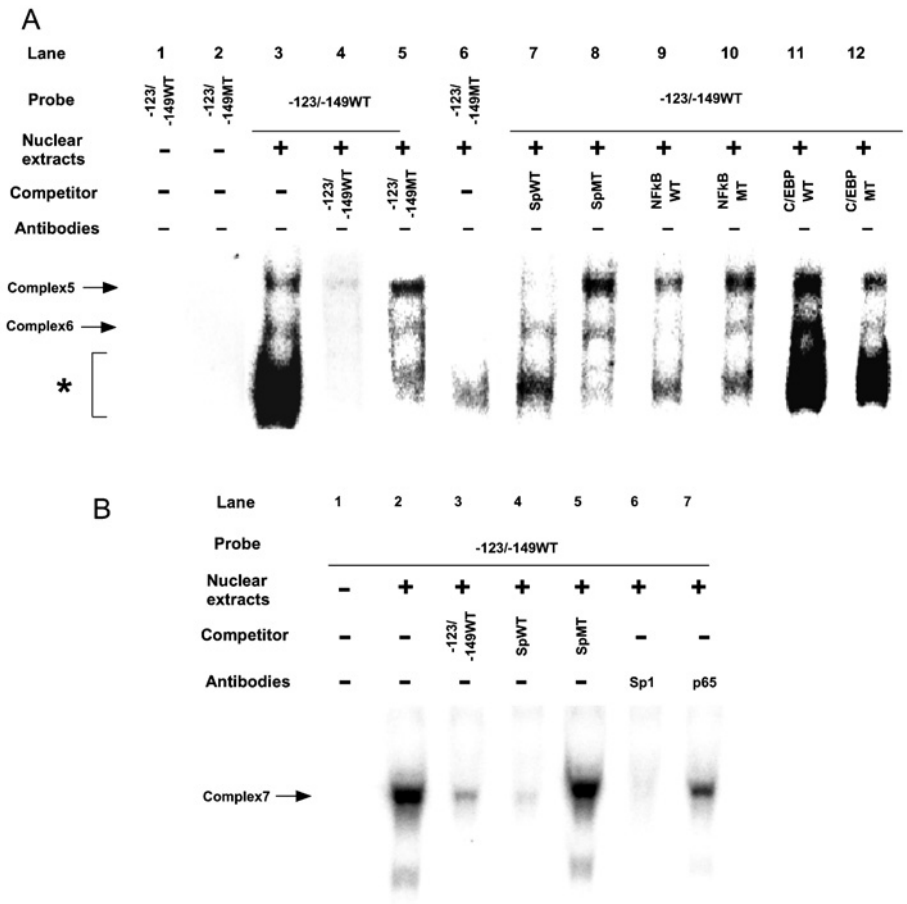

Figure 6 Identification of nuclear proteins interacting with the NFκB element in the *mCES2* gene promoter

The probes and competitors are listed in Table s1 (http://www.BiochemJ.org/bj/384/bj3840101add.htm). Symbols are explained in the legend of Figure 4. (**A**) EMSAs were performed using Hepa1–6 nuclear extracts and the following probes: – 123/– 149WT in lanes 1, 3–5, 7–12 and – 123/– 149MT in lanes 2 and 6. Oligonucleotide competitors were added with 50-fold excess amounts of the following probes: – 123/– 149WT in lane 4, – 123/– 149MT in lane 5, SpWT in lane 7, SpMT in lane 8, NFκBWT in lane 9, NFκBMT in lane 10, C/EBPWT in lane 11, and C/EBPMT in lane 12. (**B**) EMSAs were performed using nuclear extracts of COS-7 cells overexpressing Sp1. The probe – 123/– 149WT was used in all lanes. Oligonucleotide competitors were added with 50-fold excess amounts of the following probes: – 123/– 149WT in lane 3, SpWT in lane 4, and SpMT in lane 5. Anti-Sp1 antibodies (2 μg) were added to the reaction mixtures in lane 6. Anti-p65 antibodies (2 μg) were added to the reaction mixtures in lane 7. Other procedures are described in the Experimental section.

were prepared in the same way. However, the doublet bands are thought to result from mCES2 protein, since anti-mCES2 antibodies [3] could specifically eliminate both bands (results not shown). Further analysis is needed to determine how mCES2 is expressed in the small intestine, and the results of such analysis may lead to the revelation of novel physiological functions of mCES2.

Our next challenge is to determine how *mCES2* transcription is controlled. An approx. 1.4-kbp fragment of the 5′-flanking region of the *mCES2* gene shows characteristics of a TATA-less promoter, such as the absence of a TATA box, but the presence of a GC box and an E box preceding the transcription start site. A TATA-less promoter seems to be shared by other members of the CES family, since two previously reported CES promoters also do not have a functional TATA box [13,25].

The *mCES2* promoter transactivation was controlled by the synergistic action of three *cis* elements: a GC box, an E box and an NFκB element (Figures 2 and 3), but the degrees of contribution of these elements to the promoter activity were not equivalent. It is noteworthy that the E box could work as a functional *cis* element only when a functional GC box was present in the promoter (Figure 3; cf. pGCmt and pGCE, and pGCNFmt and pAllmt), indicating that there was a potential co-operative interaction between nuclear factors that bound to these two *cis* elements. These nuclear proteins were revealed to be Sp1, Sp3 and USF1 by the following experiments.

Sp family proteins and USFs are transcription factors that are ubiquitously expressed and are involved in transactivation of TATA-less promoters via binding to the GC/GT box and E box (CANNTG), respectively [26–29]. Among Sp family proteins, Sp2 is structurally different from other Sp members [30–32], and Sp4 is predominantly expressed in the brain [31,33]. Sp3 is expressed in all mammalian cells that express Sp1 [30,32]. Thus Sp1 and Sp3 were our first choice as candidates for nuclear proteins that interact with the GC box of the *mCES2* promoter. On the other hand, we chose USF1 as a candidate for nuclear proteins that bind to the *mCES2* E box, since USFs are involved in the regulation of many genes that play a role in glucose or fatty acid metabolism, such as those encoding glucokinase [34] and FAS (fatty acid synthase) [35,36].

In addition to the GC box, we also determined that Sp1 could bind to the *mCES2* NFκB element. Hirano et al. [20] demonstrated that Sp1 can bind to subsets of NFκB elements with high affinities. They proposed that Sp1 might raise the basal expression

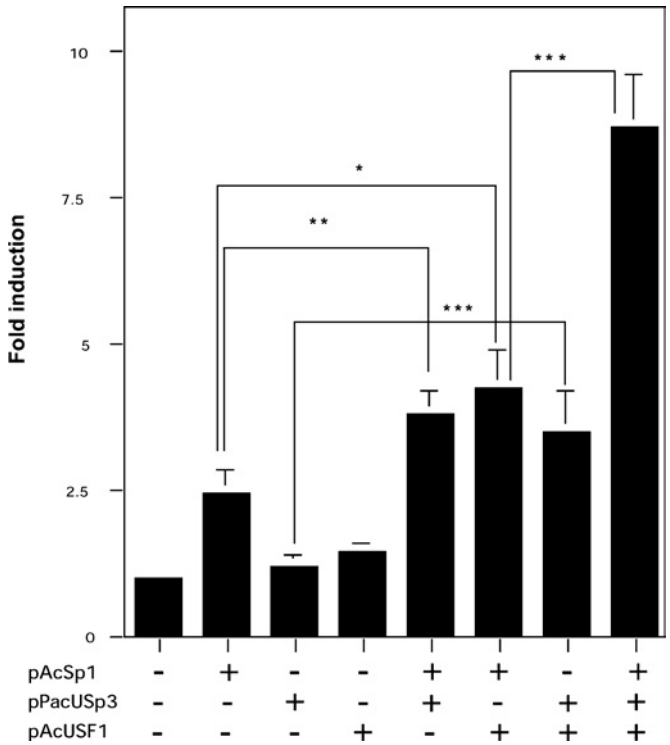

Figure 7 Co-transfection assays of the *mCES2* gene promoter

Luciferase genes regulated by the *mCES2* promoter (150 ng) were transiently co-transfected into *Drosophila* SL2 cells together with 50 ng of pAcSp1, pPacUSp3 and/or pAcUSF1. Total DNA amount was adjusted to 300 ng by using an empty pAc5.1V5/His vector. Luciferase activity was determined as relative activity (Firefly/total cellular protein). Each value is the mean ± S.D. of fold induction towards the activity obtained from the *mCES2* promoter alone (left column) for three separate experiments, each performed in duplicate. *, ** and *** indicate statistically significant differences between two transfectants indicated in the Figure ($P < 0.05$, $P < 0.01$ and $P < 0.005$ respectively).

levels of NFκB-dependent genes in the absence of activated NFκB. Although *mCES2* gene transcription is apparently not NFκB-dependent, their proposition, at least in part, supports our data, because the *mCES2* NFκB element is necessary for full promoter activity. Further analysis is needed to determine whether NFκB is involved in the regulation of *mCES2* gene transcription.

Sp family and USFs show many heterotypic interactions with different classes of nuclear proteins [37–40]. It has recently been reported that physical and functional interactions between Sp1 and USFs regulate human deoxycytidine kinase promoter activity [41]. Here, we have provided additional experimental data showing the synergistic role of the Sp family and USF1 in transactivation of the promoter. Our results from co-transfection assays are in accordance with all other results obtained in the present study. Notable findings are that transfection with USF1 alone only weakly activated the transcription, but that this was a positive activator in the presence of either Sp1 or Sp3. Although the mechanism by which Sp3 interacts with USF1 is currently unknown, these results are also in good agreement with those indicating that the E box needs the GC box to work as a functional *cis* element (Figure 3).

Unexpectedly and interestingly, Sp3 could interact with Sp1 to synergistically increase *mCES2* promoter activity rather than compete with Sp1, whereas Sp3 alone was unable to drive the transactivation. More surprisingly, Sp3 could co-operatively interact with the Sp1–USF1 complex to increase *mCES2* promoter activity further. Regarding the interaction between Sp1 and Sp3, our results are reminiscent of studies in which transcriptional regulation of the mouse hepatocyte growth factor receptor gene [42] or the rat *FAS* gene [43] was analysed. In these studies, Sp1 and Sp3 transcription factors exhibited co-operative and additive functions in gene transcription, respectively, and Zhang et al. [42] reported that physical interaction between these factors was demonstrated by co-immunoprecipitation assays. However, others reported that anti-Sp3 antibodies did not co-immunoprecipitate Sp1, or vice versa [44]. Transcriptional properties of Sp3 are so complicated [27] that it remains uncertain how Sp3 is involved in the synergism observed in our case. Further analysis is needed to elucidate the mechanism.

It is very intriguing that Sp1 and USF1 are strongly involved in transcriptional regulation of the *mCES2* gene, because there has been accumulating evidence of Sp1 and USF1 being mediators for insulin response. The current understanding of Sp1 is that it is not just a basal transcription factor, but that it also plays a role in insulin signalling cascades, as well as in other signal transduction pathways [45]. Keembiyehetty et al. [46] showed that insulin augments Sp1 expression, enhances its translocation to the nucleus, and increases its glycosylation. In the diet-induced DNase I-hypersensitive site of the rat *FAS* gene promoter, the binding of Sp1 to the GC box, which is juxtaposed with FAS insulin-responsive element 1 (FIRE1), contributed to the binding of NF-Y to FIRE1 [47]. On the other hand, Latasa et al. [48] have elegantly demonstrated *in vivo* that USF binding to the E box is required for SREBP binding and activation of the FAS promoter in refeeding states in mice. There is no information at present on hormonal or nutritional regulation of *mCES2* gene transcription. However, considering our proposition that mCES2 plays an important role in fatty acid (ester) metabolism, we are ready to test the possibility that *mCES2* gene transcription is regulated by insulin or feeding conditions through Sp1 and/or USFs.

In the present study, we have shown tissue expression profiles of mCES2 and parts of the mechanism by which transcription of the *mCES2* gene is regulated. mCES2 is expressed in the liver, kidney, small intestine, brain, thymus, lung, adipose tissue and testis. We have also shown that Sp1, Sp3, and USF1 contribute to synergistic transactivation of the *mCES2* promoter. Although the possibility of involvement of other transcription factors in the regulation of *mCES2* gene expression cannot be ruled out and further studies are needed to elucidate the mechanisms fully, our data indicate that Sp1, Sp3 and USF1 are indispensable factors for transcription of the *mCES2* gene. The results of the present study have provided some clues for understanding the molecular mechanisms regulating *mCES2* gene expression, and should be useful for studies aimed at elucidation of physiological functions of mCES2.

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