Identification of human myometrial target genes of the cAMP pathway: the role of cAMP-response element binding (CREB) and modulator (CREM α and CREM $\tau_2\alpha$) proteins

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Abstract

cAMP-response element (CRE) binding (CREB) and modulator (CREM) proteins, activated by protein kinase A-mediated phosphorylation, bind as homo- and heterodimers to promoters containing CRE and activator protein 1 (AP-1) sites to alter target-gene expression. We have previously reported differential expression of CREB and CREM splice variants CREM α and CREM $\tau_2\alpha$ in human myometrium during pregnancy and labour. Via microarray studies with cultured myometrial cells stably transfected with CREB, CREM α and CREM $\tau_2\alpha$ cDNAs, CREB affected the expression of 958 genes; 522 being up-regulated and 436 down-regulated. CREM α altered the expression of 118 genes; 71 were increased and 47 decreased. CREM $\tau_2\alpha$ affected 220 genes; 148 were activated and 72 repressed. Notably, genes affected by CREB, CREM α and CREM $\tau_2\alpha$ belong to largely discrete groups: less than 9% were affected by more than one factor. Genes involved in regulation of cell death and apoptosis, growth and maintenance, signal transduction, physiological and developmental processes, protein kinase cascades, extracellular matrix, cytoskeleton, cell-cycle regulation, transport, and a variety of enzymes, intracellular components and nucleic acid-binding proteins have been described, many of which are involved in the modulation of myometrial activity during pregnancy and parturition.

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Introduction

Many signalling pathways and cascades, comprising a wide variety of hormone-, growth factor-, stress- and inflammation-mediated stimuli, crosstalk and converge to mediate the activation of the ubiquitous cAMP-response element (CRE) binding (CREB) and modulator (CREM) proteins (De Cesare *et al.* 1999), via phosphorylation at serine residues 133 and 117 respectively (Gonzalez & Montminy 1989, de Groot *et al.* 1993). This family of transcription factors consists of a large number of protein isoforms generated from an array of alternatively spliced transcripts. Constituent exons bestow upon these splice variants different facets of functionality, including a leucine zipper domain for dimerization with other related proteins, a basic region for DNA binding, two glutamine-rich domains involved in trans-activation, and a kinase-inducible domain (termed a P-box or KID) as a site for phosphorylation and activation (Sun *et al.* 1992, Walker *et al.* 1994, Habener *et al.* 1995, Sanborn *et al.* 1997). Each CREB/CREM isoform therefore possesses trans-activation and/or trans-repression properties depending on the exonic configuration of their mRNAs. In total, seven alternatively spliced isoforms of CREB have been identified (Don and Stelzer 2002), and a

multitude of CREM isoforms arising not only by alternative splicing, but also due to alternative promoters, different transcription- and translation-initiation sites, and changes in stability due to variant poly-A sites (Sanborn 2000a).

CREB and CREM are members of the basic region leucine-zipper (bZIP) family of transcription factors (Landschulz *et al.* 1988, Ziff 1990), which also includes the activating transcription factor (ATF) proteins and Jun and Fos. These proteins are able to function as homo- and/or hetero-dimers (Sassone-Corsi 1988, Hai *et al.* 1989, Hai and Curran 1991, Borrelli *et al.* 1992), and regulate the transcription of downstream-affected genes via binding to regulatory motifs in their promoter regions such as the CRE (consensus sequence, 5'-TGACGTCA-3') and the TPA-response element (TRE) or activator protein 1 (AP-1) site (consensus sequence, 5'-TGAGTCA-3'; Xie *et al.* 1995). Binding of these motifs can occur irrespective of the phosphorylation state of the constituent dimer proteins, and indeed is one method of inhibition of transcription of cAMP-responsive genes.

Although activation of CREB and CREM can occur for example via (i) synaptic activity through calmodulin, (ii) growth factor binding through extracellular signal-

regulated kinase (ERK) and (iii) stress and inflammatory cytokine action through p38, perhaps the major determinant is the binding of hormone ligands to G-protein-coupled receptors (GPCRs; Servillo *et al.* 2002). This causes a rise in the intracellular level of cAMP due to activation of adenylate cyclase, which in turn promotes the phosphorylation of CREB and CREM factors via protein kinase A (PKA). Other components of the cAMP signaling pathway are often up- or down-regulated to affect cAMP levels, notably hormonal ligands that bind to the GPCRs (Zuo *et al.* 1994), the receptors themselves (Dong *et al.* 1999), phosphodiesterase (Kofinas *et al.* 1990) and the stimulatory protein G_{as} (Europe-Finner *et al.* 1993, 1994, Lopez-Bernal *et al.* 1995). Increased expression of the latter is known to increase constitutive as well as stimulated cAMP accumulation, and augment distal events such as transcription factor phosphorylation and cAMP-responsive gene expression (Yang *et al.* 1997). Phosphorylated dimers of the CREB/CREM family are then able to bind to the CREB-binding protein (CBP)/p300 co-activator protein, which in turn recruits the basal transcriptional machinery to initiate gene expression.

Transcripts of the CREB and CREM genes are present in all human tissues examined so far, but appear to be particularly important in the heart where they regulate cardiac myocyte function (Fentzke *et al.* 1998), in the immune system where they are involved in the development and function of T lymphocytes (Muller *et al.* 1998, Haus-Seuffert and Meisterernst 2000), in the brain where they have been implicated in the regulation of long-term memory and the circadian clock (Sassone-Corsi 1998), the testes where they orchestrate spermatogenesis (Don and Stelzer 2002) and the uterus where there is strong evidence for their roles in the regulation of uterine contractility (Bailey *et al.* 2000, 2002). In the latter context, an increased level of cAMP in human myometrial smooth-muscle cells during pregnancy has been reported (Europe-Finner *et al.* 1993, 1994), potentiated by altered expression of various components of the cAMP signalling pathway, in particular G_{as} . Subsequently, we have reported the differential expression of specific CREB/CREM isoforms in this tissue during gestation, namely phosphorylated full-length CREB, CREM $\tau_2\alpha$ and CREM α proteins (Bailey *et al.* 2000, 2002), and demonstrated their ability to bind CRE-containing oligonucleotides and activate and/or repress reporter-gene transcription. The change in expression of these factors represents a major switch from the expression of the CREB activator to the CREM α repressor protein (Fig. 1a), a switch also well characterized in the testes during spermatogenesis (Walker *et al.* 1996, Foulkes and Sassone-Corsi 1992). Furthermore, the pre-mRNA splicing mechanisms within myometrial cells appear to switch from favouring

the production of the alternatively spliced CREM $\tau_2\alpha$ activator that decreases sequentially through the non-pregnant, pregnant non-labouring and labouring phases, to the CREM α repressor protein that proceeds from zero expression in the non-pregnant uterus to a high level of expression in the labouring myometrium (Fig. 1b). To further characterize these factors, and elucidate the molecular mechanisms affecting myometrial gene expression, we have created stably transfected cell lines with plasmid constructs expressing the individual CREB and CREM proteins, and made use of microarray and semi-quantitative (SQ)/real-time reverse transcriptase (RT)-PCR experiments to identify downstream target genes that may be under their transcriptional control.

Materials and methods

Human myometrial tissue collection

Human myometrial tissue biopsies were collected from pregnant non-labouring (P) women at elective Caesarean section and from non-pregnant (NP) premenopausal women (ages 32–46 years) at hysterectomies performed for benign gynaecological disorders such as menorrhagia or dysmenorrhoea. The myometrial samples were snap-frozen in liquid nitrogen and stored at -70°C . Written consent was obtained from all women and ethical approval for the study was granted by the Newcastle and North Tyneside Health Authority Ethics Committee.

Establishment of stable myometrial cell lines

Primary cultures of human myometrial cells were initiated by treating small pieces of P myometrium with collagenase solution (1 mg/ml collagenase, 0.02 mg/ml DNase, 0.2 mg/ml trypsin inhibitor, elastase and BSA fraction V, in Hank's balanced salt solution free of Ca^{2+} and Mg^{2+} ions). Samples were incubated with gentle shaking for 4 h, then transferred to a new tube to which 5 ml complete medium plus 10% fetal calf serum had been added. After centrifugation at 1000 *g* for 10 min, the supernatant was decanted and discarded, and the cells resuspended in 15 ml fresh complete medium plus 10% fetal calf serum, and the cells seeded into flasks. After incubation at 37°C for 1 h to allow fibroblast attachment, the medium, now containing primarily myometrial cells with few contaminating fibroblasts, was transferred to new flasks and incubated for 24 h at 37°C . The growth medium was then replaced to inhibit fibroblast growth, with MEM D-Valine medium (Gibco) plus 10% fetal calf serum, containing 50 U/ml penicillin and 50 $\mu\text{g}/\text{ml}$ streptomycin. Growth medium was replaced with fresh medium every 2–3 days, and after reaching confluence cells were subcultured at a ratio of 1:3.

Figure 1 (a) Immunodetection with CREM-1 antibody (Santa Cruz Biotechnology) of CREB and CREM in human lower uterine myometrial samples from non-pregnant (NP), pregnant non-labouring (P) and spontaneously labouring (SL) women revealed three species of protein; a 43 kDa phosphorylated CREB protein highly expressed in the NP state but significantly down-regulated in P and SL tissue, a 28 kDa CREM α isoform absent from NP myometrium but up-regulated in P and further again in SL, and a 39 kDa CREM $\tau_2\alpha$ isoform chiefly expressed in NP tissue then down-regulated in P and SL. This blot reveals a dramatic switch in expression from the expression of the CREB transcriptional activator protein to the expression of the CREM α transcriptional repressor in the transition from the NP to the P state, and also illustrates a change in the RNA splicing machinery to favour the production of CREM α in preference to CREM $\tau_2\alpha$. Reproduced from Bailey *et al.* 2000. (b) Splicing mechanisms in the myometrium shift expression from the CREM $\tau_2\alpha$ trans-activator/trans-repressor in the non-pregnant uterus to the CREM α trans-repressor during pregnancy and labour. Exons are shown by rectangles and the ellipse represents a 'novel module' present in a small number of CREM splice variants, containing an in-frame stop codon. Q-rich, glutamine-rich domains involved in trans-activation; P-Box, kinase-inducible domain; P1 and P2, alternative promoters; ICERs, inducible cAMP early repressors transcribed from the P2 promoter. CREM $\tau_2\alpha$ lacks exon C and functions as an activator or repressor depending upon promoter context and cell type. CREM α lacks both exons encoding Q-rich domains (C and G), and except in exceptional circumstances acts as a repressor of transcription.

Full-length CREB was cloned into pCDNA3·1/V5 HisTOPO (Invitrogen) via RT-PCR using mRNA from pooled NP tissue ($n=6$) as a template, and primers (F) 5'-ATGACCATGGAATCTGGAGCCGAGAAC-3' and (R) 5'-TTAATCTGATTTGTGGCAGTAAAGGTC-3', where F is forward primer and R is reverse primer. CREM $\tau_2\alpha$ and CREM α plasmids were constructed as described previously (Bailey *et al.* 2002). Transfections were performed upon low-passage-number myometrial cells (passages 2 or 3) at 70% confluence using 4 μ l Mirus *TransIT-LT1* lipid-based transfection reagent (Cambridge Bioscience, Cambridge, UK) per 1 μ g plasmid. Typically, 6 μ g plasmid was used per transfection, mixed with 24 μ l LT-1 reagent and 200 μ l serum-free medium and incubated at room temperature for 5 min. This complex was added to the cells and incubated at 37 °C for 4 h, after which time it was removed and fresh medium (plus serum) added. Incubation at 37 °C was continued for 48 h, after which time the selective agent G418 was added to a final concentration of 80 μ g/ml. Colonies were subsequently cloned by limiting dilution, and maintained with selective pressure in an increased final G418 concentration of 250 μ g/ml.

Cell staining/immunocytochemistry

Cultured myometrial cells were stained to allow morphological examination using haematoxylin and eosin stains. Cells grown on coverslips were rinsed with 1 $\times$ PBS, fixed in cold methanol for 10 min, washed with water and then exposed to haematoxylin for 1 min. Following a quick water wash, cells were exposed to Scott's water (0.04 M NaCO₃/0.08 M MgSO₄) for 1 min. Another water wash was performed, then the cells were incubated in eosin stain for 2 min. After a final brief wash in water, the slips were allowed to dry and mounted in DPX resin on microscope slides for viewing.

To determine the level of fibroblast contamination in the smooth-muscle cell cultures, immunocytochemistry was performed using fibroblast antigen (Ab-1; Oncogene Research Products, San Diego, CA, USA; Saalbach *et al.* 1997) and the DAKO ChemMate APAAP detection kit with a Vector Blue Alkaline Phosphatase Substrate Kit III (Vector Laboratories, Burlingame, CA, USA). Cells were cultured on coverslips and fixed as above, then incubated in normal rabbit serum for 10 min. After a brief rinse with Tris-buffered saline (TBS), cells were incubated in Ab-1 primary antibody for 1 h at a 50 μ l/ml dilution (10 μ g/ml final concentration) at room temperature. After three 5-min rinses in 1 $\times$ TBS, cells were exposed to the secondary-link antibody for 45 min. Following three more 5-min rinses in 1 $\times$ TBS, the cells were incubated in APAAP for 20 min, followed by another round of washing in 1 $\times$ TBS. One drop of the Levamisole chromagen was then added to the cells, and

colour development was monitored microscopically. Chromagenesis was halted by rinsing with water, and fibroblast contamination was assessed by microscopic examination and cell counting.

RNA isolation

Total RNA was isolated from approximately 1×10^7 cells using TRI-Reagent (Sigma-Aldrich) according to the manufacturer's protocol. The resulting pellet was resuspended in 100 μ l RNase-free water, and incubated at 55 °C for 10 min to aid this process. This total RNA was then applied to an RNeasy mini-column (Qiagen), and subjected to RNase-free DNase treatment using the RNase-free DNase set (Qiagen). The remainder of the manufacturer's protocol was then followed for RNA cleanup, eluting with 25 μ l RNase-free water. Total RNA was quantified by spectrophotometry, and used for microarray analysis.

Confirmation of CREB/CREM expression levels in stably transfected cell lines

Levels of CREB, CREM α and CREM $\tau_2\alpha$ expression in stably transfected cell lines were examined and compared with one another by SQ RT-PCR using the Superscript one-step RT-PCR system (Invitrogen) in a total volume of 25 μ l with 5% (v/v) RNaseOUT (Invitrogen) RNase inhibitor, and 100 ng total RNA template prepared as described above. Primers for CREB and CREM were as described above, and reaction conditions as follow. Reverse-transcription step, 50 °C for 30 min, 94 °C for 2 min; amplification step, 40 cycles (CREB)/30 cycles (CREM) of 94 °C for 15 s, 63 °C (CREB) or 58 °C (CREM) for 30 s, and 68 °C for 1 min, followed by 68 °C for 9 min. 5 μ l of each reaction was subjected to agarose gel electrophoresis; RNA samples from those colonies displaying the highest CREB/CREM expression were selected for microarray analysis.

Affymetrix microarray analysis

Target preparation

A total of 10 μ g RNA were used for each target. Each sample was used as a basis to synthesize double-stranded cDNA, which in turn was used to synthesize biotin-labelled cRNA for hybridization to the microarray chips. The Affymetrix protocol was followed, and the steps are summarized briefly here.

cDNA synthesis

First-strand cDNA synthesis was achieved using the SuperScript Choice system (Invitrogen) and the

GeneChip T7-Oligo(dT) primer at 42 °C for 1 h. Second-strand cDNA was synthesized from this using *Escherichia coli* DNA ligase, polymerase I and RNase H at 16 °C for 2 h.

Synthesis of biotin-labelled cRNA

This was performed according to the protocol, using the Affymetrix Enzo BioArray High Yield RNA Transcript Labelling Kit. 20 µg of this labelled and purified cRNA was then fragmented (according to the protocol) prior to the hybridization step.

Hybridization

The hybridization cocktail was prepared according to the Affymetrix instructions, and incubated with the array at 45 °C for 16 h in rotisserie oven at 60 r.p.m.

Washing, staining and scanning

Washing and staining was performed in the Affymetrix Fluidics Station 400 according to the standard format of the single stain protocol for eukaryotic targets.

Data analysis

Data from the chip hybridizations was processed using GeneSpring 6 software (Silicon Genetics, Redwood City, CA, USA). Human U133 A microarray chips were hybridized with biotin-labelled cRNA from the following myometrial cell lines: (i) control non-transfected primary cultures (Ctrl), two replicates; (ii) control cells stably transfected with empty vector with no insert downstream of a cytomegalovirus (CMV) promoter (CMV –), three replicates; (iii) cells stably transfected with a plasmid to express CREB (CREB), three replicates; (iv) cells stably transfected with a plasmid to express CREM $\tau_2\alpha$ (CREM $\tau_2\alpha$), three replicates; (v) cells stably transfected with a plasmid to express CREM α (CREM α), three replicates. Raw-data files direct from the Affymetrix chip scanner were imported into the GeneSpring program for each chip. These data sets were transformed by converting all signal values below 0.01 to 0.01, then subjected to per-chip normalization to the 50th percentile and per-gene normalization to the median. To determine a baseline profile of gene expression with which to compare the CREB, CREM $\tau_2\alpha$ and CREM α results, an initial comparison was made between the control cell lines (i) and (ii), using normalized data from the primary non-transfected cells (i) as an initial baseline and data from the CMV – empty vector-transfected cells (ii) as the experimental set. This established which genes were affected by the stable transfection of the vector and subsequent selection process, and which therefore should be excluded from the main analyses

involving the CREB/CREM constructs. According to related literature (Mayanil *et al.* 2001, Gibellini *et al.* 2002), an appropriate cut-off point for this purpose is in the region of greater than or equal to a 1.5–2-fold change in expression; a 1.5-fold cut-off was used in our analysis. This was achieved by performing a parametric one-way ANOVA on the normalized data assuming equal variances, incorporating the Benjamini and Hochberg False Discovery Rate multiple-testing correction set at a rate of 0.05. The resultant list of genes differentially expressed between the two control classes was then filtered to include only those genes showing a fold change in expression of 1.5 or greater. These genes were then subtracted from the list of genes represented on the Affymetrix Human 133 A chip (22 283), resulting in the elimination of genes whose expression was altered by the process of stable transfection and selection. The remaining data set following this exclusion was then used as a final baseline for comparisons with CREB/CREM expression. These comparisons involved much the same process; data was normalized as above, then compared with gene expression levels in the ‘final baseline’ by ANOVA using the same criteria. The results were then filtered to include only those genes whose expression altered by a fold change of 2 or greater. Often, results from microarray experiments are also filtered using the criteria of their Absolute and Difference calls; only Absolute calls of Present and Difference calls of Increased or Decreased are included. However, certain reports in which there were a number of known genes that were expected to show altered expression under experimental conditions have suggested that this methodology results in the erroneous rejection of true-positive results. Consequently, Absolute calls were not used as a basis for exclusion in our analyses.

SQ RT-PCR

In order to support the results obtained from the microarray experiments, a selection of candidate genes were examined for differential expression as a result of the over-expression of CREB, CREM $\tau_2\alpha$ and CREM α by SQ RT-PCR. The primers used for this were as follows, where F is forward primer and R is reverse primer. All sequences are shown 5' to 3'. glyceraldehyde-3-phosphate dehydrogenase (GAPDH), F, CTGCCGTCTAGAAAAACC; R, CCACCTTTCGTTGTCATACC; matrix metalloproteinase 1 (MMP1), F, CCTCGCTGGGAGCAAACAC; R, AAGGCTTCTCAATGGCATGGTC; PTPRB, F, TGCCCTACCTTTCGGATAGACA; R, GCAGGAGGTAAAGGATCTGTTTG; PRKCA, F, CCTTTGGAGTTTCGGAGCTGA; R, AGTTCCATGTTTCCTTCCTCGTC; PLA2R1, F, CAGGTGGTGGAGACATTTGTG; R, CGTGGGTGTTCCCTTTGATT; CRSP8, F, TCCAGGACAACTTACATTCGGTC; R, TCTCAGATG

GCTTGCCTACCA; SMARCA1, F, TGCGGAAC TCATTGCAGTGTT; R, TGAAGTTCCACGTCT TGGTGTG; CDKN1C, F, GAGAAGTCGTCGG GCGATG; R, GGCTCTTTGGGCTCTAAATTGG; GPR30, F, CAACATCTGGACGGCAGGTAC; R, CTCCTCACACCGGCATGGT; CDC42EP2, F, ACGCTCCTCAGCCCTGGAC; R, GCCAGAAAGG TAGGAAGTGTGTG; FMOD, F, CAAGGCAATAG GATCAATGAGTTC; R, CTTGATCTCGTTCCC GTCCA; HUMNK1A, F, ACCGCTACCACGAGCA AGTC; R, TGCACACCACGACAATCATCA; SUI1, F, CCGAGGATTTCAGCAGCCT; R, AAAACCCAT GAACCTTCAGC; MTCO1, F, TTCGCCGACC GTTGACTATT; R, TACGGGTTCTTCGAATGT GTG; Palladin, F, GCGAGGTATAAAGCCCGA; R, GCTTAGATCTGGGGTTGGTAA; CTGF, F, CAAGGGCCTCTTCTGTGACTT; R, TGCCTCCT CTTTGCAAACAA; CDKN1A, F, AGCTGCCG AAGTCAGTTTCT; R, AACTAGGGTGCCCTTCT TCTT; gp(96)1, F, TGGAGGTGTGAGGATCCGAA; R, GCAAGACGTGTTCGATTCTGA; COL4A1, F, ACGGTGCGTATCGCTGGAA; R, TGCCCTGGG AAACCTATTTCT; LIM, F, GAGTCACTTGTCAG CCCTTGT; R, ACATTGTTCCGAATGGGCTT.

SQ RT-PCR was performed using the SuperScript OneStep RT-PCR system. 50 ng DNase-treated total RNA from stably transfected cells, or 5 ng mRNA from pooled ($n=6$) NP or P myometrial tissue was used as template according to the manufacturer's protocol, and a total of 28, 30 and 32 cycles of PCR performed at an appropriate annealing temperature for each particular primer pair to ascertain the best quantitative signal for each target sequence following agarose gel electrophoresis (2% E-gels; Invitrogen) of the products. Image analysis and band intensities were analysed with the Intelligent Quantifier program (Bio Image Systems, Jackson, MI, USA).

Real-time RT-PCR analysis

Forward primers were designed using LUX primer-design software (www.invitrogen.com) and were as follows: CREB PRKCA, 5'-GACGAGAGTTCCAT GTTTCCTTCCTCGC-3' and CREM α CDKN1C, 5'-CATCGGAGAAAGTCGTCGGGCGAG-3'. Reverse non-LUX-labelled primers were as above for SQ RT-PCR. Reactions were performed using the SuperScript III Platinum One-Step Quantitative RT-PCR System (Invitrogen). Reactions were set-up in 25 μ l volumes according to the manufacturer's protocol, and cycled as follows: 50 °C for 15 min, 95 °C for 2 min, then 45 cycles of 95 °C for 15 s and 60 °C for 30 s. Commercially available LUX primers (Invitrogen) were used to amplify GAPDH as a reference gene. Melting-curve analysis was performed to ensure reaction specificity, and relative quantitation was determined by standard curve analysis.

Results

Stable transfection and microarray data

Following the stable-transfection and selection process, cell lines were examined to verify that (i) they were predominantly composed of myometrial smooth muscle cells and that fibroblast contamination had been successfully kept to a minimum, and (ii) the cells had not been damaged in any way, i.e. were in a healthy state with no gross morphological changes. Fig. 2a and b show the results of these examinations following the appropriate cell-staining procedures, indicating not only a low-level presence of fibroblasts in the cultures, but also a normal morphology in the stably transfected cell lines when compared with the non-transfected control. The degree of fibroblast contamination was estimated by microscopic cell counting of slide-mounted and stained cultures, and was consistently less than 1%. This was achieved via the use of D-valine (substituting L-valine) growth medium, which has long been known to inhibit the growth of fibroblasts (Gilbert & Migeon 1975, 1977, White *et al.* 1978, Frauli & Ludwig 1987, Sordillo *et al.* 1988, Hongpaisan 2000).

Agarose gel electrophoresis of CREB and CREM RT-PCR products from stably transfected cell lines revealed a wide range of expression levels between isolated clones (Fig. 2c). Total RNA was isolated and purified from those colonies showing the highest levels of expression, and amplified and labelled prior to microarray hybridization and analysis according to the methods described below. The microarray data derived from Chip Sequence files for all hybridizations including the cell-only control, two replicates of the empty-vector control and three replicates of each of the CREB, CREM $\tau_2\alpha$ and CREM α experimental samples, have been submitted to the NCBI gene expression and hybridization array data repository GEO (<http://www.ncbi.nlm.nih.gov/geo/>), under GEO numbers GSM 17039, GSM 17040, GSM 17041, GSM 17042, GSM 17059, GSM 17060, GSM 17061, GSM 17062, GSM 17063, GSM 17064, GSM 17065 and GSM 17066. Following hybridization and scanning, chip data were imported into the GeneSpring software for normalization and manipulation. Rather than simply comparing gene expression between the stably transfected cell lines and the control empty-vector cell line directly, we derived a 'final baseline' of gene expression in cultured myometrial cells with which to compare the experimental data from the stably transfected cells, as detailed in the Materials and methods section, which takes into account expression differences induced by the transfection and selection processes. Although this may increase the incidence of false negatives in the form of genes genuinely altered by the expression of the transfected factors and not by transfection and selection, it drastically reduces the incidence of false positives, i.e.

Figure 2 (a) Immunocytochemistry of human myometrial and fibroblast cultured cells using anti-fibroblast specific antigen (Ab-1) mAb ASO2 (Oncogene Research Products). (i) Note intense anti-ASO2 reactivity with fibroblast cells, in contrast to the light staining with myometrial cells in (ii). Negative controls, omitting the primary ASO2 mAb, are shown in (iii) and (iv) for fibroblast and myometrial cells respectively. (b) Haematoxylin and eosin staining of stably transfected myometrial cells expressing (i) CREB, (ii) CREM α and (iii) CREM $\tau_2\alpha$. Morphology was compared with control non-transfected (iv) and empty-vector stably transfected (v) cells, and showed no differences. (c) RT-PCR analysis of isolated stably transfected colonies to determine abundance of CREB, CREM α and CREM $\tau_2\alpha$ mRNAs. Total RNA was isolated from these cell lines, and used as a template for RT-PCR of these CREB/CREM isoforms. Expression levels were compared with control non-transfected (–) and empty-vector stably transfected (C) cells. Those with the highest degrees of expression (*) were used as the three replicate RNA populations for the microarray analysis.

Table 1 Number of genes affected by stable expression of CREB, CREM α and CREM $\tau_2\alpha$ in cultured myometrial cells according to data from Affymetrix Human 133 A microarray chips and GeneSpring data mining. Normalized gene-expression levels for stable cell lines transfected with expression constructs for the CREB, CREM α and CREM $\tau_2\alpha$ genes were compared to normalized baseline levels as described in the Material and methods section. Out of a possible 20 966 genes, the number of genes whose expression was altered by 1.5-fold or greater is shown, along with the number of genes up- and down-regulated

	No. of downstream genes affected	Affected genes per chip (%)	No. of genes	
			Up-regulated	Down-regulated
Stably- transfected gene				
CREB	958	4.57	522	436
CREM α	118	0.56	71	47
CREM $\tau_2\alpha$	220	1.05	148	72

genes affected by transfection and selection and not by the transfected factors themselves. This process indicated that the expression of a total of 1317 genes (5.9% of the total) was affected significantly (greater than or equal to 1.5-fold) by the transfection and selection process, of which 879 were up-regulated and 438 down-regulated. This total does not seem excessive: similar studies have shown 16% of genes to be affected in this way (Mayanil *et al.* 2001). These genes were subtracted from the 22 283 represented on the microarray chip, to give a final baseline of 20 966 genes. All subsequent analyses of gene expression in the stably transfected cell lines included data for these genes only, and the mean levels of expression in this final baseline were used as the basis for these comparisons, the outcome of which is summarized in Table 1, which presents the numbers of genes up- and down-regulated by the three bZIP factors under investigation. Due to the comprehensive nature of this study utilizing microarrays on which over 20 000 genes are represented, the volume of information in our results was substantial. Consequently, these results have been split into categories for presentation: Fig. 3a lists those genes affected by the three bZIP factors that are directly attributable to uterine function on the basis of current knowledge. The remainder of affected genes are listed in online supplementary Fig. 3b–d (see <http://jme.endocrinology-journals.org/content/vol34/issue1/>) for CREB, CREM α and CREM $\tau_2\alpha$ respectively, plus an additional figure (online Supplementary Fig. 3e) containing those genes affected by CREB that are classified only as clones, hypothetical proteins, expressed sequence tags (ESTs) and open reading frames (ORFs), and also annotated genes that have not, as yet, been linked to significant myometrial activity.

The expression of a number of the genes determined by microarrays was altered by more than one of these three bZIP factors, as summarized in Fig. 4. From a total of 1296 genes found to be significantly altered as a result of CREB, CREM α or CREM $\tau_2\alpha$ expression, 112 were regulated by more than one of these three factors. Of

these 112, eight were affected by all three, 31 were affected by CREB and CREM α , 65 were affected by CREB and CREM $\tau_2\alpha$, and eight were affected by CREM α and CREM $\tau_2\alpha$. The genes in these lists were classified and grouped according to their functions as determined by the GeneSpring software; where genes belonged to more than one functional group, the results tables were edited manually to prevent multiple entries of these particular genes.

Genes affected by CREB, CREM α or CREM $\tau_2\alpha$ expression, classified according to GeneSpring

The results revealed a diverse range of gene categories in terms of biological processes, cellular components and molecular functions that are regulated by these bZIP factors. The most pertinent genes and groups of genes known to be involved in the function of the human uterus were selected from our results and are presented in Fig. 3a. Due to the number of genes included, their functions, properties and links to human uterine activities cannot be elaborated upon in detail here: however, for each gene or group of genes, Fig. 3a contains appropriate comments or citations to relevant publications that support their inclusion. In addition to these genes, other examples include those responsive to oestrogen, cAMP and calcium; splicing factors and genes involved in their control, hormone receptors, A-kinase anchoring proteins (AKAPs), transcription factors, zinc-finger and LIM-domain proteins, ion channels, cytoskeletal proteins, adhesion molecules, extracellular components, histones and histone-modification genes, kinases, GPCRs, chemokines and chemokine receptors and proteoglycans. Full listings of results further to those in Fig. 3a can be found in online Supplementary Fig. 3b–e.

SQ and real-time RT-PCR

The validity of the microarray results was examined by performing a panel of SQ/real-time RT-PCR reactions.

(a)	Gene(s)	↑/↓	References/comments
Cell surface linked signal transduction			
	Integrins $\alpha 4$, $\beta 2$, $\beta 4$, $\beta 8$	↑	Taylor <i>et al.</i> 1996; Terpe <i>et al.</i> 1994
	Voltage-dependent Na^+ & K^+ channels	↓	Sanborn 2000b; Sperelakis <i>et al.</i> 1992; Toro <i>et al.</i> 1990
	Regulators of G protein signalling (RGS) 7, 10, 11, 12, 14 & 20	↑/↓	Suarez <i>et al.</i> 2003; Park <i>et al.</i> 2002
Cytokines, hormones & receptors			
	Interleukins (IL8 & 18) & IL-receptor 17C Chemokine (C-C motif) ligands	↑/↓	Keelan <i>et al.</i> 2003; Tribe <i>et al.</i> 2003; Osman <i>et al.</i> 2003; Bowen <i>et al.</i> 2002; Chan <i>et al.</i> 2002; Helmer <i>et al.</i> 2002; Friebe-Hoffman <i>et al.</i> 2001; Rauk <i>et al.</i> 2001; Schmid <i>et al.</i> 2001; Elliot <i>et al.</i> 2000; Hatthachote & Gillespie 1999
	Prostaglandin F, LH/hCG, & oxytocin receptors	↑	Al-Matubsi <i>et al.</i> 2001; Ou <i>et al.</i> 2000; Sparey <i>et al.</i> 1999; Zuo <i>et al.</i> 1994; Moonen <i>et al.</i> 1986; Fuchs <i>et al.</i> 1984
Cytoskeletal proteins			
	Supervillin CBP/p300-interacting transactivator Tropomyosin 1α	↑	Chen <i>et al.</i> 2003 AP2 co-activator Component of actin filaments of myofibrils
Extracellular matrix			
	Collagen (types XIII $\alpha 1$, XI $\alpha 2$, V $\alpha 1$, VI $\alpha 2$, XVI $\alpha 1$, IV $\alpha 2$, VI $\alpha 1$, IV $\alpha 3$, XVIII $\alpha 1$)	↑/↓	Weston <i>et al.</i> 2003; Dziadek <i>et al.</i> 1995; Goranova <i>et al.</i> 1993; Nishinaka & Fukuda 1991
	Matrix metalloproteinases (MMPs) 1 & 23A	↑	Ma & Chegini 1999; Dumin <i>et al.</i> 1998
Intracellular signalling			
	Voltage gated Na^+/K^+ channel peptides	↑	
	Rho GTPase activating proteins 1 & 6	↓	Prakash <i>et al.</i> 2000
Protein kinases			
	Protein kinase C isoforms α , $\beta 1$ & ζ	↑	Eude <i>et al.</i> 2000; Morrison <i>et al.</i> 1996
Transport			
	Genes involved in transport of K^+ , Ca^{2+} , Na^+ , Zn^{2+} & glucose-6-phosphate	↑/↓	Esplin <i>et al.</i> 2003; Curley <i>et al.</i> 2002; Sanborn 2000b; Murugesan <i>et al.</i> 1989; Honore <i>et al.</i> 1986; Kelly & Abel 1983

Figure 3 (a).

11 randomly selected genes were chosen from the results of the CREB, CREM α and CREM $\tau_2\alpha$ microarray experiments, and, by SQ RT-PCR, their mRNA levels assessed in control cultured myometrial cells transfected with empty vector, the stably transfected myometrial cell

line used for the array hybridization, pooled NP tissue and pooled P tissue (Fig. 5). In each case, the level of expression in stably transfected cells compared with control cells reflected the results from the microarrays: those genes found to be up- or down-regulated by

(b)

PregnancyPregnancy-specific glycoproteins 3, 6 & 9 ↑ Snyder *et al.* 2001**Non-classified/other**Calpain-10 ↑ Belt *et al.* 1999Transient receptor potential channels (Trp) C4 & C5 ↑/↓ Dalrymple *et al.* 2002MEF2D (component of smooth-muscle specific complex) ↑ Katoh *et al.* 1998CALCRL (calcitonin receptor-like gene) ↓ Dong *et al.* 1999

Smag-64 (smooth-muscle activation-specific gene) ↑ Preferentially expressed in neointimal smooth muscle cells

Corticotropin releasing hormone receptors CRH-R1 & CRH-R2 ↑/↓ Grammatopoulos & Hillhouse 1999

Suppressor of cytokine signalling SOCS1 ↑ Blumenstein *et al.* 2002 α 2-adrenoceptors α 1A-AR & α 2A ↑/↓ Involved in myometrial contraction
 β 2-adrenoceptor β 2-AR ↑ Increase cAMP: myometrial relaxationProstaglandin receptors D2 (DP) & EP3 ↓ Keelan *et al.* 2003**Figure 3 (b).**

Figure 3 (a) Total RNA samples were isolated from stably transfected cell lines expressing CREB, CREM α and CREM $\tau_2\alpha$. Human U133 A array chips were hybridized with biotin-labelled cRNA representing the mRNA populations of cultured myometrial cells in the form of (i) non-transfected controls, (ii) empty-vector stably transfected controls and (iii) stably transfected experimental lines over-expressing the CREB, CREM $\tau_2\alpha$ or CREM α bZIP transcription factors. Details are given in the Data analysis and first Results sections. Due to the comprehensive number of genes involved, the resultant lists are published as online supplementary Fig. 3b–e (see <http://jme.endocrinology-journals.org/content/vol34/issue1>). The most germane of these are listed here, comprising genes from several functional groups. All are attributable to the control of uterine/myometrial function, and references as such are included in the figure to minimize the length of elaboration in this report. In each case, the effect of CREB/CREM on the expression of the gene/genes is indicated by arrows, where ↑, up-regulation, ↓, down-regulation, and ↑/↓, both up- and down-regulation for groups of genes, or where a specific gene was affected differently by more than one of the bZIP factors.

expression of the CREB and CREM factors by microarray analysis also showed increased or decreased expression respectively in the stable cell lines compared with control cells by RT-PCR. In addition, the expression of these genes was found to be significantly altered between the pooled NP and P tissue, reflecting the *in vivo* biological situation. Of the 11 genes examined, six were expressed to a greater degree in NP tissue compared with P tissue, four were expressed at a higher level in P tissue in comparison to NP tissue, and one gene showed no significant difference in expression levels. Real-time RT-PCR analysis of two of these genes, PRKCA and CDKN1C, indicated significant up- and down-regulation respectively of their expression in stable cell lines in accordance with the microarray data, and also increased expression in NP tissue compared with P tissue reflecting the *in vivo* situation.

Discussion

We have previously identified and described a cAMP-responsive genetic component in the human myometrium during pregnancy, in the form of the temporal and spatial expression of a number of specific splice variants of ubiquitous bZIP transcription factors. In an attempt to discover the downstream genes affected by these factors, we examined gene expression in myometrial cell lines stably transfected with expression constructs of CREB, CREM α and CREM $\tau_2\alpha$, and compared it to control cells, by way of isolation of RNA from these cells and hybridization of biotin-labelled cRNA copies to Affymetrix human microarray chips.

After confirming that our cell cultures comprised less than 1% contaminating fibroblasts, we derived a baseline of expression with which to compare the expression

Figure 4 (a).

profile of the stably transfected bZIP cell lines. This was achieved by comparing gene expression in control cells stably transfected with empty vector against control non-transfected cells. Although a relatively uncommon approach (most studies simply compare experimental expression cell lines with empty-vector controls), we believe that accounting for changes in gene expression

due to the biochemically stressful transfection and selection processes is essential to obtain more-robust results. While this essentially produces a number of false negatives in the form of genes genuinely altered by the expression of the transfected factors and not by transfection and selection, it drastically reduces the incidence of false positives, i.e. genes affected by transfection and

(b)

Gene	Genbank	CREB	CREM α	CREM $\tau_2\alpha$
Genes affected by CREB, CREMα & CREM$\tau_2\alpha$				
SMARCA1	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily 1-like 1	NM_014140	5,3 ↑	5,2 ↑ 7,6 ↑
HIST1H2BI	ESTs, Highly similar to uncharacterized hypothalamus protein HT010 (Homo sapiens) [H.sapiens]	AF119892.1	4,1 ↑	4,3 ↑ 5,6 ↑
KCNH2	H2B histone family, member K	NM_003525	3,6 ↑	4,7 ↑ 5,3 ↑
RPS20	potassium voltage-gated channel, subfamily H (eag-related), member 2	AB044806.1	2,5 ↓	2,8 ↓ 2,8 ↓
E46L; DKFZP586H2219	fibulin 1	AL576654	3,2 ↓	2,8 ↓ 3,3 ↓
MGC10848		Z95331	9,1 ↓	3,1 ↓ 9,0 ↓
GAS1	hypothetical protein MGC10848	NM_030569	25,0 ↓	33,3 ↓ 34,4 ↓
	growth arrest-specific 1	AW611727	33,3 ↓	33,3 ↓ 51,1 ↓
Genes affected by CREB & CREMα				
PTENP1	phosphatase and tensin homolog (mutated in multiple advanced cancers 1), pseudogene 1	AF023139.1	9,3 ↑	11,8 ↑ -
D21S2056E	DNA segment on chromosome 21 (unique) 2056 expressed sequence	NM_003683	8,1 ↑	8,1 ↑ -
SNRK	SNF-1 related kinase	NM_017719	7,3 ↑	5,1 ↑ -
FANCF	Fanconi anemia, complementation group F	NM_022725	5,8 ↑	7,8 ↑ -
TFAM	transcription factor A, mitochondrial	NM_012251	5,1 ↑	10,4 ↑ -
GDF2	growth differentiation factor 2	NM_016204	4,9 ↑	3,7 ↑ -
POMT2	putative protein O-mannosyltransferase	NM_013382	4,9 ↑	6,3 ↑ -
ITGB8	integrin, beta 8	NM_002214	4,5 ↑	2,8 ↑ -
EVA1	epithelial V-like antigen 1	AF275945.1	4,0 ↑	2,7 ↑ -
CLFLAR	CASP8 and FADD-like apoptosis regulator	AF005775.1	3,3 ↑	3,2 ↑ -
EMP1	epithelial membrane protein 1	NM_001423	3,9 ↑	4,7 ↑ -
	Homo sapiens mRNA; cDNA DKFZp434P2435 (from clone DKFZp434P2435), mRNA sequence	AL117520.1	3,9 ↑	4,0 ↑ -
	Homo sapiens cDNA: FLJ22209 fs, clone HRC01496, mRNA sequence	AK025862.1	3,3 ↑	2,8 ↑ -
	Homo sapiens cDNA FLJ13754 fs, clone PLACE3000362, mRNA sequence	AK023816.1	2,8 ↑	2,4 ↑ -
SOC51	suppressor of cytokine signaling 1	AB006043.1	2,2 ↑	3,6 ↑ -
MGC2508	hypothetical protein MGC2508	AA639585	2,7 ↑	2,9 ↑ -
SMT3H2	SMT3 suppressor of mit two 3 homolog 2 (yeast)	AI971724	2,5 ↑	3,0 ↑ -
	Homo sapiens cDNA: FLJ21533 fs, clone COL06072, mRNA sequence	BE965029	2,4 ↑	2,1 ↑ -
YKT6	SNARE protein Ykt6	NM_006555	2,2 ↑	2,1 ↑ -
PSG3	pregnancy specific beta-1-glycoprotein 3	AB015670.1	3,0 ↑	2,5 ↑ -
FLJ10661	hypothetical protein FLJ10661	NM_018172	2,1 ↑	2,5 ↑ -
SPHK2	sphingosine kinase 2	AF245447.1	2,4 ↑	2,7 ↑ -
WBSR5	Williams-Beuren syndrome chromosome region 5	AF257135.1	2,4 ↑	3,8 ↑ -
		BE892698	3,1 ↑	8,7 ↑ -
KIAA0363	KIAA0363 protein	AB002361.1	3,7 ↑	3,1 ↑ -
SEMA3B	sema domain, immunoglobulin domain (lg), short basic domain, secreted, (semaphorin) 3B	NM_004636	2,2 ↓	3,1 ↓ -
BRDG1	BCR downstream signaling 1	NM_012108	4,2 ↓	5,9 ↓ -
GRT1	hypothetical protein FLJ22474	NM_024719	5,6 ↓	2,3 ↓ -
PACE4	paired basic amino acid cleaving system 4	D28514.1	7,7 ↓	7,1 ↓ -
IL26	interleukin 26	NM_018402	8,3 ↓	9,1 ↓ -
KIAA0987	erythrocyte membrane protein band 4.1-like 3	NM_012307	14,3 ↓	11,7 ↓ -
Genes affected by CREB & CREM$\tau_2\alpha$				
FLJ20060	Homo sapiens clone IMAGE:248602, mRNA sequence	AF339805.1	66,2 ↑	- 49,1 ↑
GAD2	hypothetical protein FLJ20060	NM_017645	39,7 ↑	- 31,0 ↑
	glutamate decarboxylase 2 (pancreatic islets and brain, 65kDa)	M81882.1	3,3 ↓	- 5,0 ↓
CYP2C8	cytochrome P450, subfamily 11C (mephenytoin 4-hydroxylase), polypeptide 8	NM_030878	7,7 ↓	- 7,1 ↓
	ESTs, Moderately similar to hypothetical protein FLJ20378 [Homo sapiens] [H.sapiens]	AW576871	8,2 ↓	- 7,2 ↓
	Homo sapiens cDNA clone IMAGE:3943734 5', MRNA sequence	BE794962	5,7 ↑	- 5,9 ↑
CDK5R1	Human 28S ribosomal RNA gene, complete cds	M11167	3,5 ↑	- 15,7 ↑
COIL	cyclin-dependent kinase 5, regulatory subunit 1 (p35)	AL567411	5,3 ↑	- 5,4 ↑
FGF18	collin	BG391060	5,1 ↑	- 3,9 ↑
CIQB	fibroblast growth factor 18	AB007422.1	5,0 ↑	- 5,4 ↑
	complement component 1, q subcomponent, beta polypeptide	NM_000491	4,9 ↑	- 4,8 ↑
		M27830	4,7 ↑	- 9,2 ↑
LSR7	lipopolysaccharide specific response-7 protein	NM_018559	4,6 ↑	- 3,3 ↑
COL13A1	collagen, type XIII, alpha 1	M59217.1	4,0 ↑	- 7,0 ↑
FGF17	fibroblast growth factor 17	NM_003867	4,2 ↑	- 7,6 ↑
Genes affected by CREB & CREM$\tau_2\alpha$ cont'd				
Clorf16	chromosome 1 open reading frame 15	AF288395.1	3,9 ↑	- 6,5 ↑
LAMB3	laminin, beta 3	L25541.1	3,6 ↑	- 3,9 ↑
IL-17RC	interleukin 17 receptor C	BF112057	3,4 ↑	- 3,8 ↑
TSSC3	tumor suppressing subtransferable candidate 3	AF001294.1	3,3 ↑	- 6,6 ↑
CD86	CD86 antigen (CD28 antigen ligand 2, B7-2 antigen)	L25259.1	3,3 ↑	- 2,8 ↑
ALK	anaplastic lymphoma kinase (Ki-1)	U66559.1	3,2 ↑	- 3,2 ↑
FER	fer (fps/tes related) tyrosine kinase (phosphoprotein NCP94)	NM_005246	3,1 ↑	- 3,5 ↑
QJB3	gap junction protein, beta 3, 31kDa (connexin 31)	AF099730	3,1 ↑	- 3,7 ↑
DKFZP434G226	hypothetical protein DKFZp434G226	NM_031217	3,0 ↑	- 2,2 ↑
SGT	small glutamine-rich tetratricopeptide repeat (TPR)-containing cyclin-dependent kinase inhibitor 2A (melanoma, p16, inhibits CDK4)	NM_003021	3,0 ↑	- 3,5 ↑
CDKN2A		U38945.1	2,9 ↑	- 3,5 ↑
SNRPD1	small nuclear ribonucleoprotein D1 polypeptide 16kDa	NM_006938	2,7 ↑	- 2,5 ↑
F3	coagulation factor III (thromboplastin, tissue factor)	NM_001993	2,7 ↑	- 2,3 ↑
SLC16A4	solute carrier family 16 (monocarboxylic acid transporters), member 4	NM_004696	2,6 ↑	- 4,1 ↑
KRAS2	v-Ki-ras2 Kirsten rat sarcoma 2 viral oncogene homolog BF673699	U87555.1	2,6 ↑	- 2,7 ↑
SCN2B	sodium channel, voltage-gated, type II, beta polypeptide U87555.1	NM_000903	2,4 ↑	- 4,6 ↑
NQO1	NAD(P)H dehydrogenase, quinone 1	NM_000667	2,4 ↑	- 2,9 ↑
ADH1A	alcohol dehydrogenase 1A (class I), alpha polypeptide	AW582267	2,4 ↑	- 2,2 ↑
RPL29	ribosomal protein L29	NM_004168	2,4 ↑	- 3,1 ↑
SDHA	succinate dehydrogenase complex, subunit A, flavoprotein (Fp)	AI909331	2,3 ↑	- 4,7 ↑
PPP1R7	protein phosphatase 1, regulatory subunit 7	NM_002663	2,3 ↑	- 4,7 ↑
S100A7	S100 calcium binding protein A7 (psoriasis 1)	NM_002064	2,3 ↑	- 2,7 ↑
GLRX	glutaredoxin (thioltransferase)	AI968626	2,3 ↑	- 2,9 ↑
HUS1	HUS1 checkpoint homolog (S. pombe)	M62898.1	2,3 ↑	- 3,4 ↑
ANXA2P2	annexin A2 pseudogene 2	NM_004094	2,3 ↑	- 2,6 ↑
E1F2S1	eukaryotic translation initiation factor 2, subunit 1 alpha	AL545523	2,2 ↑	- 2,3 ↑
PSMC3	proteasome (prosome, macropain) 26S subunit, ATPase, 3	NM_016041	2,2 ↑	- 2,6 ↑
F-LAN-1	CGI-101 protein	NM_006553	2,1 ↑	- 2,4 ↑
HFL-EDDG1	erythroid differentiation and denucleation factor 1	NM_003966	2,1 ↑	- 2,3 ↑
SEMA5A	sema domain, seven thrombospondin repeats (type 1 and type 1-like), transmembrane domain (TM) and short cytoplasmic domain, (semaphorin) 5A	NM_000024	2,0 ↑	- 3,0 ↑
ADRB2	adrenergic, beta-2-, receptor, surface	AV716798	2,0 ↑	- 2,2 ↑
HT010	uncharacterized hypothalamus protein HT010	NM_003107	2,2 ↑	- 3,7 ↑
	Homo sapiens mRNA full length insert cDNA clone 1	NM_002888	2,7 ↑	- 14,7 ↑
	EUROIMAGE 1977059, mRNA sequence	AL109674.1	2,7 ↑	- 6,7 ↑
	retinoic acid receptor responder (azartore induced) 1	AA827892	2,7 ↓	- 3,4 ↓
	Homo sapiens mRNA full length insert cDNA clone	AW974666	2,9 ↓	- 4,5 ↓
	EUROIMAGE 48866, mRNA sequence	AF070536.1	3,4 ↓	- 5,0 ↓
	Homo sapiens cDNA FLJ14113 fs, clone	AK024175.1	3,7 ↓	- 3,0 ↓
	MAMMA1001715, mRNA sequence	AI799984	3,8 ↓	- 5,4 ↓
	golgi associated, gamma adaptin ear containing, ARF binding protein 2	NM_003674	4,5 ↓	- 2,9 ↓
	cyclin-dependent kinase (CDC2-like) 10	NM_021219	4,5 ↓	- 6,8 ↓
CDK10		M30894.1	5,6 ↓	- 4,8 ↓
JAM2	junctional adhesion molecule 2	NM_002023	5,9 ↓	- 8,8 ↓
TRG0	T cell receptor gamma locus	AL110126.1	6,3 ↓	- 8,8 ↓
FMOD	fibromodulin	AL050321.1	6,3 ↓	- 4,2 ↓
	Homo sapiens mRNA; cDNA DKFZp564H1916 (from clone DKFZp564H1916), mRNA sequence	NM_001766	7,7 ↓	- 2,4 ↓
	Homo sapiens mRNA; cDNA DKFZp566F1224 (from clone DKFZp566F1224), mRNA sequence	BF344265	10,0 ↓	- 6,0 ↓
CD1D	CD1D ligand, d polypeptide	AF031587.1	12,5 ↓	- 10,1 ↓
HIPI	hematopoietic PBX-interacting protein	NM_020380	16,7 ↓	- 6,3 ↓
CC15	chemokine (C-C motif) ligand 15			
AF15Q14	AF15Q14 protein			
Genes affected by CREMα & CREM$\tau_2\alpha$				
GPR30	G protein-coupled receptor 30	U63917.1	-	4,3 ↑ 10,7 ↑
NAP1L1	nucleosome assembly protein 1-like 1	BC002387.1	-	2,4 ↑ 2,2 ↑
SUCLG1	succinate-CoA ligase, GDP-forming, alpha subunit	NM_003849	-	2,2 ↑ 2,9 ↑
OSTF1	osteoclast stimulating factor 1	NM_012383	-	2,1 ↑ 2,3 ↑
CDCL2	cell division cycle 2-like 2	U04819.1	-	2,0 ↑ 2,3 ↑
DKFZP586H111	DKFZP586H111 protein	AL050131.1	-	2,4 ↑ 2,1 ↑
	ESTs	BF228395	-	2,5 ↑ 2,2 ↑
KRT7	keratin 7	AI920978	-	4,0 ↑ 4,8 ↑

Figure 4 (b).

Figure 4 (a) Overlap between the groups of genes regulated by CREB, CREM α and CREM $\tau_2\alpha$: almost exactly one-third of the genes affected by CREM $\tau_2\alpha$ are also affected by CREB, and the same is true for CREM α . The genes that are commonly affected by CREB and CREM $\tau_2\alpha$ do differ markedly from those commonly affected by CREB and CREM α , however, and the proportion of genes commonly affected by the CREM $\tau_2\alpha$ and CREM α proteins is only 13% and just under 7% of the totals respectively. In summary, the majority of genes affected by these transcription factors belong to discrete groups, and are affected by one factor only. (b) A number of genes had their levels of expression altered by more than one of the CREB, CREM α and CREM $\tau_2\alpha$ factors. These genes were classified with respect to which of these factors affected their expression, and are grouped accordingly in the above table. The three columns on the right of each panel indicate the degree of up- or down-regulation in CREB, CREM α and CREM $\tau_2\alpha$ stably transfected cell lines compared with the previously derived baseline expression levels; for example, 3.5 ↑ denotes a 3.5-fold increase in gene expression. In the majority of cases these values are similar for each specific gene, although there are exceptions. The physiological relevance of this with reference to the temporal and spatial patterns of expression of the bZIP factors is discussed in the text.

Figure 5 SQ and quantitative RT-PCR was performed to verify differential expression of a random selection of genes revealed in the microarray analyses. Comparisons were made between (i) total RNA templates isolated from control empty-vector stably transfected cell lines and cell lines stably transfected with CREB, CREMα and CREMτ2α constructs, and (ii) mRNA templates isolated from pooled (n=6) non-pregnant (NP) and pregnant non-labouring (P) myometrial tissue samples. Details are given in the Materials and methods section. The level of expression in stably transfected cells compared with control cells reflected the results from the microarrays: those genes found to be up- or down-regulated by expression of the CREB and CREM factors by microarray analysis also showed increased or decreased expression respectively in the stable cell lines compared with control cells by RT-PCR. In addition, the expression of these genes was found to be significantly altered between the pooled NP and P tissue, reflecting the *in vivo* biological situation. Of the 11 genes examined, six were expressed to a greater degree in NP tissue compared with P, four were expressed at a higher level in P tissue in comparison to NP, and one gene showed no significant difference in expression levels. Real-time RT-PCR analysis of two of these genes, PRKCA and CDKN1C, indicated significant up- and down-regulation respectively of their expression in stable cell lines in accordance with the microarray data, and also increased expression in NP tissue compared with P tissue, reflecting the situation *in vivo*.

selection and not by the transfected factors themselves. In our hands, these manipulations affected the expression of 1317 genes (5.9% of the total represented on the microarray chip) greater than or equal to 1.5-fold. This total does not seem excessive: similar studies have shown 16% of genes to be affected in this way (Mayanil *et al.* 2001). These genes were subtracted from the 22 283 represented on the microarray chip, to give a final baseline of 20 966 genes for our comparisons.

Rigorous statistical treatment of the resultant microarray data, in triplicate for each stable cell line, showed the expression of a total of 1296 genes to be affected by the three bZIP factors, representing 6.2% of genes examined. CREB, the transcriptional activator whose myometrial expression we have found to be almost

entirely arrested in the transition from the non-pregnant to the pregnant state, altered the expression of 958 genes; CREMα, the transcriptional repressor completely absent from NP myometrium but up-regulated in P and labouring tissue, altered the expression of 118 genes; CREMτ2α, able to function as an activator and a repressor of transcription, affected the transcription of 220 genes. These results, confirmed via SQ RT-PCR/real-time RT-PCR of RNA isolated from stably transfected cell lines and myometrial tissues of a panel of genes from the resultant gene lists, showed the altered expression of a wide variety of genes over a broad range of classifications known to be intrinsically involved in the development, control and overall function of the uterus and its component proteins and molecules. The

most germane of these are listed in Fig. 3a, alongside appropriate citations of reports describing their particular involvement in uterine function. Pertinent examples to elaborate upon include the following. Regulator of G-protein signalling RGS2: responsive to oxytocin and progesterone; increases during pregnancy then falls again prior to labour in the rat. Prostaglandin F receptor: increases during pregnancy and again at onset of labour. Luteinizing hormone (LH)/human chorionic gonadotropin (hCG) and oxytocin receptors: temporally and spatially regulated in myometrium during pregnancy. Chemokines and cytokines: interleukin (IL)-1 β increases in myometrium during pregnancy and negatively regulates oxytocin receptor; IL-8 increases during term labour; IL-1 β and IL-6 increase myometrial oxytocin secretion; IL-1 and IL-8 receptors decrease as gestation progresses. Cytoskeletal proteins: supervillin mediates actin/myosin organization; tropomyosin-1 α is a component of actin filaments/myofibrils; CITED2 is a co-activator of AP-2. Collagens: nine different collagens were affected, some of which are known to be up-regulated in the mouse myometrium during pregnancy. Protein kinases, including mitogen-activated protein kinases (MAPKs), cAMP-dependent protein kinases and three protein kinase C (PKC) isoforms thought to mediate endothelin-induced uterine contractions. Ion-transport channels, thought to be important in augmenting uterine contractility in labour. Affected examples include the α 3 sodium pump which decreases in human myometrium at labour, and ATP-sensitive potassium channels which decrease in pregnancy/labour. Pregnancy-specific glycoproteins (PSGs) 3, 6 and 9, which regulate the production of anti-inflammatory cytokines. Calpain-10: calpains may facilitate nuclear factor κ B (NF- κ B) translocation, cyclooxygenase expression and prostaglandin synthesis. Transient receptor potential channels (Trp): components of store-operated calcium entry (SOCE) channels that are involved in myometrial calcium homeostasis. Corticotropin-releasing hormone (CRH) receptors R1 and R2: CRH promotes myometrial quiescence via cAMP generation. CRH receptors are desensitized at term, and a contractile state ensues. Suppressor of cytokine signalling (SOCS) 1: SOCS proteins are differentially regulated in gestational tissues; reduction is associated with onset of labour. α 2 and β 2 adrenoreceptors: associated with contractile and relaxatory processes respectively. Prostaglandin receptors F, D2 (DP) and EP3: critical in the maintenance of pregnancy and initiation and progression of labour; differentially expressed in the myometrium and associated with regulation of cytokines.

In addition to the genes listed in Fig. 3a, many other genes affected by the expression of CREB, CREM α and CREM $\tau_2\alpha$ belonging to functional gene groups intimately involved in uterine function were found as a result of this investigation. These are listed in online

supplementary Fig. 3b–e due to the fact that they have not yet been characterized in the human myometrium, though many of these genes may turn out to be of real importance in the regulation of myometrial contractility in the future.

By determining a genomic profile of the genes that are regulated by the over-expression of CREB/CREM transcription factors in myometrial cells, we have provided further evidence for their involvement in the regulation of myometrial gene expression and function during pregnancy. We have previously shown these factors to be differentially expressed in human myometrial tissues, with a dramatic switch between the expression of phosphorylated CREB transactivator in the non-pregnant state to the CREM α repressor isoform in the pregnant and particularly labouring states, with an accompanying steady reduction in the expression of the CREM $\tau_2\alpha$ transcriptional activator. Here we have shown that these factors alter the expression of a wide range of genes, many of which are or may be involved in the control of uterine growth and differentiation, and maintaining the balance between the contractile and relaxatory states of the myometrium throughout pregnancy and in labour. The three factors show a high degree of exclusivity in the downstream genes whose expression they affect (Fig. 4b) despite their ability to form heterodimers with common interaction partners from the bZIP transcription factor family, which suggests that some other complex-specific co-factors may be involved in their mechanisms of action.

The results presented here show a high degree of similarity to those from a similar study involving the closely related bZIP transcription factors and heterodimerization partners ATF2 and a novel splice variant ATF2-small, which are also differentially expressed in the human myometrium in a spatially as well as temporally dependent manner (Bailey *et al.* 2000, Bailey & Europe-Finner 2004). Taken together, these findings demonstrate the importance of five interacting bZIP factors in controlling myometrial gene expression, much of which is linked to gestational physiological processes such as tissue, extracellular matrix and cytoskeletal remodelling, signal transduction, activity of cytokines, hormones and their receptors, and of course muscular contractility. They also highlight the importance of interplay between the MAPK pathways involved in ATF2 activation and activity, and the PKA pathways involved in CREB and CREM activity.

In conclusion, specific forms of the cAMP-responsive bZIP transcription factors (CREB, CREM α and CREM $\tau_2\alpha$) have previously been shown by us to be temporally differentially expressed in the human myometrium throughout gestation, and to possess trans-activation and trans-repression properties with regard to downstream genes containing CREs in their promoter regions. We show here that these potent factors act in

human myometrial cells to affect the expression of genes with a wide range of roles attributable to uterine activity. This supports our hypothesis that the cAMP/PKA signalling pathway, acting via the cAMP-responsive transcription factors CREB, CREM α and CREM $\tau_2\alpha$, plays an important role in the control of uterine contractility during human pregnancy and labour.

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