The Switch in Alternative Splicing Of Cyclic AMP Response Element Modulator Protein CREM\textsubscript{τ2α} (Activator) To CREM\textsubscript{α} (Repressor) In Human Myometrial Cells Is Mediated By SRp40

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Running Title: Regulation of CREM by SRp40

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The transcription factor cAMP response element modulator protein, CREM, plays a major role in cAMP-responsive gene regulation. Biological consequences resulting from the transcriptional stimuli of CREM are dictated by the expression of multiple protein isoforms generated by extensive alternative splicing of its precursor mRNA. We have previously shown that alternative splicing enables the expression of the CREM gene to be ‘switched’ within the human myometrium during pregnancy from the production of CREM\textsubscript{τ2α}, a potent transcriptional activator to the synthesis of CREM\textsubscript{α}, a transcriptional repressor. Furthermore we have recently reported that this change in the expression of CREM spliced variants is likely to have important ramifications on the regulation of downstream CRE-responsive target genes involved in uterine activity in gestation.

We have investigated the splicing factors involved in controlling the expression of myometrial CREM splice variants. Data presented here from transient transfections indicates that the switch in the synthesis of CREM\textsubscript{τ2α} to CREM\textsubscript{α} that occurs during pregnancy is regulated primarily by an SR protein family member, SRp40. We also show that expression of this splicing factor is tightly regulated in the myometrium during pregnancy. SRp40 regulates the splicing of CREM via its interactions with multiple ESE motifs present in the alternatively exons of CREM. In vitro splicing and electrophoretic mobility shift assays were employed to confirm the functionality of the SRp40-binding ESEs, thus providing a mechanistic explanation as to how SRp40 regulates the switch in splicing from production of CREM\textsubscript{τ2α} to CREM\textsubscript{α}.

Transcriptional activation orchestrated by the CREB/CREM/ATF–family of transcription factors and cAMP–response elements (CRE) represents an important mechanism of cAMP-responsive gene regulation (1, 2, 3). One mechanism of CRE-mediated transcriptional activation is the PKA-dependent phosphorylation of CREM (Cyclic-AMP response element modulator) (4, 5). CREM is a member of the basic region-leucine zipper (bZIP) family of transcription factors, which also includes the cyclic AMP binding protein (CREB) and the activating transcription factor (ATF) proteins, as well as Jun and Fos (6, 7). These transcription factors function as homo- and/or hetero-dimers (8,9), and regulate the transcription of downstream-target genes via binding to the CRE elements (CRE, consensus sequence 5’-TGACGTCA-3’). Genes containing CRE motifs within their promoters are involved in a wide range of cellular and molecular processes, including cell signaling, immune regulation, gene transcription and cell cycle/survival (2, 10, 11).

The CREM gene undergoes extensive alternative splicing involving one or more of its internal cassette exons (5, 12, 13). Each CREM protein isoform possesses trans-activation and/or trans-repression properties depending on the exon content of their mRNAs (see Fig 1a). For example, CREM proteins derived from mRNAs containing one or more of the exons encoding the trans-activation domains generally serve as transcriptional activators, whereas CREM isoforms derived from mRNAs with the DNA-binding domain intact but the exons encoding the trans-activation domains spliced out, predictably serve as transcriptional repressors (2, 13). CREM repressors lack the functional domains which mediate transcriptional activation, but can still
have the capacity to bind to CREs as a homo-/hetero-dimer, suppressing transcriptional activation by displacing functionally active dimers from the CRE (2). In addition, CREM protein isoforms are not only generated from alternative splicing, but also from the use of alternative promoters, transcription and translation initiation sites, together with changes in stability due to variant poly-A sites (5, 14).

One major mechanism of CREM/CRE-mediated transcriptional activation is the binding of hormone ligands to G-protein coupled receptors (GPCRs, 15) resulting in an increase in the intracellular level of cAMP due to activation of adenylyl cyclase, which in turn promotes the phosphorylation of CREM via protein kinase A (PKA). Various components of the cAMP signaling pathway are often up or down-regulated to potentiate cAMP levels, notably hormonal ligands that bind to the GPCRs (16), the receptors themselves (17), phosphodiesterase (18) and the stimulatory protein Goαs (19, 20, 21). Increased expression of Goαs is known to increase constitutive as well as stimulated cAMP accumulation, and enhance distal events such as transcription factor phosphorylation and cAMP-responsive gene expression (22).

There is extensive evidence for the physiological relevance of CREM-mediated gene regulation. CREM appears to be particularly important in the pathophysiology of the heart, where it plays a role in cardiac gene regulation (23), in the brain, where it has been implicated in the regulation of long-term memory and the circadian clock (2), in the testes, where it orchestrates spermatogenesis (24), in the liver, where it plays a role in hepatocyte regeneration (25), and the uterus, where there is strong evidence for its role in the regulation of uterine contractility (26, 27). Europe-Finner et al (19, 20) reported an increased level of cAMP in human myometrial smooth-muscle cells during pregnancy, potentiated by altered expression of various components of the cAMP signaling pathway, in particular the G protein Goαs. We have previously reported differential expression of specific CREM protein isoforms within the myometrium tissue throughout pregnancy, namely CREMατα and CREMα (Fig 1B) and demonstrated their ability to bind CRE-containing oligonucleotides and activate and/or repress reporter gene transcription (27). Furthermore, through micro-array studies we have recently shown that these potent factors act in myometrial cells to affect the expression of a plethora of genes with defined roles associated with uterine activity during pregnancy (28). Pre-mRNA splicing mechanisms within myometrial cells appear to switch the production of the alternatively spliced CREMατα activator that decreases sequentially through the non-pregnant, pregnant non-laboring and laboring phases, to the synthesis of CREMα repressor protein that proceeds from zero expression in the non-pregnant uterus to a high level of expression in the pregnant and laboring myometrium (Fig 1B). Thus, alternative splicing is responsible for altering the biological consequences resulting from the transcriptional stimuli of either CREMατα or CREMα within the human myometrium during fetal maturation.

Alternative pre-mRNA splicing is a tightly regulated RNA processing event mediated by a complex interplay of specific cis elements and trans-acting factors (29, 30, 31). The cis elements include the 5′ and 3′ splice sites at exon/intron boundaries, and the branch site and polypyrimidine tract preceding the 3′ splice site (30, 31). Additional regulatory cis elements include exon splicing enhancers (ESEs) or exon splicing silencers (ESSs) which have been identified in precursor mRNA sequences from various tissue-specific or developmentally regulated genes (32-34). ESEs serve as splicing enhancers by contributing to the accuracy and efficiency of alternative splicing when recognised and bound by specific trans-acting factors (34, 35). Numerous trans-acting basal or regulatory splicing factors compete and interact with the various cis-elements, and indeed with each other, including the ubiquitously expressed U small nuclear ribonucleoprotein particles (U snRNPs), the serine-arginine (SR) family of nuclear phosphoproteins, and the heterogeneous ribonucleoproteins (hnRNPs), among many others (29, 36, 37). Variations in concentrations and ratios of splicing factors in different cell types and tissues can influence pre-mRNA processing (36-40) and individual splicing factors can exhibit unique specificity for particular pre-mRNAs or the tissue type in which they are expressed (29,30, 37, 38, 41). We have previously reported that the splicing factors SF2/ASF and hnRNPA1, are both spatially and temporally regulated within the human myometrium during pregnancy (Fig 2A) and secondly that these factors are involved in regulating alternative splicing of the GTP-binding
protein Gaα (42, 43). This finding further supports the premise that concentration ratios of trans-splicing factors in vivo may therefore be critical in defining the expression of specific protein isoforms in different tissues. Consequently, in this study we employed CREM mini-gene splicing constructs which incorporate the regulatory cis-elements associated with the alternative splicing of CREM in vivo, and employed transient transfections and in vitro splicing together with RNA electrophoretic mobility shift assays (EMSAs) to investigate the trans- and cis-acting factors and elements involved in regulating the switch in the expression of myometrial CREMτ2α to CREMα that occurs throughout pregnancy.

EXPERIMENTAL PROCEDURES

Construction of CREM mini-genes
A CREM mini-gene, pcDNA3.1 CREM-1 was generated from human genomic DNA using CREM-specific PCR primers, containing specific restriction sites, for the individual exon/intron fragments of CREM to facilitate the sequential insertion of each fragment into the pcDNA3.1(-) expression vector (Invitrogen, Life Technologies). These primers, detailed in Table 1, amplify CREM exons E and F (which encodes the phosphorylation domains), exon G (encoding the second glutamine-rich trans-activation domain) and the downstream exon H, together with truncated versions of their adjacent intronic sequences. PCR products were initially cloned into the TOPO®-TA cloning vector (Invitrogen, Life Technologies), and sequenced to confirm exon/intron DNA sequences. Each CREM fragment was then subcloned sequentially into pcDNA3.1 by repeated restriction digestion and ligation using T4 DNA ligase (Promega) to generate the complete pcDNA3.1CREM-1 splicing construct that is under transcriptional control of the CMV promoter and contains the bGH polyadenylation signal. A second CREM mini-gene construct pcDNA3.1CREM-2 which consists of exons C (encoding the first glutamine-rich trans-activation domain) and exons E/F/G/H (and their flanking intronic regions) was also generated in order to reproduce the regulated splicing of the CREM gene in myometrium and ensure that the regulatory factors that splice CREM precursor mRNA into CREMτ2α are present in this model system. Plasmids encoding splicing factors SF2/ASF (pCG-SF2), SRp20 (pCG-20), SRp40 (pCG-40), SRp55 (pCG-55), 9G8 (pCG-9G8) and hnRNPA1 (pCG-A1) have been described (37).

Tissues and cell culture
Human primary myometrial smooth-muscle cell cultures and HeLa cells (LGC-ATTC) were used in this study. Samples of upper and lower segment myometrial tissue were obtained from non-pregnant, pregnant non-labouring, and spontaneous labouring women undergoing surgery. Written consent was obtained from all women, and ethical approval granted by the Newcastle and North Tyneside Health Authority Ethics Committee. Primary myocyte isolation was undertaken essentially as described by (44). Primary myocytes were cultured with complete MEM-D-valine medium (Cell Culture Technologies), which inhibits fibroblast growth, supplemented with 10% fetal calf serum (FCS), penicillin (1 U/ml) and streptomycin (1ng/ml) and cultured under standard conditions at 37°C with 5% CO2.

Transient transfection of cultured myometrial cells
Transfection experiments on primary myometrial cells (obtained from pregnant tissue samples) were undertaken on sub-culture passages 2-3. Myometrial and HeLa cells (used as a control) were co-transfected, at 60-70% confluency (in the absence of antibiotics) using Mirus LT-1 (Cambridge Bioscience) cationic-lipid transfection reagent with 0.5 µg pcDNA3.1CREM-1 or pcDNA3.1CREM-2 and 1.5 µg pCG-SF2, pCG-A1, pCG-20, pCG-40, pCG-55, pCG-9G8 or a pCG control vector. Transfection efficiencies were in the range of 25-30% for all experiments, as determined by transfection with a β-galactosidase encoding plasmid, pcDNA3.1 LacZ (Invitrogen) (data not shown). Cells were harvested either 48 hours (HeLa cells) or 72 hours (myometrial cells) after transfection. Confirmation that the CREM mini-gene plasmids contain the necessary cis-acting regulatory signals for efficient pre-mRNA splicing was obtained by run-off transcription using a mMESSAGE-mMACHINE capping/transcription kit (described below) and RT-PCR of the CREM spliced products from total RNA extracted from myometrial cells post-transfection.

Western blot immunodetection
Myometrial tissue homogenates were prepared as previously described (42). Protein concentration
was assayed using the DC protein assay kit (Bio-Rad) and SDS-PAGE performed using 200 μg total protein from each homogenate resolved on 12% polyacrylamide gels. Immuno-reactive bands were detected by enhanced chemiluminescence, ECL (Amersham) and data obtained where a linear relationship existed between the amount of protein loaded and the intensity of the ECL signal from the immunoblots. Transfection efficiencies were also confirmed in individual experiments by Western immunoblotting using specific monoclonal antibodies to SF2/ASF (anti-SF2, Zymed), hnRNPA1 (4B10, Abcam) and a polyclonal antibody to SRp40 (anti-SRs Zymed). Note the increased protein expression of SF2/ASF, hnRNPA1, SC35 and SRp40 on transfection with their respective plasmids (Fig 2B) and endogenous levels of these proteins in myometrial cells were also calculated by this procedure (data not shown).

Expression of SRp40 protein
To generate human SRp40 protein, the full length cDNA coding sequence of the pCG-40 plasmid (described above) was amplified by PCR using Pfx DNA polymerase (Invitrogen Life Technologies). SRp40-specific sense and anti-sense primers were used that also contain Xba-1 and Bcl-1 restriction sites, respectively, to facilitate the subcloning into a pcDNA3.1 expression vector that has an upstream T7 RNA promoter. SRp40 protein was produced using a transcription/transcription (TnT) procedure using T7 RNA polymerase (Promega). 2 μl of the TnT reaction was resolved on 12% polyacrylamide gels and production of SRp40 protein verified by observation of an intense 40kDa band after coomassie blue staining (Fig 2C, lane 1). Production of SRp40 protein was also confirmed after Western immunoblotting using the polyclonal antibody to SRp40 (anti-SRs Zymed) as shown in Fig C, lane 2.

CREM mRNA splicing analysis by RT-PCR
CREM mRNA spliced variants generated from the mini-genes in transfected cells were analysed by RT-PCR. Total RNA was isolated from individual transfection experiments using SV total RNA isolation kit (Promega) or Tri-Reagent (Sigma) and first-strand cDNA synthesised from 1-μg total RNA using 20U Superscript III reverse transcriptase (Invitrogen, Life-Technologies) with 100ng oligo-dT16 as primer. PCR amplification was carried out using 2μl of cDNA template with CREM-specific primers (detailed in Table 2). PCR analysis was performed with 25 cycles as detailed in Fig 3. PCR products were sequenced to confirm their identity.

ESE-electrophoretic-mobility-shift-assay (ESE-EMSA)
Oligonucleotides containing the individual CREM exon splicing enhancer motifs were designed to use in RNA–electrophoretic mobility shift assays (EMSA). Sequences containing the ESE-motifs were placed downstream from a T7 RNA promoter to facilitate transcription. Sequences for the oligonucleotides are shown in Table 3. GpppG-capped and [α-32P]UTP-labelled RNA ESE-oligonucleotides were synthesised by run-off transcription for 4 hours at 37°C using the T7 capped transcription kit as described above. 3μl of radiolabelled RNA was incubated for 15 minutes at 30°C with either 3μl SRp-40 protein, 5 units of SF2/ASF or 5 units of SC35 (or 5 μl nuclear-extract) together with 3-5 μl Buffer B (ProteinOne). Both HeLa nuclear extracts (ProteinOne, USA) and nuclear extracts prepared from cultured myometrial cells were used. Myometrial nuclear extracts were prepared using a nuclear extract kit and protocol obtained from Active-Motif. In super-shift experiments the nuclear extract and transcribed oligonucleotides were supplemented with 1μg anti SRs pAb (ProteinOne) or 1μg anti –hnRNPA1 mAb (Abcam). RNA band shifts were then analysed by non-denaturing 10% agarose gel electrophoresis followed by autoradiography.

In vitro splicing assays
To generate transcripts for in vitro splicing, the pcDNA 3.1CREM mini-gene constructs which also harbor a T7 promoter were first linearised with Pme I. GpppG-capped and [α-32P]UTP-labelled pre-mRNA substrate was synthesised by run-off transcription using a mMESSAGE-mMACHINE capping/transcription kit with T7 RNA polymerase (Ambion, Inc.). In vitro splicing assays were undertaken essentially as described (45). Briefly, 25-30 fmoles of radiolabelled precursor mRNA was incubated for 3 hours at 30°C with 10 μl HeLa or myometrial nuclear extract supplemented with 15-25 pmol of SF2/ASF or SC35 (ProteinOne), recombinant hnRNPA1 protein (41, 46) or SRp40 protein which was produced using a coupled transcription/translation (TnT) system (Promega) as detailed above (Fig 2C). Reactions were
stopped by the addition of proteinase K and then phenol extracted, ethanol precipitated as described (Mayeda, 1999). RNA products generated from splicing in vitro were then fractionated by denaturing 6% polyacrylamide electrophoresis followed by autoradiography.

RESULTS

Effect of over-expression of SR proteins on CREM isoform expression
Transient co-transfections were undertaken on primary myometrial cell cultures (and HeLa cells) to evaluate the effect of increased levels of several SR proteins and hnRNPA1 on the expression of spliced variants of CREM. CREM mRNA spliced variants generated from the pcDNA3.1-CREM plasmids co-transfected with SF2/ASF, hnRNPA1, SC35, SRp40, SRp20, SRp55 or 9G8 were analysed by RT-PCR. Detection of the exogenous CREM mRNA variants CREM\(\tau_2\alpha\) and CREM\(\alpha\) was undertaken using sense primers for the pcDNA3.1 vector or exon F together with antisense primers for exon F or exon H (which also contained 9 nucleotides of vector). All primers are detailed in Table 2. Data represented here is based on each experiment being performed in triplicate. Representative RT-PCR analyses of transfection experiments are shown in Fig 3. Co-transfection of pcDNA3.1CREM-1 and pcDNA3.1CREM-2 with the pCG-SRp40 plasmid resulted in increased expression of CREM\(\tau_2\alpha\) mRNA spliced variants which contain the alternatively spliced exon G, as reflected in the intensity of the 615bp band compared with the 426bp band (Fig 3 A (i) lane 4). The retention of exon G as a consequence of over-expression of SRp40 was further confirmed by RT-PCR using specific CREM sense and antisense primers for exons F and H as detailed in Fig 3 A & B, (iii) lanes 4. However, increased levels of SRp40 did not appear to affect the inclusion of exon C into CREM mRNAs as was observed when the pcDNA3.1CREM-2 plasmid (containing exon C) was transfected. This was reflected by the similar intensities of the 762-bp and 615-bp PCR bands in Fig 3 B (i), lane 4 and also the 378-bp and 231-bp bands in Fig 3 B (ii), lane 4 using the CREM sense and antisense primers for exons F and H. Note that in cultured myometrial cells the basal splicing pattern for pcDNA3.1CREM-1 and -2 in the presence of the control pCG plasmid was similar to the splicing of endogenous CREM in the pregnant myometrium (Fig 1) in that CREM\(\tau_2\alpha\) and CREM\(\alpha\) were both expressed, as indicated by the similar intensities of the bands representing these two isoforms (Fig 3, A & B, lanes 8).

In HeLa cells the basal splicing pattern for pcDNA3.1CREM-1 and -2 appeared to be different from that found in myometrial cells. RT-PCR analysis of HeLa cells using pcDNA3.1CREM-1 and -2 and the control plasmid pCG indicated that the predominant CREM mRNA variant expressed in these cells is the small CREM\(\alpha\) isoform, as indicated by the low intensity of the band representing CREM\(\tau_2\alpha\) and high intensity of the band representing CREM\(\alpha\). However, a change in the splicing pattern of the CREM minigenes was observed as a consequence of over-expression of SRp40, consistent with the results obtained from myometrial cells, as indicated by an increase in the intensity of a 482-bp band shown in Fig 3 C, lanes 4 & 9. Increased levels of SF2/ASF also appeared to affect the splicing of exon G in HeLa cells, but only when the pcDNA3.1CREM-1 construct was co-transfected (Fig 3 C, lane 6).

Expression profiles of SRp40 and SC35 in the myometrium during pregnancy
The expression of SRp40 and SC35 was evaluated by immunoblotting using both non-pregnant and upper and lower myometrium tissues from pregnant and labouring patients. Quantification by densitometric analysis demonstrated that the levels of SRp40 were consistent in all non-pregnant samples (Fig 4 A, lanes 1-4). However, during pregnancy, myometrial SRp40 expression decreased significantly in both the lower and upper uterine segments (P<0.01) and then decreased further to undetectable levels at the onset of labour. A different expression profile was observed for SC35. Quantification indicated that SC35 protein levels were comparable in non-pregnant myometrium and the upper uterine region in both pregnancy and labour. However, there was a decrease, though not significant, in the expression of SC35 in the lower uterine segments in both pregnancy and labour.

Identification of multiple ESEs in the alternatively spliced exons C and G of CREM
We analysed the alternatively spliced CREM exon sequences using ESEfinder (47;...
http://rulai.cshl.edu/tools/ESE/) to identify putative ESE motifs recognised by SF2/ASF, SC35, SRp40 or SRp55. We found multiple high-score motifs for all four SR proteins in both the alternatively spliced exons C and G of CREM, as shown in Fig 5A. One heptameric high-score motif (CAGAAGA) for SF2/ASF was identified in exon C, which overlaps with a heptameric SRp40 motif. A second ESE motif for SF2/ASF (CCCAGGA) was identified in exon G. Three octameric motifs for SC35 (GATTTCCTA, GACTGCAG, GGTTGTTG) were found in the exon G sequence, and one hexameric ESE motif for SRp55 (CGCGAGC) was also present in exon G. Interestingly, there were seven SRp40 motifs (CCAGAAG, CCACCAGC, ACACACC, ATTCAGG, CCACACGC, ACACAGC, TCCCAGG), the first three in exon C and the last four in exon G, as shown in Fig 5A. To determine the functionality of these ESEs in SR-protein binding, we designed ribo-oligonucleotides containing the ESE motifs for use in RNA electromobility shift assays as detailed in Table 3. To confirm that ESE:SR-protein binding was specific, control oligonucleotides with the ESE motifs abolished were also designed. The mutant sequences were designed such that no new SR–protein motifs for SF2/ASF, SC35, SRp40 or SRp55 were introduced, according to ESEfinder.

**SRp40 functionally binds seven heptameric exon splicing enhancers in CREM**

SRp40 was assayed for binding to the individual ESE motif sequences by electrophoretic mobility shift assay. The results demonstrate that SRp40 bound to all seven of the heptamer ESE motifs present in exons C and G of CREM. ESE:SRp40 binding was observed after individual wt-ESE-oligonucleotides were incubated with the SRp40 protein, as reflected by the band shifts observed in Fig 5B. Of note, the intensity of the band shifts for ESE oligonucleotide 6 (which contains two SRp40 binding sites) was particularly strong. SRp40 protein did not bind the control oligonucleotides which had the ESE motifs abolished. SR protein:ESE binding was also observed when the wt-oligonucleotides 4 and 5 were incubated with recombinant SF2/ASF and/or SC35 (Fig 5B). Similar results were seen when the recombinant proteins were replaced by nuclear extract prepared from cultured myometrial cells. In contrast, when the oligonucleotides were incubated with HeLa nuclear extract, band shifts were observed in some cases when either the wt or mutated ESE oligonucleotides were used (Fig 5C). It is possible that there are additional unidentified protein binding sites or exon splicing silencer elements (ESSs) present within the sequences of the oligonucleotides. These may bind to other protein factors (such as the SR-protein antagonist hnRNPA1) that are present in HeLa nuclear extracts. Alternatively, the mutant sequences may have fortuitously introduced binding sites for other RNA-binding proteins. Sequence-specific binding of SRp40 was also observed when the SRp40:ESE complexes were supershifted by using an anti-SR antibody (Fig 5C). A similar result was observed when the SRp40 protein was replaced with nuclear extracts prepared from cultured myometrial cells (data not shown). hnRNPA1 binding was also observed when both wt- and mutated ESE oligonucleotides 1 (contains overlapping ESE motifs for SRp40 and SF2/ASF) and 4 (contains motifs for both SC35 and SF2/ASF) were used in electromobility shift assays. hnRNPA1 binding was also confirmed when the reactions were incubated with an anti-hnRNPA1 antibody.

**SRp40 switches the splicing pattern of CREM spliced variants**

We further evaluated the role of SRp40 in regulating the alternative splicing of CREM by *in vitro* splicing. Pre-mRNA transcripts from the pcDNA3.1CREM-1 and -2 constructs were spliced in HeLa nuclear extract supplemented with recombinant SF2/ASF, hnRNPA1, SC35 or SRp40 (Fig 6A). CREM pre-mRNA was spliced under these experimental conditions generating CREM spliced products with and without the inclusion of exons C and G (Fig 6A). The addition of SRp40 protein to splicing reactions containing precursor RNA from pcDNA3.1CREM-1 generated mRNA products in which the intron between exons F and G was removed and the exon G retained, corresponding to CREM τα mRNA. Conversely, when the nuclear extract was supplemented with hnRNPA1, the band representing this intron was absent (Fig 7, lane 2) indicating exon G was spliced out. This was further evidenced by the presence of the higher molecular weight band representing the lariat intron- exon G complex, which was present only when hnRNPA1 was added, but not with SF2/ASF, SC35 or SRp40. Nuclear splicing reactions supplemented with transcribed, unlabelled ESE 6 RNA were also included to further verify the relevance and
functionality of the CREM ESEs. ESE 6 was initially chosen because it contains two high-score motifs for SRp40. Indeed, ESE 6 competed out the effect of SRp40 and reduced the levels of spliced mRNAs containing exon G (Fig 7 B). Similar results were also observed for ESE-oligonucleotides 8 and 9, which also contain high-score SRp40 ESE motifs (data not shown). When the ESE 6 control oligonucleotide which has the ESE motif mutated was used, no competition was observed, suggesting that the SRp40 effect is specific.

DISCUSSION

Alternative splicing promotes a switch in the expression of CREM$\tau_2\alpha$, an activator, to CREM$\alpha$, a repressor, within the human myometrium throughout pregnancy and labour. We have recently shown that this change in the expression of spliced variants of CREM is relevant to the regulation of downstream CRE-responsive target genes involved in uterine activity during gestation and parturition (28). In this present study we unravel the splicing mechanisms that control the expression of CREM spliced variants in human myometrial cells. We provide evidence to indicate that the switch from synthesis of CREM $\tau_2\alpha$ to CREM$\alpha$ that occurs throughout pregnancy and labour is orchestrated by the downregulation of an SR protein family member, SRp40.

Our data from transfection of CREM minigenes in myometrial, and HeLa cells, indicates that overexpression of SRp40 favoured the proximal alternative 5' splice site promoting mRNA transcripts in which exon G were included, thus, stimulating the synthesis of CREM$\tau_2\alpha$ within these cells. Overexpression of other members of the SR protein family SF2/ASF, SC35, SRp20, SRp55 and 9G8 did not appear to affect the splicing patterns of CREM within myometrial cells. In contrast to SRp40, and in keeping with its previously defined role in splice site selection, over-expression of hnRNPA1 favoured the selection of distal 5' splice sites such that exon G was skipped out resulting in the synthesis of CREM$\alpha$.

We have previously reported that the endogenous alternative splicing pattern of another gene, the adenyl cyclase stimulatory G-protein $\alpha_s$, is different in myometrial cells compared with HeLa cells (43). In myometrial cells the principal spliced variant was $\alpha_s$, long whereas in HeLa cells the short isoform of $\alpha_s$, resulting from the skipping of an internal exon 3, was predominant. A similar pattern of splicing was also observed for CREM in that in myometrial cells the principal CREM variant appeared to contain exon G whereas in HeLa cells the predominant mRNAs of CREM had exon G skipped out. We propose that the preference for exon skipping in HeLa cells with both $\alpha_s$ and CREM conforms to the abundance of hnRNPA1 6-7 x 10^7 copies/cell in these cells (37).

Data presented here suggests that SRp40 regulates splice site selection via its interactions with exon splicing enhancers. This is evidenced by the presence and functionality of multiple ESEs present in both of the alternatively spliced exons C and G of CREM. In exon C there are three SRp40 binding sites, all of which are recognised and bound by SRp40. In exon G there are four high scoring ESE motifs which specifically bind SRp40 supporting a model in which SRp40 binding is required for the recruitment of the splicing complex to a 5' proximal splice site. The ESEs present in exon G appear to be particularly important in the splice site selection thus providing a mechanistic explanation as to how SRp40 regulates the switch in CREM$\tau_2\alpha$ to CREM$\alpha$ (Fig 7).

SRp40 has previously been studied for its role in alternative splicing and has been shown to select both proximal and distal splice 5' sites in a substrate-specific manner (29, 39). Our findings indicate that SRp40 appears to concurrently activate the selection of both proximal and distal 5' splice sites within the same precursor mRNA transcript. We suggest that, in vivo, in the non-pregnant state when levels of SRp40 are high, SRp40 promotes the skipping of exon C and inclusion of exon G, resulting in the production of CREM$\tau_2\alpha$. Conversely as levels of myometrial SRp40 decrease throughout pregnancy and labour there is a parallel decrease in the expression of CREM$\tau_2\alpha$ and an increase in CREM$\alpha$. This raises the question as to how SRp40 works to regulate the switch in the expression of CREM spliced variants when it is down-regulated. We propose that SRp40 and multiple ESEs present in the exons C and G work in a bi-directional manner to regulate CREM spliced variants. SRp40 via its interactions with ESEs in exon C promotes the proximal splice site resulting in the inclusion of exon C. Conversely, SRp40 via its interactions with ESEs in exon G promotes the selection of a
proximal splice site resulting in the inclusion of exon G. The down-regulation of SRp40 that occurs throughout pregnancy suggests that when there are only negligible levels of SRp40 present, there is no SRp40:ESE complex formation, and consequently both the alternatively spliced exons C and G are skipped out of the precursor mRNA transcript, thus generating CREMα. One could speculate that hnRNPA1, a well characterised antagonist to the SR proteins (36) may be a contributory factor and have a silencing affect in regulating CREM. However, hnRNPA1 is clearly spatially regulated in the myometrium during pregnancy whereas CREM is not. We have previously shown that hnRNPA1 levels are moderate in the non-pregnant myometrium (42) as shown in Fig 2 A and as such hnRNPA1 may well contribute to the skipping of exon C that occurs in the non-pregnant state. However, throughout pregnancy and at the onset of labour there is a dramatic switch in the spatial expression of hnRNPA1 within the functionally distinct upper and lower regions of the uterus (Fig 2A), whereas the expression and switch in the synthesis of CREMτ2α to CREMα that occurs is not spatially regulated within the different uterine regions. Moreover, since the expression patterns for SC35, and SF2/ASF, within the non-pregnant and pregnant human myometrium are not comparable with the expression of CREM spliced variants it is unlikely they regulate the switch in spliced variants of myometrial CREM that occurs in pregnancy. However, SC35 may possibly be a candidate splicing factor in regulating CREM spliced variants in other tissues as there are three functional SC35-specific ESE motifs present in CREM. Moreover, it could be reasoned that other unidentified protein factors may also be involved. A previous study by Cowper (48) identified SR protein-like repressor factors, named SRrp40 and SRr35. These factors appear to antagonise authentic SR proteins SF2/ASF and SC35 by selecting the most distal splice sites in the adenovirus pre-mRNA in an activity similar to hnRNPA1.

ESEs have been reported to promote either 5’ or 3’ splice site selection and there is recent evidence to indicate that in a few examples a single ESE can concurrently promote recognition of both upstream and downstream 5’ and 3’ splice sites, thus serving as a bi-directional splicing enhancer (49, 50). Caputi et al (51) reported a novel bi-directional ESE that regulates the expression of the HIV-env, vpu and nef mRNAs and interestingly both SRp40 and SF2/ASF were shown to bind and activate this ESE. What appears to be novel with the CREM gene is the observation that different sets of SRp40 ESEs within different exons within the same gene appear to work bi-directionally to promote distal and proximal 5’ splice site selection. To the best of our knowledge this is this first example of a human gene where multiple ESEs present in two alternatively spliced exons work bi-directionally resulting in the synthesis of two functionally distinct protein isoforms namely CREMτ2α and CREMα.

It is widely accepted that alternative splicing is a fundamental mode of gene regulation in the generation of structurally and functionally distinct protein isoforms (52,53). Furthermore, the accuracy of the splicing machinery is essential for developmental and tissue-specific control of gene expression (24, 53-55). In previous studies we have demonstrated that alternative splicing regulates expression of different components of the cAMP signaling pathway within the developmentally regulated myometrium throughout pregnancy and labour, namely Gaα and CREM (20, 26). In addition, we have reported that two key splicing factors SF2/ASF and hnRNPA1 are differentially expressed within the myometrium during gestation (42) and our findings also showed that these pivotal splicing factors regulate the expression of Gaα (43). Data presented here describes the temporal/spatial expression profiles for two more splicing regulators SRp40 and SC35 within the myometrium during pregnancy and labour. In this study we show that the molecular mechanisms controlling the switch in the expression of a potent transcription activator CREMτ2α to that of a transcription repressor CREMα involve the differential expression of SRp40. Hence our studies emphasize the importance of alternative splicing in controlling the expression of functionally distinct protein isoforms associated with uterine activity during gestation and labour.

Evidence from previous studies shows that subtle fluctuations in the concentrations of specific splicing factors can define the formation of different spliced mRNA isoforms derived from a number of precursor mRNA species and this switching of splice sites also occurs after over-expression of SR proteins in vivo, when tested with a range of reporter genes, promoting aberrant
exon skipping and inclusion (29, 38, 39). In this study we show that the down-regulation of SRp40 in pregnancy is important for the switch in the splicing pattern of CREM. As the specificity of SR proteins in regulating the efficiency or pattern of alternative splicing of different genes is attributed in part to the recognition of ESEs (30, 34, 41), then it is worthy to note that CREM exons contain multiple copies of SR-protein-specific ESE motifs, which raises the question of whether different sets of SR proteins are involved in regulating CREM in vivo.

To conclude, our study provides strong evidence that the switch in the alternative splicing of the human CREM gene in the human myometrium during fetal maturation is controlled by SRp40 and involves the use of multiple ESEs. We provide evidence to indicate that the switch in the splicing of CREM\(\tau_2\alpha\) to CREM\(\alpha\) is controlled by the complex interplay of at least seven SRp40-specific bi-directional splicing enhancers in addition to the consensus splicing cis-elements. In addition, this is the first study to characterise the expression profiles for SRp40 and SC35 in a human organ. We demonstrate that expression of endogenous SRp40 is tightly regulated throughout pregnancy. Moreover, we show that the reduced expression of SRp40 that occurs within the myometrium throughout pregnancy correlates with the levels of CREM\(\tau_2\alpha\) and CREM\(\alpha\) within these cells. Importantly, differential expression of SRp40 and other members of the SR-protein family in various types of tissues, such as the heart, liver and brain, may under normal physiological and pathophysiological conditions define the expression of functionally distinct isoforms of CREM and their subsequent transcriptional effects on downstream target genes.

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The abbreviations used are: CREM, cyclic AMP response element modulator protein; CRE, cyclic AMP-response elements; CREB, cyclic AMP binding protein; ATF, activating transcription factor; bZIP, basic region-leucine zipper; bGH, bovine growth hormone; EMSAs, electrophoretic mobility shift assays; ESE, exon splicing enhancer; pAb, polyclonal antibody; mAb, monoclonal antibody; TnT, Transcription/Translation.

FIGURE LEGENDS

Fig. 1. Schematic diagram of the human CREM gene showing the two myometrial CREM spliced variants CREM$_{\alpha}$ and CREM$_{\alpha}$. (A) Alternative splicing involves the exons C and G exons which encode the Q-rich, glutamine-rich trans-activation domains; P-box= kinase inducible domain; PI and P2 are the alternative promoters; ICERS, inducible cAMP early repressor transcribed from promoter 2. (B) Western immunoblot analysis illustrating the switch in the expression of CREM$_{\alpha}$ to CREM$_{\alpha}$ that occurs during pregnancy. CREM$_{\alpha}$, a potent transactivator/weak repressor, lacks exon C whereas CREM$_{\alpha}$, a repressor of transcription, lacks both exons C and G which encode the Q-rich trans-activation domains.

Fig. 2. Temporal and spatial expression of myometrial SF2/ASF and hnRNPA1 during human gestation, controls for transient transfection and production of SRp40 protein. (A) Western immunodetection and subsequent densitometric analysis of SF2/ASF and hnRNPA1 in the upper (US) and lower (LS) myometrium as described in Pollard et al (42). (B) Western immunoblotting demonstrating over-expression of SF2/ASF, hnRNPA1, SC35 and SRp40 after transient transfection. Lanes 1, transfection with pCG control vector, lanes 2, transfection with pCG-SF2, pCG-A1, pCG-35 or pCG-40. (C) Production of SRp40 protein by coupled transcription/translation TnT reaction). Strong staining was observed at ~ 40kDa after 2 µl TnT produce was separated by SDS-PAGE and stained with coomassie blue (lane 1). (ii) immunoblotting using anti-SRs mAb to verify the expression of SRp40 protein by TnT as reflected by the intensity of a single band of ~ 40kDa.

Fig. 3. Effect of SR proteins and hnRNPA1 on CREM isoform expression. RT-PCR was employed using RNA from transient co-transfection of (A & B) myometrial cells and (C) HeLa cells using pcDNA3.1-CREM-1 and pcDNA3.1-CREM-2 together with plasmids encoding SF2/AF, hnRNPA1, SC35, SRp40, SRp20, SRp55, 9G8 or the pCG control vector. PCR reactions were performed with an initial hot start cycle at 94 °C (4 min), 55 °C (30 s) and 72°C (1 min) followed by 20-25 cycles at 94 °C (1 min), 55 °C (30 s) and 72°C (1 min). PCR reactions were carried out using (i) sense primers for the pcDNA 3.1 vector together with antisense primers for exon H, plus 9 nucleotides of vector to identify CREM spliced variants expressed from the pcDNA3.1 vector. Splicing patterns were consequently confirmed by using (ii) the vector sense primers with anti-sense primers of the exon F of CREM and (iii) sense primers for exon F with anti-sense primers for exon H. Note over-expression of SRp40 results in the inclusion of exon G (as indicated by *). GAPDH controls for each cell type are shown below.

Fig. 4. Expression profiles for SRp40 and SC35 within the human myometrium during pregnancy. (A) Western immunoblotting and densitometric analysis of SRp40 protein expression. Detection of SRp40 was undertaken using an anti-SR pAb (Zymed) which recognizes a range of SR proteins but does not react with SR proteins of a similar molecular weight to SRp40. A significant decrease in the levels of SRp40 was observed in the upper and lower uterine regions in all pregnant and laboring samples. Data are shown as the mean ±SEM (n = 12 for all non-pregnant (NP), pregnant (P), and laboring (SL) samples. NP vs P/US and vs P/LS = <0.01; NP vs SL/US and SL/LS = < P 0.001. (B) Detection of SC35 was undertaken using an anti-SC35 mAb (ProteinONE, USA). SC35 protein levels were similar in all non-pregnant, upper uterine pregnant and laboring samples (n = 12 for each group). SC35 levels decrease though not significantly in the lower segments in both pregnancy and labour.
Fig. 5. Functionality of CREM ESEs by electrophoretic mobility shift assays (EMSAs).
(A) EMSAs of SRp40 binding to ESE motifs within the exons C and G of CREM. GpppG-capped and [α^{32}P] UTP-labelled RNA oligonucleotides were synthesised by run-off transcription and incubated with either SRp40 TnT reaction or recombinant SC35. RNA: protein complexes were then resolved on non-denaturing 10% agarose gels. Bandshifts representing the protein:ESE complexes were observed for all wt-oligonucleotides containing ESE motifs for SRp40 and SC53. (B) Protein: RNA binding was also observed when the oligonucleotides were incubated with myometrial or HeLa nuclear extracts. (C) Further verification of RNA: protein complexes was accomplished by supershift assays using SRp40 protein with the anti-SR protein antibody.

Fig. 6. SRp40 switches the splicing pattern of CREM spliced variants
(A) In vitro splicing of CREM in myometrial nuclear extracts. pcDNA3.1-CREM-1 and pcDNA3.1CREM-2 GpppG-capped and [α^{32}P] UTP-labelled precursor-mRNA was supplemented with recombinant SF2/ASF, hnRNPA1, SC35 or SRp40 (TnT reaction). RNA was resolved on 6% PAG with 8M urea. Precursors, intermediates and final spliced products are indicated. (B) Nuclear splicing reactions supplemented with transcribed, unlabelled ESE 6 RNA were also included to further verify functionality of the CREM ESEs. ESE 6 competed out the effect of SRp40 and reduced the levels of spliced mRNAs containing exon G suggesting that the SRp40 effect is specific.

Fig. 7. Model proposing how SRp40 regulates the switch in alternative splicing of CREMτ to CEMα that occurs in pregnancy and labour. In the non-pregnant state when levels of SRp40 are high, SRp40 promotes the skipping of exon C and inclusion of exon G, resulting in the production of CREMτ. Conversely, as levels of myometrial SRp40 decrease throughout pregnancy and labour (Fig 4, A) there is a parallel decrease in the expression of CREMτ and an increase in CREMα (Fig 1, B). Our data indicates that the multiple ESE elements present in CREM affect splicing through initiation by SRp40.

Table 1. PCR primers for the construction of CREM mini-genes. *Restriction sites are shown in italics.

Table 2. RT-PCR primers for the amplification of CREM spliced variants.

Table 3. Sequences for oligonucleotides containing ESE motifs in the exons C and G of CREM. Shaded sequence represents the individual ESE motifs. There are overlapping ESE motifs for SRp40 (shaded) and SF2 (boxed) in oligonucleotide 1. Control oligonucleotides with the ESE motifs abolishes were also designed. The first 17 bases of each oligonucleotide (in italics) consist of a T7 RNA promoter sequence to facilitate in vitro transcription.
### Table 1.

| Exon      | Restriction site | DNA sequence (5’-3’)                      |
|-----------|------------------|------------------------------------------|
| sense C   | Nhe 1            | GCTAGCTATTAGTGAGTGTTATTACTTA             |
| antisense C | Xho 1         | CTCGAGCGGTATAAGCACTTCATA                 |
| sense E/F | Xho 1            | CTCGAGCTATTGCTAGTTGCTTC                 |
| antisense E/F | EcoR V    | GATATCGATACAGTTTAGTTAGTAATTAATGA        |
| sense G   | EcoR V           | GATATCTTCTCAATTCAGCATAGGA               |
| antisense G | BamH 1    | GGATCCCTGTGATATAGTAAATCCATAG           |
| sense H   | BamH 1          | GGATCCTCAAGATCACCCTTATAG                |
| antisense H | Pme 1     | GTTTAAACTCATTAGCCTAGCTCTC               |

### Table 2.

| Primer name | Target site | DNA sequence (5’-3’)                      |
|-------------|-------------|------------------------------------------|
| pcDNA31 sense | exon E     | CTCACTATAGGGAGACCCAAGC                    |
| sense E1    | exon E     | TGCAGAGACAGATGAATCTGCAG                   |
| sense E2/V  | exon E     | CTAGACTCGAGGTAGCAGCAATTGC                |
| sense F     | exon F     | GAATGAACTGTCCTCTGATGTGC                  |
| antisense F | exon F     | GGTACTGCCATGGTAGCAATAC                   |
| antisense H/V | exon H     | GTTTAAACCTGTTTTTCATTAGCCTC               |

Tyson-Capper *et al*
Table 3.

| Number and position | CREM exon | SR protein | oligonucleotide sequences |
|---------------------|-----------|------------|---------------------------|
| 1 23/24             | C         | SRp40/SF2  | TAATACGACTCTACTATAAGGCAAGGAAGGCTCC |
| 1                   | C         | -          | TAATACGACTCTACTATAAGGCAAGGCTCC |
| 2 103               | C         | SRp40      | TAATACGACTCTACTATAAGGCAAGGCTCC |
| 2                   | C         | -          | TAATACGACTCTACTATAAGGCAAGGCTCC |
| 3 135               | C         | SRp40      | TAATACGACTCTACTATAAGGCAAGGCTCC |
| 3                   | C         | -          | TAATACGACTCTACTATAAGGCAAGGCTCC |
| 4 29/38             | G         | SC35/SF2   | TAATACGACTCTACTATAAGGCAAGGCTCC |
| 4                   | G         | -          | TAATACGACTCTACTATAAGGCAAGGCTCC |
| 5 61                | G         | SC35       | TAATACGACTCTACTATAAGGCAAGGCTCC |
| 5                   | G         | -          | TAATACGACTCTACTATAAGGCAAGGCTCC |
| 6 85/99             | G         | SRp40/SRp40| TAATACGACTCTACTATAAGGCAAGGCTCC |
| 6                   | G         | -          | TAATACGACTCTACTATAAGGCAAGGCTCC |
| 7 125               | G         | SRp55      | TAATACGACTCTACTATAAGGCAAGGCTCC |
| 7                   | G         | -          | TAATACGACTCTACTATAAGGCAAGGCTCC |
| 8 147               | G         | SRp40      | TAATACGACTCTACTATAAGGCAAGGCTCC |
| 8                   | G         | -          | TAATACGACTCTACTATAAGGCAAGGCTCC |
| 9 163/176           | G         | SRp40/SC35 | TAATACGACTCTACTATAAGGCAAGGCTCC |
| 9                   | G         | -          | TAATACGACTCTACTATAAGGCAAGGCTCC |

A.J. Tyson-Capper et al.
Figure 1

A

Alternative splicing

CREM\(_{\tau_2\alpha}\)
(39 kDa)

CREM\(_{\alpha}\)
(28 kDa)

B

CREM \(_{\tau_2\alpha}\)

CREM\(_{\alpha}\)

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Figure 3

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Figure 4

A

NP | P | SL
---|---|---
US | LS | US | LS | US | LS

SRp40

40kDa

US | LS | US | LS | US | LS

non-pregnant | pregnant | labouring

B

NP | P | SL
---|---|---
US | LS | US | LS | US | LS

SC35

35kDa

US | LS | US | LS | US | LS

non-pregnant | pregnant | labouring

A.J.Tyson-Capper et al
Figure 5

A

ESE 1 wt ESE 1 abol ESE 2 wt ESE 2 abol ESE 3 wt ESE 3 abol ESE 4 wt ESE 4 abol ESE 5 wt ESE 5 abol ESE 6 wt ESE 6 abol ESE 8 wt ESE 8 abol ESE 9 wt ESE 9 abol

SRp40 SC35 SRp40

bandshifts transcribed RNA oligos

B

ESE 6 wt ESE 6 abol ESE 7 wt ESE 7 abol ESE 8 abol ESE 8 abol ESE 9 wt ESE 9 abol

myometrial nuclear extracts

ESE 6 wt ESE 6 abol ESE 7 wt ESE 7 abol ESE 8 abol ESE 8 abol ESE 9 wt ESE 9 abol

HeLa nuclear extracts

bandshifts

C

ESE 2 wt ESE 2 abol ESE 6 abol ESE 6 abol ESE 8 wt ESE 8 abol ESE 9 wt ESE 9 abol

1 2 3 4 5 6 7 8

supershift transcribed RNA
Figure 6

A

CREM1 pre-mRNA
substrate

Intron before exon G

B

Intron before exon G

Figure 6

A.J.Tyson-Capper et al
non-pregnant                  pregnant

SRp40

CREM\(\alpha\) repressor

SRp40

CREM\(\tau_2\alpha\) activator

CREM\(\alpha\) repressor

PKA

CBP/p300 + basal transcription machinery

activation/repression of downstream target genes

Figure 7

A.J. Tyson-Capper et al
The switch in alternative splicing of cyclic AMP response element modulator protein CREM $\alpha$ (activator) to CREM $\alpha$ (repressor) in human myometrial cells is mediated by SRp40.

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