Research article

THE TRANSCRIPTIONAL REGULATION AND CELL-SPECIFIC EXPRESSION OF THE MAPK-ACTIVATED PROTEIN KINASE MK5

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Abstract: The mitogen-activated protein kinase (MAPK) cascades regulate important cellular processes, including growth, differentiation, apoptosis, embryogenesis, motility and gene expression. Although MAPKs mostly appear to be constitutively expressed, the transcript levels of some MAPK-encoding genes increase upon treatment with specific stimuli. This applies to the MAPK-activated protein kinases MK2 and MK3. By contrast, the transcriptional regulation of the related MK5 has not yet been studied. The MK5 promoters of mouse, rat and human contain a plethora of putative transcription factor sites, and the spatio-temporal expression of MK5 suggests inducible transcription of the gene. We examined the transcription pattern of MK5 in different tissues, and studied the kinetics of MK5 expression at the transcriptional and/or translation level in PC12 cells exposed to arsenite, forskolin, KCl, lipopolysaccharide, spermine NONOate, retinoic acid, serum, phorbol ester, temperature shock, and vanadate. Cells exposed to forskolin display a transient increase in MK5 mRNA, despite their unaltered MK5 protein levels. The MK5 promoters of human, mouse and rat contain a cAMP-responsive element that binds the cAMP-responsive element-binding protein (CREB) *in vitro*. Luciferase reporter constructs containing an 850-base pair human MK5 promoter fragment

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Abbreviations used: bp – base pairs; CRE – cAMP-response element; CREB – CRE-binding protein; HSF – heat shock factor; LPS – lipopolysaccharide; MAPK – mitogen-activated protein kinase; miRNA – microRNA; MK – MAPK-activated protein kinase; MNK – MAPK-interacting kinases; MSK – mitogen- and stress-activated kinases; PKA – protein kinase A or cAMP-dependent protein kinase; RA – retinoic acid; RSK – ribosomal S6 kinase; siRNA – small interfering RNA; TPA – tetradecanoyl phorbol acetate

encompassing the CRE showed a basal activity that was 10-fold higher than the corresponding construct in which the CRE motif was deleted. siRNA-mediated depletion of CREB had no effect on the endogenous MK5 protein levels. Several binding motifs for heat shock factor are dispersed in the mouse and rat promoter, and temperature shock transiently enhanced the MK5 transcript levels. None of the other tested stimuli had an effect on the MK5 mRNA or protein levels. Our results indicate an inducible regulation of MK5 transcription in response to specific stimuli. However, the MK5 protein levels remained unaffected by all the stimuli tested. There is still no explanation for the discrepancy between the increased mRNA and unchanged MK5 protein levels.

Key words: Mitogen-activated protein kinase-activated protein kinase, MK5, Promoter, CREB, Heat-shock, Oxidative stress.

INTRODUCTION

Protein phosphorylation is a major post-translational modification that dictates the function of a protein. This reversible process is governed by protein kinases, which catalyse the transfer of the γ-phosphoryl group of ATP to the hydroxyl groups of specific serine, threonine and/or tyrosine residues, while protein phosphatases remove phosphate groups. More than 500 protein kinase-encoding genes and more than 150 protein phosphatase-encoding genes have been identified in the human genome [1].

One major group of protein kinases is the mitogen-activated protein kinases (MAPKs). Initially, the MAPK cascade seemed to be a tripartite module in which the most upstream protein kinase, the MAPK kinase kinase (MAPKKK or MEKK), activates a MAPK kinase (MAPKK or MEK), which in turn activates the MAPK. This MAPK then phosphorylates non-protein kinase substrates. However, the discovery of protein kinases that act as substrates for MAPKs blurred the nomenclature of the protein kinases in this tripartite cascade. To avoid having to rename the MAPKs, these newly discovered protein kinases were called MAPK-activated protein kinases (MAPKAPKs). Based on the homology of the kinase catalytic domain, the MAPKAPKs are divided into four subfamilies: RSKs (ribosomal S6 kinases or MAPKAPK1), MSKs (mitogen- and stress-activated kinases), MNKs (MAPK-interacting kinases), and MKs (MAPKAPK). At least four typical MAPK cascades exist in mammalian cells. The Raf-MEK1/2-ERK1/2 pathway mainly transmits responses to growth factors and cytokines, while stress-inducing signals activate the MEKK1/4-MKK4/7-JNK and the MEKK1/4-MKK3/6-p38 pathways. The MEKK2/3-MEK5-ERK5 pathway can respond to both growth factor- and stress-induced signals. A fifth atypical pathway, composed of the ERK3/4-MK5 module, has yet to be fully characterized and its upstream activators and signals remain to be identified [2, 3].

Since many *mapk* genes display constitutive expression, the promoters and transcriptional regulation of the MAPK-encoding genes have scarcely been

studied. However, the transcript levels of some MAPK are inducible. The expressions of the rice MAPK genes OsBIMK1 and BWMK1 are affected by abiotic and biotic stresses [4, 5], while cold stress and high salt stress increase the transcript levels of OsMAPK2, and sugar starvation increases those of OsMAPK4 [6, 7]. Transcript profiling of the tissues derived from untreated and stress-exposed *Arabidopsis thalania* revealed 2,715 differentially expressed genes, including the MAPKs MKK9 and MAPKKK14 [8]. The expression of three other *Arabidopsis thalania* MAPK genes was also induced by temperature and salt stress [9]. In *Xenopus laevis* oocytes, steroid hormone treatment enhanced the transcript levels of MOS, the upstream activator of MEK1 [10]. Less is known about the transcriptional regulation of mammalian MAPKs. Patients suffering from surgical trauma displayed enhanced ERK5 (= MAPK7) transcript levels, but the exact stimuli and mechanisms for increased transcription of this gene are not known [11]. Microarray studies comparing platelet-derived growth factor-treated NIH3T3 cells and lung tissue from rats exposed to diesel exhaust particles with cells and tissue from control animals revealed elevated transcript levels of MK2 [12, 13]. MK2, MK3 and MK5 form a subfamily of closely related MAPKAPKs [14]. The effects of several stimuli on MK2 and MK3 transcript levels have been assessed. MK2 and MK3 mRNA levels increase in rat ovaries after the injection of human chorionic gonadotropin [15]. MK2 mRNA levels were strongly increased in the PC12 pheochromocytoma cell line when exposed to KCl, forskolin, the calcium ionophore A23187, and ATP, while phorbol ester exposure triggered a moderate increase. Interleukin-6, nerve growth factor and epidermal growth factor did not have any effect on the MK2 transcript levels. Kainic acid also increased MK2 mRNA levels in the rat brain. None of the stimuli modulated the mRNA levels of MK3 in PC12 cells, and the mRNA levels of the third subfamily member, MK5, were not examined [16]. In this study, we examined the MK5 mRNA and protein levels in different cell lines exposed to 10 different stimuli, and studied the induction of the 5' flanking region of the *mk5* gene. Our results show a tissue-specific transcription pattern and an inducible transcription of the *mk5* gene in response to specific stimuli. However, none of the stimuli tested here affected the MK5 protein levels. The molecular mechanism responsible for the discrepancy between the increased mRNA levels and the unchanged protein levels remains unknown, as does the biological relevance of this discrepancy.

MATERIALS AND METHODS

Materials
Forskolin, retinoic acid, LPS, sodium arsenite, vanadate and 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA) were all purchased from Sigma Aldrich (St. Louis, MO, USA). Spermine NONOate was obtained from Calbiochem, Merck, and KCl was purchased from Merck (Merck Chemicals Ltd, Nottingham, UK). ^{32}P α-dCTP was obtained from Amersham/GE Healthcare (Buckinghamshire,

UK). The anti-PRAK (A-7) antibodies were purchased from Santa Cruz Biotechnology Inc (Santa Cruz, CA, USA), and the anti-CREB (#9104), anti-ERK1/2 (#4695) and anti-FOS 9F6 (#2250) antibodies from Cell Signaling (Beverly, MA). Anti-GFP (ab290) was purchased from AbCam (Cambridge, UK). The actin, alkalic phosphatase-conjugated secondary antibodies sheep anti-mouse IgG, and anti-rabbit IgG were from Sigma Aldrich. Oligonucleotides were purchased from Sigma Aldrich or Eurogentec (Seraing, Belgium). Purified CREB protein was obtained from Active Motive (Carlsbad, CA, USA).

Plasmids
The plasmids pTAL-LUC and pCRE-LUC were obtained from Clontech (Clontech, Takara Bio Inc, Shiga, Japan). The plasmid pCREMK5-LUC was generated as follows: the complementary oligonucleotides encompassing three copies of the proximal CRE of the human MK5 promoter, 5'-GTA-<u>TGA-CGT-CA</u>T-TAC-TA<u>T-GAC-GTC-A</u>TT-ACT-A<u>TG-ACG-TC</u>A-TTA-3' and 5'-TCG-ATA-A<u>TG-ACG-TCA</u>-TAG-TAA-<u>TGA-CGT-CA</u>T-AGT-AA<u>T-GAC-GTC-A</u>TA-CGT-AC-3' (the CRE motifs are underlined), were annealed and ligated into the *Kpn*I/*Xho*I sites of pTAL-LUC. A minimal promoter and three copies of the MK5 CRE drive the transcription of the luciferase reporter gene. The control plasmid pCRE-LUC contains the same promoter, but has three copies of the consensus CRE motif. The expression plasmid for VP16 was obtained from Clontech, and the expression plasmid for CREB-VP16 was generated by cloning rat CREB cDNA sequences into the *Eco*RI/*Bam*HI sites of the VP16 expression plasmid. For the luciferase plasmid containing the human MK5 promoter fragment, human genomic DNA was obtained from whole blood, and a fragment was amplified by PCR using the forward primer: 5'-GAC-CTC-CAC-AGA-TCC-TCT-CAG-AAG-GAG-3' (nucleotides 50645-50670 in GI:29126252) combined with the reverse primer starting from the MK5 start codon: 5'-GCT-TTG-TCC-ATG-TCG-CTC-TCC-TCC-GAC-3'. This yielded a PCR product of 850 bp, which was cloned into the TA vector pCR2.1 (Invitrogen). Positive clones were sequenced to verify the presence of the CRE motif. The 850-bp fragment was then subcloned into the *Kpn*I/*Xho*I sites of the luciferase reporter plasmid pGL3-Basic (Promega). The CRE motif was mutated from TGACGTCA to TCACAAAA using the following primers: 5'-GAG-TCC-GGG-CCT-ATC-ACA-AAA-TTA-GCG-CAG-CGC-CAT-3' and 5'-ATG-GCG-CTG-CGC-TAA-TTT-TGT-GAT-AGG-CCC-GGA-CTC-3'. This mutation was previously shown to prevent CREB binding and to abrogate the cAMP response [17]. All the newly generated plasmids and mutations were confirmed by sequencing.

Cell culture and transfection
PC12 cells, donated by Dr. Jaakko Saraste (University of Bergen, Norway), were maintained in RPMI 1640, supplemented with 15% horse serum (Gibco), 2.5% foetal bovine serum, 2 mM L-glutamine, 110 units/ml penicillin and 100 μg/ml streptomycin. The human neuroblastoma cell line SK-N-DZ (CRL-2194) was

from ATCC (LGC Promochem; Middlesex, United Kingdom) and was maintained in Dulbecco's modified Eagle's medium, supplemented with 10% foetal bovine serum. HEK293 cells were purchased from the European Collection of Cell Cultures (cat. no. 85120602; Salisbury, Wiltshire, UK) and kept in Eagle's Minimum Essential Medium supplemented with 10% foetal calf serum, 2 mM L-glutamine, 110 units/ml penicillin and 100 μg/ml streptomycin. COS-1 cells (ATCC CRL1650) were kept in Dulbecco's modified Eagle's medium, supplemented with 10% foetal bovine serum.

Multi-tissue blot hybridization

The human Multiple Tissue Expression (MTE^{TM}) Array was purchased from Clontech (Cat. No. 636901) and the mRNA Array Human Tumor, Array II (192 spots/47 pairs) was from BioChain Institute (Hayward, CA, USA; cat. No.H3235713-1). pcDNA-MK5$_{WT}$ was digested with *Eco*RI/*Xho*I to extract the MK5 fragment, after which 50 ng of the fragment was used for probe labelling with the Random Prime Labelling Module (Amersham) according to the manufacturer's instructions. The MTE array was prehybridized in a hybridization buffer (5xSSC, 0.1% SDS, 5% dextrane sulphate, 1:20 Liquid block; Amersham) for 30 min at 45ºC. Hybridization of the probe and the array was done overnight at 45ºC. The next day, the array was washed 4 times in washing solution 1 (1xSSC, 0.05% SDS) at 60ºC for 15 min, then twice for 30 min at 48ºC in solution 2 (0.5xSSC, 0.5% SDS), and finally the array was rinsed in diluent buffer (0.1 M TrisBase pH 9.5, 0.3 M NaCl) at room temperature. Next, the array was incubated for 1 h at room temperature in a blocking buffer (a 1:10 dilution of liquid block in diluent buffer), then incubated with anti-fluorescein-AP/BSA for 1 h at room temperature. Subsequently, the array was washed 3 times for 10 min in Tween20 washing solution (0.3% Tween in diluent buffer) and rinsed in diluent buffer. For detection, CDP-star was added to the membrane. Detection was achieved by exposure to a photosensitive film (Kodak) for 3 h.

RNA isolation and reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA from cells was isolated using the Nucleospin RNA II purification kit (Clontech, Takara Bio Inc, Shiga, Japan) according to the manufacturer's protocol. The RNA concentrations and purity were checked by spectrometry using a Nanodrop ND-1000 (NanoDrop Technologies Inc., Wilmington, DE, USA). 2 μg of RNA was reverse transcribed using an iScript cDNA synthesis kit (BioRad, Biocompare, San Francisco, CA), and subjected to PCR. The primers for APRT and the PCR conditions were previously described [18]. The sequences of the hMK5 primers are: 5'-CCC-TAC-ACT-TAC-AAC-AAG-AGC-TGT-G-3' and 5'-CTT-TAT-CTG-TGA-ATC-CAC-GGC-CAT-TC-3'. The primers for GADPH are: 5'-TGG-CCA-AGG-TCA-TCC-ATG-ACA-AC-3' and 5'-CAT-GAG-GTC-CAC-CAC-CCT-GTT-GCT-GTA-3'. 30 cycles of 30 sec at 94ºC, 30 sec at 62ºC and 30 sec at 72ºC were run to amplify hMK5 and

GADPH transcripts. To amplify CREB cDNA, the following primers were used: 5'-ATG-GAA-TCT-GGA-GCC-GAC-AAC-3' and 5'-TCC-TGT-AGG-AAG-GCC-TCC-TGG-3'. 28 cycles of 30 sec at 94ºC, 30 sec at 60ºC and 30 sec at 72ºC were used.

Electrophoresis mobility shift analysis (EMSA)
EMSA was performed as previously described [19]. Briefly, complementary oligonucleotides were annealed in 10xTEN buffer (100 mM TrisCl pH 7.5, 10 mM EDTA, 1 M NaCl) and then labelled with ^{32}P α-CTP (3,000 Ci/mmol) with Klenow polymerase. The sequences of the complementary oligonucleotides used in EMSA are (the CRE motifs are underlined): MK5-CRE: 5'-GTA-CGC-CTA-TGA-CGT-CAT-TAG-CGC-A-3' and 5'-TCG-ATG-CGC-TAA-TGA-CGT-CAT-AGG-3'; c-*fos* CRE: 5'-CTA-GGA-GCC-CGT-GAC-GTT-TAC-ACT-C-3' and 5'-GAT-CGA-GTG-TAA-ACG-TCA-CGG-GCT-C3'; NFκB: 5'-CGA-GAG-TTG-AGG-GGA-CTT-TCC-CAG-GC-3' and 5'-CGG-AGC-CTG-GGA-AAG-TCC-CCT-CAA-CT-3'; SIE: 5'-CTA-GGA-GCA-GTT-CCC-GTC-AAT-CCC-3' and 5'-GAT-CGG-GAT-TGA-CGG-GAA-CTG-CTC-3'; AP1: 5'-CGC-TTG-ATG-ACT-CAG-CCG-GAA-3' and 5'-TTC-CGG-CTG-AGT-CAT-CAA-GCG-3'; and MK5-mutCRE: 5'-GTA-CGC-CTA-TGA-CGT-CAT-TAG-CGC-A-3' and 5'-TCG-ATG-CGC-TAA-TGA-CGT-CAT-AGG-3'. In the competitive EMSA, a 100-fold molar excess of unlabelled competitor oligonucleotide was used. The labelled oligonucleotides and CREB protein were mixed in a total volume of 20 μl containing 25 mM HEPES, pH 7.5; 12.5 mM MgCl$_2$; 100 mM KCl; 1 mM DTT; 10 μM ZnSO$_4$; 20% glycerol; 0.1% NP-40; 200 ng/μl PolydI-dC (GE Healthcare/Amersham); 0.5 μg/μl calf-thymus DNA; and 300 μg/ml BSA. 2 ng/μl labelled oligonucleotides and 250 ng/μl recombinant CREB protein (Active Motive) were used. The DNA-protein complexes were allowed to form for 30 min on ice. Then, 6x loading buffer (40% glycerol, 50 mM EDTA, 0.1% bromophenol blue, 3x TBE) was added to a final concentration of 1x before the samples were loaded on a 5% non-denaturating acrylamide gel. The gels were run for 5 h at 4ºC at 10 V/cm in 0.5xTBE, and subsequently dried and subjected to autoradiography.

Transient transfection and luciferase assay
Cells were plated at 3x10^5 cells per well in a 6-well plate. Cells were transfected using Lipofectamine 2000 according to the manufacturer's instructions. COS cells were transfected by the DEAE/dextrane method as previously described [20]. Luciferase assays were performed as previously described [18].

Western blotting
To detect specific proteins, cells were collected in lysis buffer, heated for 10 min at 70ºC and sonicated. Samples were then analyzed by SDS-PAGE with NuPage 4-12% Bis-Tris SDS-PAGE (Invitrogen) according to the manufacturer's protocol, and blotted onto a 0.45 μm PVDF membrane (Millipore, Billerica,

MA, USA). Immunoblotting was performed by first blocking the membrane with PBS-T (PBS with 0.1% Tween-20 (Sigma Aldrich) containing 10% (w/v) dried skimmed milk for 1 h and subsequently incubating the membrane overnight at 4ºC with primary antibody. After 4 washes, the membrane was incubated with the appropriate secondary antibody for 1 h. Visualization of the proteins was achieved by using CDP Star (Tropix, Bedford, MA, USA) substrate and Lumi-Imager F1 from Roche (Basel, Switzerland).

Small interfering RNA (siRNA)-mediated depletion of CREB

HEK293 cells were plated at $3x10^5$ cells per well into a 6-well dish, and validated CREB-directed siRNA or scrambled siRNA (Ambion Inc., Austin, TX, USA) was transfected by Lipofectamine 2000 (Invitrogen, Life Technologies Corporation, Carlsbad, CA, USA) according to the manufacturer's instructions using 100 nM siRNA/10^6 cells. RNA was purified 24 and 48 h after transfection and subjected to semi-quantitative RT-PCR to monitor the CREB and MK5 transcript levels. 72 h after transfection, the cells were harvested and the CREB protein levels were assessed via Western blotting.

Densitometry

Densitometry was performed using a BioRad Model GS-700 Imaging Densitometer and the Multi-Analyst version 1.1 Software.

Statistical analysis

Student's t-test was performed and differences with p-values < 0.05 were considered significant. All the data is presented as means $\pm$ SD.

RESULTS

MK5 mRNA levels vary in different organs

The original paper describing the identification of MK5 revealed that the *mk5* gene seems to be transcribed in most of the tissues tested, but the steady-state transcript levels varied in different organs, with a high level of expression in the brain, heart, liver, skeletal muscle, and kidneys, and low mRNA levels in the spleen [21, 22]. MK5 transcripts and protein were also detected in ovulating oocytes, in 2-, 4-, and 8-cell stages, and in morula and blastocysts during murine preimplantation development [23, 24]. We extended the investigation of the tissue distribution of MK5 transcripts by monitoring MK5 mRNA in multiple adult and foetal tissues by dot blot hybridization. Fig. 1 shows the MK5 expression pattern in adult tissue and in some human cell lines. The *mk5* gene seems to be transcribed in many adult tissues. Fig. 2 compares the MK5 transcript levels in normal and tumour samples. We did not always obtain the same results with multiple RNA samples of the same tissue on the same multiple tissue arrays, e.g. MK5 mRNA was not always discovered in parallel samples of colon, kidney, lymph node and rectum mRNA that were placed on different areas of the blot (see Fig. 2). We also investigated the foetal expression of MK5

in the brain, heart, kidneys, liver, lungs, spleen, and thymus, and found MK5 transcripts only in the foetal kidneys (Fig. 1, C11).
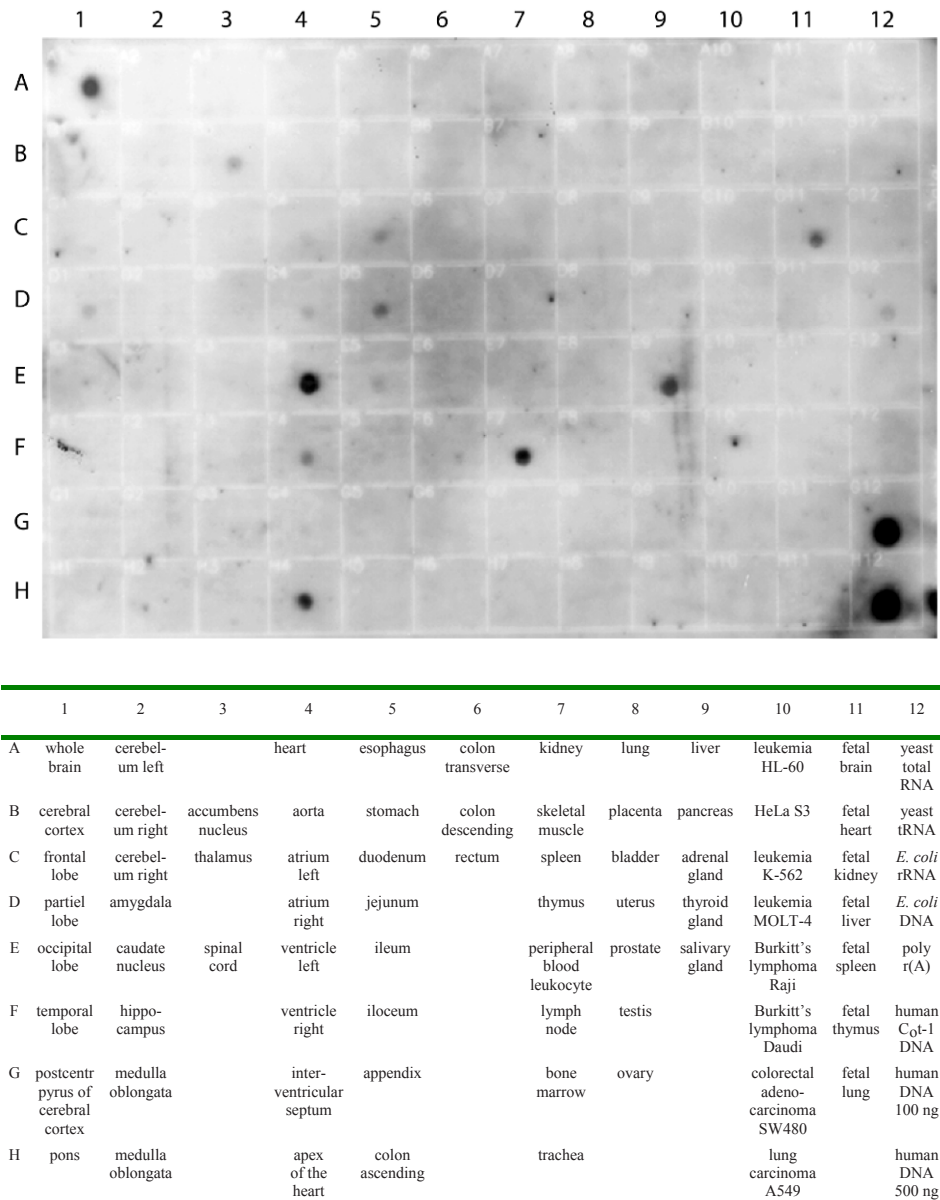

|	1	2	3	4	5	6	7	8	9	10	11	12
A	whole brain	cerebel-um left		heart	esophagus	colon transverse	kidney	lung	liver	leukemia HL-60	fetal brain	yeast total RNA
B	cerebral cortex	cerebel-um right	accumbens nucleus	aorta	stomach	colon descending	skeletal muscle	placenta	pancreas	HeLa S3	fetal heart	yeast tRNA
C	frontal lobe	cerebel-um right	thalamus	atrium left	duodenum	rectum	spleen	bladder	adrenal gland	leukemia K-562	fetal kidney	*E. coli* rRNA
D	partiel lobe	amygdala		atrium right	jejunum		thymus	uterus	thyroid gland	leukemia MOLT-4	fetal liver	*E. coli* DNA
E	occipital lobe	caudate nucleus	spinal cord	ventricle left	ileum		peripheral blood leukocyte	prostate	salivary gland	Burkitt's lymphoma Raji	fetal spleen	poly r(A)
F	temporal lobe	hippo-campus		ventricle right	iloceum		lymph node	testis		Burkitt's lymphoma Daudi	fetal thymus	human C_0t-1 DNA
G	postcentr pyrus of cerebral cortex	medulla oblongata		inter-ventricular septum	appendix		bone marrow	ovary		colorectal adeno-carcinoma SW480	fetal lung	human DNA 100 ng
H	pons	medulla oblongata		apex of the heart	colon ascending		trachea			lung carcinoma A549		human DNA 500 ng

Fig. 1. The tissue-specific expression of MK5. A multi-tissue blot with RNA isolated from different tissues was examined for the presence of MK5 transcripts. The top panel shows the results of the hybridization with an MK5-specific probe, and the bottom panel indicates from which particular tissues the RNA on the blot was derived. RNA was isolated from normal adult and embryonic tissues.
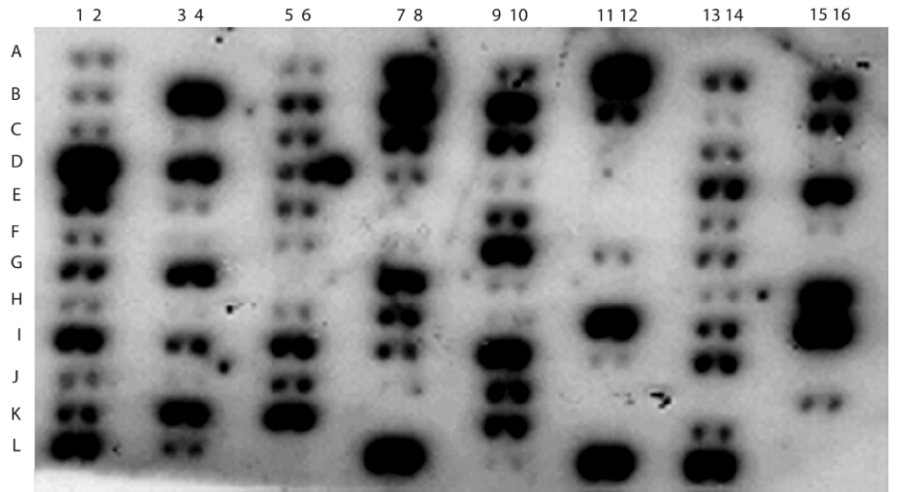
	1 and 2	3 and 4	5 and 6	7 and 8	9 and 10	11 and 12	13 and 14	15 and 16
A	positive control	negative control	adrenal tumor	normal adrenal	adipose tumor	normal adipose	bladder tumor	normal bladder
B	bladder tumor	normal bladder	breast tumor	normal breast	breast tumor	normal breast	breast tumor	normal breast
C	breast tumor	normal breast	colon tumor	normal colon	colon tumor	normal colon	colon tumor	normal colon
D	esophagal tumor	normal esophagus	esophagal tumor	normal esophagus	gal bladder tumor	normal gal bladder	kidney tumor	normal kidney
E	kidney tumor	normal kidney	kidney tumor	normal kidney	liver tumor	normal liver	liver tumor	normal liver
F	liver tumor	normal liver	liver tumor	normal liver	lung tumor	normal lung	lung tumor	normal lung
G	lung tumor	normal lung	lung tumor	normal lung	lymphoma	normal lymph node	lymphoma	normal lymph node
H	ovary tumor	normal ovary	ovary tumor	normal ovary	ovary tumor	normal ovary	ovary tumor	normal ovary
I	prostate tumor	normal prostate	prostate tumor	normal prostate	prostate tumor	normal prostate	rectum adeno-carcinoma	normal rectum
J	rectum adeno-carcinoma	normal rectum	rectum adeno-carcinoma	normal rectum	small intestine tumor	normal small intestine	soft tissue tumor	normal soft tissue
K	soft tissue tumor	normal soft tissue	stomach tumor	normal stomach	stomach tumor	normal stomach	stomach tumor	normal stomach
L	thymus tumor	normal thymus	thyroid tumor	normal thyroid	thyroid tumor	normal thyroid	uterus tumor	normal uterus

Fig. 2. The transcription profile of the *mk5* gene in normal and tumour samples. An MK5-specific probe was used in the hybridization and the results are shown in the top panel. The bottom part of the figure shows a schematic representation of the type of tissues located on the blot.

The promoters of mouse, rat and human MK5 contain conserved binding motifs for transcription factors

This spatio-temporal distribution pattern of MK5 mRNA suggests specific regulation of the *mk5* gene at the transcriptional level. Therefore, we examined the published MK5 promoter region sequences for the presence of consensus

binding sites for transcription factors. Computer-aided screening of the ~3300-bp genomic sequence upstream of the ATG start codon revealed several putative transcription factor binding sites for the mouse, rat and human *mk5* gene (see Fig. 3; [25]). Only those sites that displayed > 95% identities with the consensus sites are indicated. The presence of such binding sites should be interpreted with care because of the ambiguity of the genomic MK5 sequences deposited in the gene bank, and because proteins may bind to non-consensus motifs. All three promoters are GC rich, with regions containing > 90% G or C. This may explain
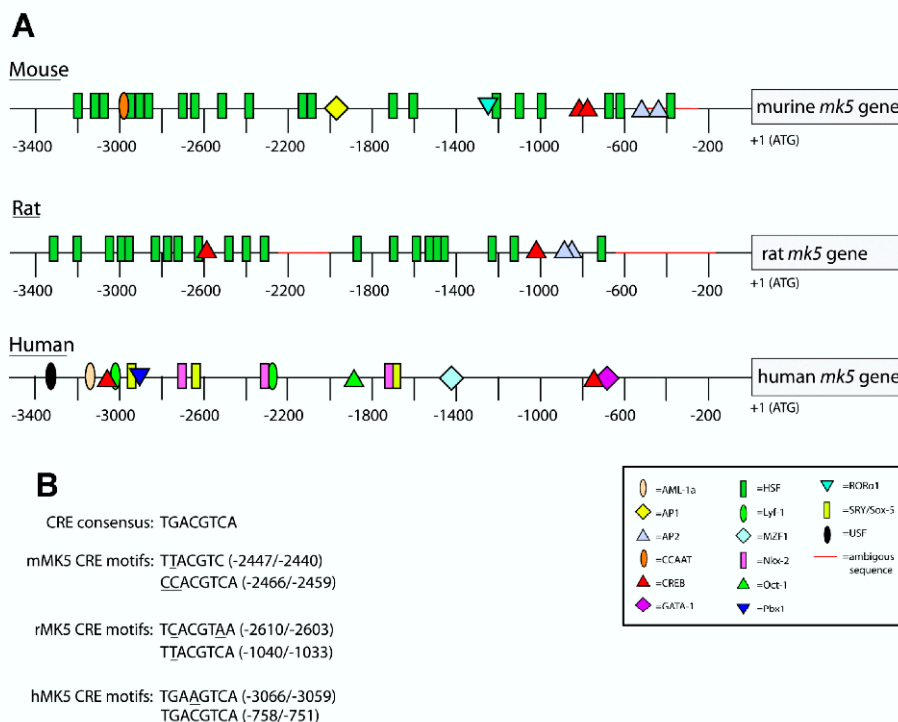
Fig. 3. Putative binding sites for transcription factors in the mouse, rat, and human MK5 promoter. A – A region of approximately 3,300 bp upstream of the start codon of the *mk5* gene was screened for putative binding sites for transcription factors using a transcription factor prediction program. Transcription factor motifs that showed 95% or more identity to known binding consensus sequences are indicated. The sequence of the MK5 promoters is based on available sequences in the GenBank [50]. A thick red line indicates regions in the mouse and rat promoter that have not been sequenced or are ambiguous. This ambiguity is probably the result of the very high GC contents of the promoters. B – The sequences and the locations in relation to the start codon of conserved CRE motifs in the MK5 promoter of the three species are shown.

why the reported mouse and rat sequences in the gene bank contain ambiguous sequences. MK5 transcripts are readily detected in tissues expressing cell-specific transcription factors (e.g. AML-1a in the thymus, Nkx-2 in the neurons

and heart, SRY/Sox-5 in the testes) for which putative binding sites are present in the *mk5* promoter. It remains to be established whether these factors are involved in MK5 expression. The MK5 promoters of all three species lack a canonical TATA box, but they share two putative cAMP response element (CRE) binding sites. The proximal CRE in the human MK5 (hMK5) promoter has the consensus sequence, while all the others have 1 or 2 mismatches (Fig. 3B). The mouse and rat promoters each possess two adjacent putative AP-2 sites. Strikingly, these promoters also contain numerous heat shock factor (HSF) binding sites, while no such sites appear to be present in the human promoter. As the presence and relative position of the CRE was conserved in all three promoters, we first explored the functionality of this element.

CREB binds specifically to the MK5 promoter *in vitro*
We investigated whether the proximal CRE in the hMK5 promoter could bind CREB *in vitro* using the electrophoretic mobility shift assay (EMSA). Fig. 4 shows that purified CREB protein binds the human MK5 CRE motif (lanes 2 in
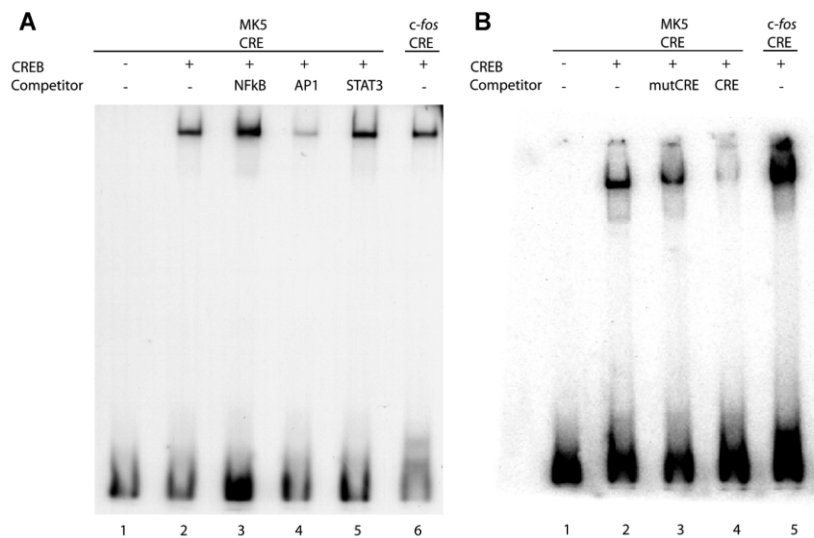
Fig. 4. CREB binds *in vitro* to the consensus CRE motif in the human MK5 promoter. Purified CREB protein was incubated with radioactive labeled oligonucleotides with the MK5 CRE motif in the presence or absence of competitor DNA, and DNA-protein complexes were analyzed on a polyacryl amide gel. A – Lane 1: free probe, lanes 2-5: probe plus recombinant CREB protein. In lanes 3-5, a 100-fold molar excess of cold competitor oligonucleotides was added. Lane 3: oligonucleotide with NFκB binding motif; lane 4: oligonucleotide with AP1 motif; lane 5: oligonucleotide with STAT3 motif. Lane 6: c-*fos* CRE plus recombinant CREB. B – Lane 1: free probe, lanes 2-4: MK5 CRE plus CREB; lane 3: as lane 2, but with a 100-molar excess of oligonucleotide with mutated CRE as a competitor; lane 4: as lane 2, but with a 100-molar excess of oligonucleotide with consensus CRE as a competitor. Lane 5: c-*fos* CRE plus recombinant CREB.

Fig. 4A and B), and the c-*fos* CRE motif (lane 6 in Fig. 4A and lane 5 in Fig. 4B) *in vitro*. This binding was not disrupted by competing double-stranded oligonucleotides with binding sites for NFκB (Fig. 4A, lane 3), STAT3 (Fig. 4A, lane 5), or a mutated MK5 CRE motif (lane 3, Fig. 4B), while an oligonucleotide with an AP-1 binding site strongly interfered with CREB-MK5 CRE binding (Fig. 4A, lane 4). This is not unexpected, as the CREB protein has been shown to bind AP-1 motifs as well [26].

MK5 transcript levels are transiently increased in response to the cAMP-elevating agent forskolin

CREB bound to its cognate CRE motif mediates transcription upon phosphorylation at Ser-133. The major CREB kinase is cAMP-dependent protein kinase (PKA), which is activated by cAMP. Forskolin, an activator of adenylate cyclase, increases the intracellular concentrations of cAMP, which induces PKA activation [reviewed in 27]. We compared the MK5 mRNA levels in untreated and forskolin-treated cells over time. A time-dependent increase in MK5 transcript levels was observed in SK-N-DZ and PC12 cells exposed to forskolin (Fig. 5A and 5B, respectively), although with different kinetics in the two cell lines. In PC12 cells, the MK5 mRNA levels peaked 30 min after forskolin treatment, remained elevated for 60 min, and then returned to their basal levels. In SK-N-DZ cells, an increase in the transcript levels occurred as early as 15 min after treatment, and gradually continued until the highest levels were reached 3 h after forskolin stimulation. 6 h after forskolin exposure, the mRNA levels had returned to their basic levels. The transcript levels of the cAMP response genes (positive control) c-*fos*, Nur77, and leukaemia inhibitory factor [28] were transiently upregulated after forskolin treatment (data not shown), whereas the APRT transcript levels remained unaltered (negative control).

These increases in MK5 mRNA levels could be the result of enhanced CREB-mediated MK5 promoter activity. Alternatively or additionally, cAMP may stabilize MK5 transcripts, which has been shown for other mRNA species [reviewed in 29]. To distinguish between the two mechanisms, we monitored the forskolin-induced MK5 mRNA levels in the presence of actinomycin D, an inhibitor of transcription. Prior to the addition of forskolin, we pre-treated PC12 cells with actinomycin D for 30 min, then added forskolin and subsequently determined MK5 mRNA levels at different time points. The levels of forskolin-induced MK5 mRNA decreased over time in the presence of actinomycin D (Fig. 5C), thus suggesting that the forskolin-induced increase in MK5 transcripts was caused by enhanced transcription rather than cAMP-mediated stabilization.
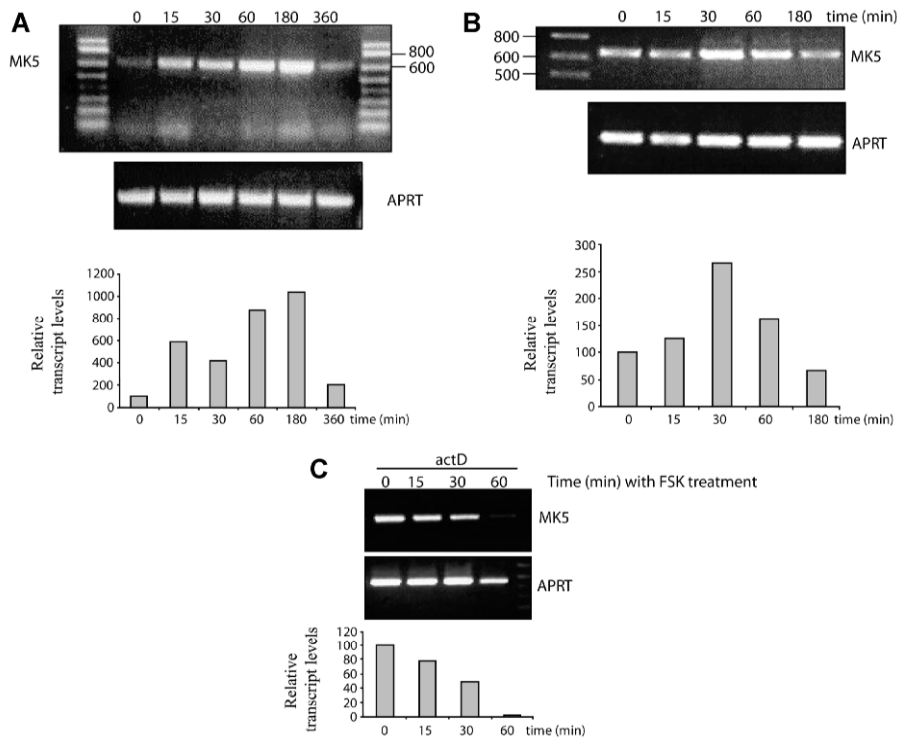
Fig. 5. Stimulation of the PKA signalling pathway by forskolin induces a transient increase in MK5 transcript levels. SK-N-DZ (A) or PC12 (B) cells were exposed to 10 μM forskolin and mRNA was isolated at different time points after treatment. The mRNA was reverse transcribed and MK5 or APRT transcripts were amplified by PCR with specific primers. The PCR products were visualized by electrophoresis on an agarose gel followed by ethidium bromide staining. A size marker was run on the gel to confirm the correct size of the expected PCR fragment. The time of forskolin stimulation is shown in minutes. C – The forskolin-induced increase in MK5 mRNA results from increased transcription and not stabilization of the transcripts. SK-N-DZ cells were pretreated with 10 μM actinomycin D for 30 min before 10 μM forskolin was added. RNA was subsequently isolated at the time points indicated and reverse transcribed into cDNA. The presence of MK5 transcripts was assayed by PCR with MK5-specific primers. The transcript levels of MK5 were compared with the APRT levels. The PCR products were run on an agarose gel and visualized by ethidium bromide staining. Densitometry was performed and the ratio of the densitometry values of the MK5:APRT signals were calculated. The ratio of the MK5:APRT value for untreated cells (0 min forskolin) was arbitrarily set as 100%, and the other values were correlated to this.

The isolated MK5 CRE motif does not mediate the cAMP response
As the proximal hMK5 CRE bound CREB *in vitro*, we examined if the isolated CRE motif of the MK5 promoter was able to elicit a cAMP response when fused to a minimal promoter. Three copies of the MK5 CRE motif were cloned in the pTAL-LUC reporter plasmid to generate the plasmid pCREMK5-LUC. The

TAL-LUC plasmid contains a minimal promoter consisting of a TATA-box element. A corresponding plasmid containing three copies of the consensus CRE motif TGACGTCA (pCRE-LUC) was used as a control. Cells were transfected with one of these two constructs and treated with forskolin. Our results show that forskolin treatment resulted in a > 5-fold increase in luciferase activity in the cells transfected with pCRE-LUC, but no increase in luciferase activity was observed in the cells transfected with pCREMK5-LUC. Co-transfection with an expression plasmid for the catalytic $C\alpha$ subunit of PKA tagged with a nuclear localization signal provoked a very potent increase (> 100-fold) in luciferase activity in the cells transfected with pCRE-LUC, while only a ~5-fold increase was measured in the cells transfected with pCREMK5-LUC. The expression of a kinase-inactive $C\alpha$ mutant (K72H) had only minor (~2.5-fold) effects on the strength of the two promoters, indicating that an active $C\alpha$ is required to enhance transcription from the consensus CRE-containing promoter (Fig. 6).
Phosphorylation of CREB at Ser-133 by PKA is necessary, but not sufficient to activate CREB and stimulate a cAMP-responsive promoter [29, 30]. It is
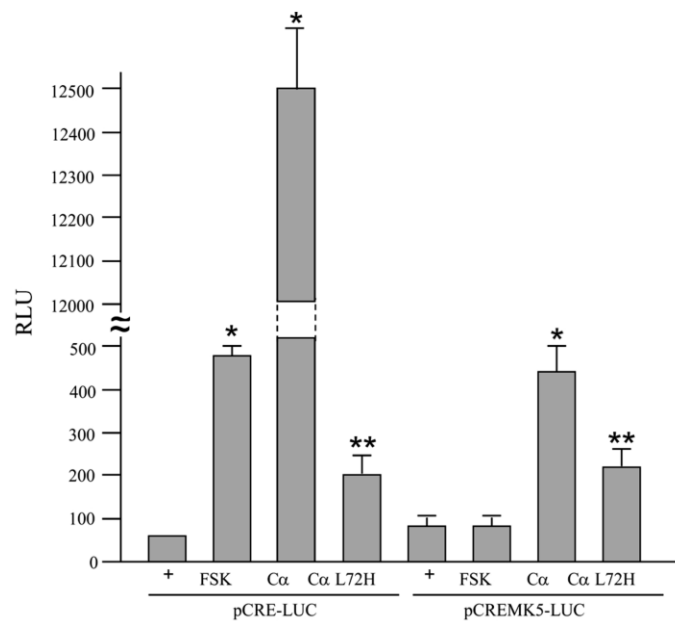

Fig. 6. The canonical proximal CRE motif of the hMK5 promoter is a poor mediator of cAMP/PKA-induced transcription. Cells were transiently transfected with a luciferase reporter plasmid and treated with 10 μM forskolin for 3 h, or they were cotransfected with an expressing plasmid encoding the catalytic $C\alpha$ subunit of PKA ($C\alpha$) or a kinase dead mutant ($C\alpha$ L72H). The luciferase reporter plasmid contained three copies of the consensus CRE motifs fused to a minimal promoter (pCRE-LUC) or three copies of the proximal hMK5 CRE motif (pCREMK5-LUC). The results show the average (± SD) of three independent parallels. RLU = relative luciferase units. *p < 0.001, **p < 0.05; Student's t-test.

plausible that the inability of PKA to enhance the transcriptional strength of the promoter in the pCREMK5-LUC reporter plasmid results from the lack of PKA to activate CREB. To test this, we co-transfected cells with the CRE-driven luciferase plasmids (either pCRE-LUC or pCREMK5-LUC) and an expression plasmid encoding CREB-VP16 fusion protein. This protein will bind to CRE through its CREB moiety, while the transactivation domain of VP16 will augment the transcription activity of the promoter independently of CREB. As depicted in Fig. 7, both plasmids have comparable basal activity (compare the luciferase activity in the presence of VP16). We observed a moderate but significant ($p < 0.001$) stimulation of transcription driven by a minimal promoter containing 3 copies of consensus CRE motifs (pCRE-LUC; 2-fold), as well as the promoter with three copies of the MK5 proximal CRE binding sites (pCREMK5-LUC; 1.6-fold) by CREB-VP16. These results demonstrate that the CREB-VP16 fusion protein transactivates the promoters containing either consensus CRE or MK5 CRE motifs, although somewhat more weakly for the promoter containing the latter motifs. This may suggest that both CRE elements are functional, but that the MK5-CRE cannot be stimulated by forskolin.
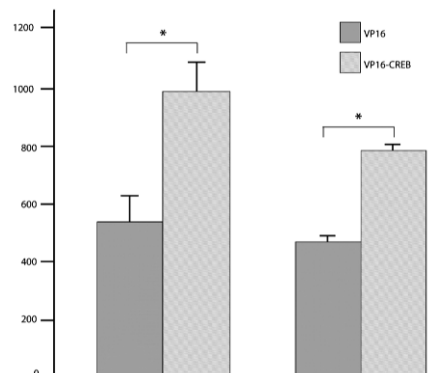

Fig. 7. The VP16-CREB fusion protein moderately stimulates transcription from a minimal promoter containing three copies of the MK5 CRE motif. Cells were co-transfected with a luciferase plasmid containing three copies of the consensus CRE motif (pCRE-LUC) or the corresponding plasmid with three copies of the proximal human MK5 CRE (pCREMK5-LUC), and an expression vector for either the transactivator domain of VP16 or a fusion protein contain this VP16 moiety and the DNA-binding domain of CREB (VP16-CREB). The results show the average ($\pm$ SD) of three independent parallels. *p < 0.001, Student's *t*-test.

The proximal consensus CRE motif in the hMK5 promoter is necessary for basal promoter activity, but cannot mediate PKA-induced transcription
The functionality of a CRE may be affected by adjacent sequences or binding motifs for other transcription factors [reviewed in 29]. This prompted us to examine the functionality of the CRE motif in the context of the MK5 promoter.
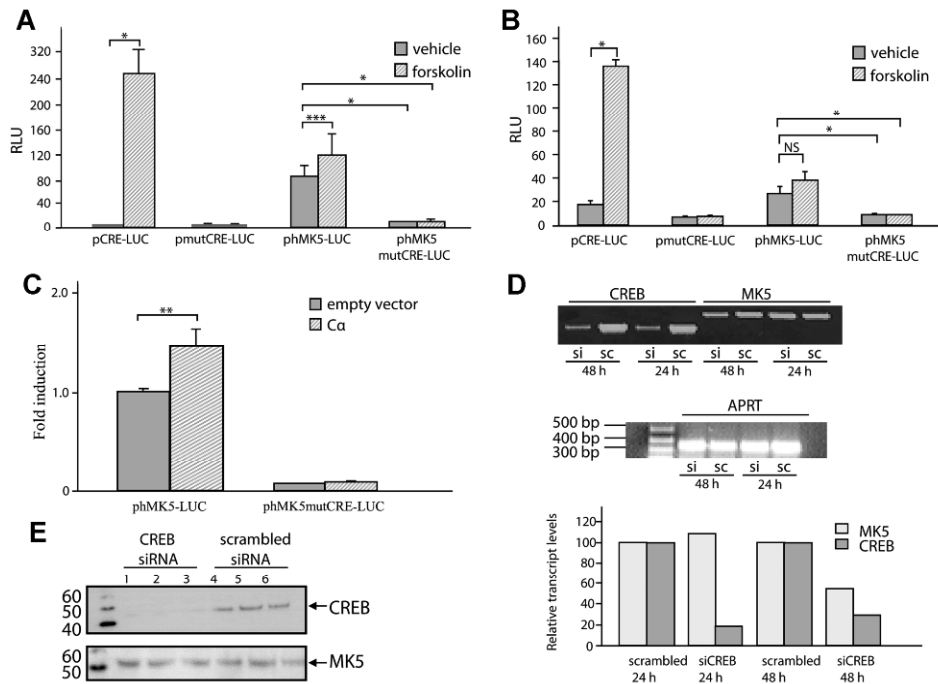
Fig. 8. The consensus CRE motif in the human MK5 promoter is required for basal promoter activity, but does not mediate cAMP responsiveness. A – SK-N-DZ cells were transiently transfected with either a luciferase plasmid containing three copies of the consensus CRE motif (pCRE-LUC) or the corresponding plasmid with mutated CRE (pmutCRE-LUC), or with a reporter plasmid containing an 850-bp fragment of the hMK5 promoter with consensus CRE (phMK5-LUC) or mutated CRE (phMK5mutCRE-LUC). Cells were either treated with vehicle or 10 μM forskolin for 3 h and luciferase activity was determined. The results show the average (± SD) of three independent parallels. Similar results were obtained in independent experiments. *p < 0.001, NS is not significant, Student's *t*-test. B – As in (A), but COS-1 cells were transfected. *p < 0.001; NS is not significant, Student's *t*-test. C – PC12 cells were co-transfected with a vector encoding NLS-tagged catalytic subunit (Cα) of PKA or an empty vector (pRcCMV), and the reporter plasmid containing an 850-bp fragment of the hMK5 promoter with consensus CRE (phMK5-LUC) or mutated CRE (phMK5mutCRE-LUC). Luciferase activity was monitored 24 h after transfection, and the results show the average of two independent experiments. Similar results were obtained in independent experiments. The luciferase activity measured in the extracts of cells transfected with the phMK5-LUC construct in the presence of an empty vector was arbitrarily set as 1.0, and the activities in the other cell extracts are shown as fold-induction. *p < 0.005, Student's *t*-test. D – Cells were transfected with either scrambled siRNA or CREB-targeting siRNA and RNA was isolated 24 or 48 h after transfection. Semi-quantitative RT-PCR was run with primers against CREB, MK5 and APRT, and the PCR products were analyzed on an agarose gel. Densitometry was performed and the ratio of the densitometry values of MK5:APRT and CREB:APRT, respectively, were calculated. The ratio of these values for scrambled siRNA was arbitrarily set as 100% and the values for siRNA CREB-treated cells were correlated. E – Knockdown of CREB protein levels had no effect on the MK5 protein levels. Cells were transfected with scrambled or CREB siRNA, and cells were harvested three days after transfection. The expressions of CREB and MK5 protein were monitored by western blotting. The molecular mass (in kDa) of the protein marker is shown. Three parallel wells were performed for each transfection.

Due to the ambiguity of the genomic sequences in the GenBank and possibly also due to the very high GC content of the promoter, we could only clone an 850-bp fragment of the hMK5 promoter encompassing the proximal consensus CRE (see Fig. 3A and 3B). Transient transfection studies with this promoter fragment linked to the luciferase reporter gene were performed in different cell lines, including PC12, SK-N-DZ, COS-1 and A549 cells. The cloned fragment induced transcription of the luciferase gene, but remained refractory to forskolin in all the cell lines tested. Mutation of the putative CRE motif in this MK5 promoter fragment consistently reduced luciferase activity 5- to 10-fold compared to the luciferase activity measured in extracts of cells transfected with the wild-type MK5 promoter fragment (Fig. 8A, B and C, and results not shown). This may indicate that CREB is involved in basal MK5 promoter activity. To test this, we monitored the MK5 transcript in cells in which CREB expression had been depleted by treating cells with siRNA directed against CREB mRNA. Semi-quantitative RT-PCR demonstrated a 70-80% reduction in CREB transcripts 24 and 48 h after the transfection of HEK293 cells with siRNA directed against CREB, while CREB mRNA levels remained unaffected in cells treated with scrambled siRNA. MK5 transcript levels were reduced by almost 50% 48 h after transfection with CREB siRNA, but not with scrambled siRNA (Fig. 8D). Thus, depletion of CREB in non-treated cells reduced the transcription levels of the endogenous *mk5* gene. These results confirm the reporter assay study, i.e. CREB may be involved in basal transcription. Western blot analysis showed a very strong reduction in the amount of CREB protein in PC12 cells 72 h after transfection with siRNA targeting CREB mRNA, but not in cells treated with scrambled siRNA. (Fig. 8E), nor in HEK293 cells from 48 h after transfection (results not shown). However, similar MK5 protein levels were observed between scrambled and CREB siRNA-treated cells (Fig. 8E). Hence, reducing MK5 transcripts by depleting CREB did not correlate with changes in MK5 protein levels.

MK5 protein levels are not increased in FSK-treated cells
Next, we examined the levels of MK5 protein in forskolin-treated cells. No increase in the MK5 levels were observed in forskolin-exposed cells after 2, 4, 6, 8, 24, 48 or 96 h compared to the levels for untreated control cells (Fig. 9). C-FOS protein levels were increased (or "protein level changes were induced") by forskolin (top panel of Fig. 9) and c-*fos* transcripts were also transiently increased by forskolin (results not shown). Moreover, ERK1/2 became transiently phosphorylated under these conditions. All these findings support the concept that forskolin and the cAMP/PKA pathway are functional in these cells. The increase in MK5, CREB and ERK2 levels in vehicle- and forskolin-treated cells at 48 and 96 h was probably due to the increased number of cells due to proliferation.
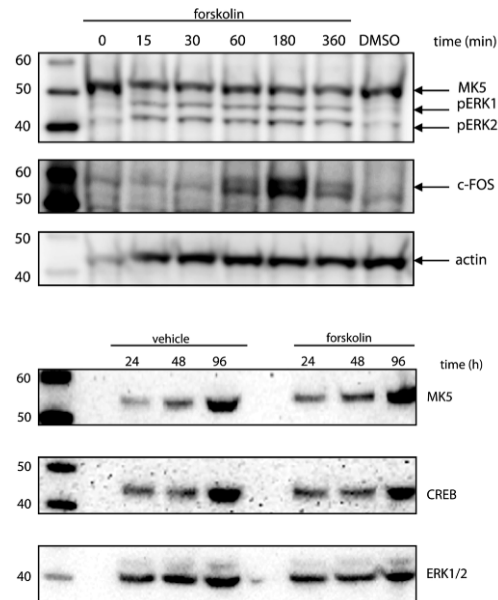
Fig. 9. Stimulation of the PKA signalling pathway by forskolin does not induce MK5 protein levels. PC12 cells were exposed to a vehicle or 10 μM forskolin for the times indicated. The amount of MK5 protein was visualized by western blot. Top panel: MK5 levels and phosphoERK1/2 levels were monitored in the time span of 0 to 360 min. The expression of the cAMP-responsive c-FOS was used as a control. Bottom panel: extended exposure of PC12 cells to forskolin (up to 4 days) did not stimulate MK5 expression. To assure equal sample loading, the blot was stripped and re-probed with antibodies against actin, CREB and ERK2. The molecular mass (in kDa) of the protein marker is shown.

The protein levels of MK5 in PC12 cells remain unchanged after exposure to growth factors, stress, phorbol ester, lipopolysaccharides and retinoic acid
The promoters of the rat, mouse and human *mk5* genes contain binding sites for transcription factors that are targets for several different pathways, including the MKK6/p38 MAPK signalling cascade. This stress-responsive pathway has been the only identified pathway until now that can induce MK5 activity *in vitro* and *in vivo* [21]. This prompted us to study the effect of stimuli such as arsenite, spermine NONOate, LPS, vanadate and KCl on the expression levels of MK5, as all these stimuli can activate the p38 MAPK pathway. In addition, stimuli that target transcription factors with putative binding sites in the MK5 promoters were investigated. These included the phorbol ester TPA, which activates the transcription factors AP1 and AP2 [31, 32], and retinoic acid (RA), which regulates the expression of AP2 and activates CREB in human tracheobronchial epithelial cells, and reduces ERK1 expression levels in squamous cell carcinoma [33-35]. Moreover, growth factors present in the serum may modulate the activity of transcription factors such as AP-1, CREB, GATA-1 and USF [36-39]. As shown in Fig. 10A-E, none of the stimuli tested changed the MK5 protein
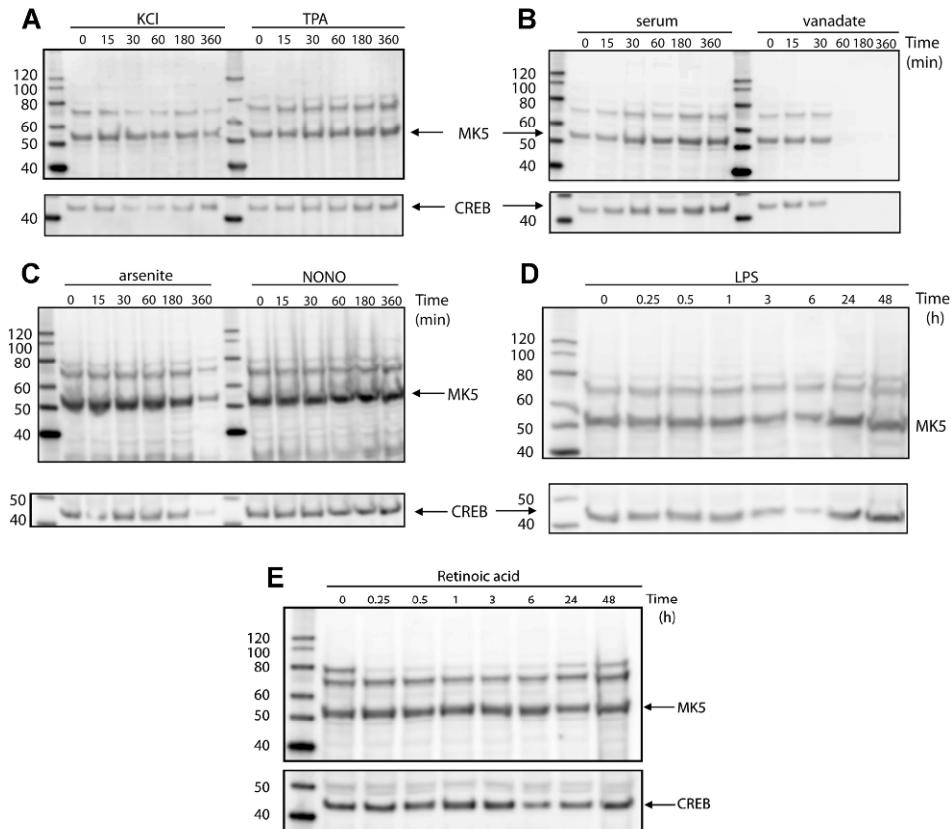
Fig. 10. The levels of the MK5 protein are not affected by the several stimuli tested. PC12 cells were serum-starved for 16 h and then treated with different stimuli for the time indicated. Cell extracts were prepared and the amount of MK5 protein was determined by western blot. Membranes were stripped and re-probed with antibodies against CREB to ensure equal loading of the gels. The position of MK5 (~55 kDa) and CREB (~43 kDa) is indicated by an arrow. The molecular mass of the protein marker (M) is indicated. A – PC12 cells were treated with 60 mM KCl or 10 ng/ml (16 nM) TPA. B – Serum-starved cells were treated with 2.5% foetal bovine serum + 15% horse serum or with 75 μM vanadate. C – Cells treated with 250 μM arsenite or 500 μM spermine NONOate. D – Serum-starved cells treated with 500 ng/ml lipopolysaccharides (LPS). E – Serum-starved PC12 cells exposed to 10 μM retinoic acid (RA).

levels compared to those in the untreated cells. It should be noticed that cells treated with vanadate detached shortly after treatment. Due to the discrepancy in MK5 transcript levels and protein levels after forskolin stimulation, we assayed the amounts of MK5 mRNA in untreated cells and in cells treated with 60 mM KCl or 16 nM TPA (Fig. 11). Because of the peculiar characteristic of the multiple HSF bindings sites in the mouse and rat MK5 promoter, MK5 transcript levels were also examined in cells that were exposed to 44ºC for 20 min, and thereafter incubated at 37ºC. MK5 transcript levels gradually increased for at

least 3 h after the temperature shock (Fig. 11A). A decrease in the levels of MK5 transcripts was measured after prolonged KCl treatment (1 h or longer), but the levels of the housekeeping glyceraldehyde-3-phosphate dehydrogenase (GADPH) gene also diminished at these time points (Fig. 11B). The reduction may result from reduced viability of the treated cells. Although an initial reduction in MK5 mRNA levels was observed 15 min after TPA treatment, the mRNA levels remained similar for the next 3 h (Fig. 11C).
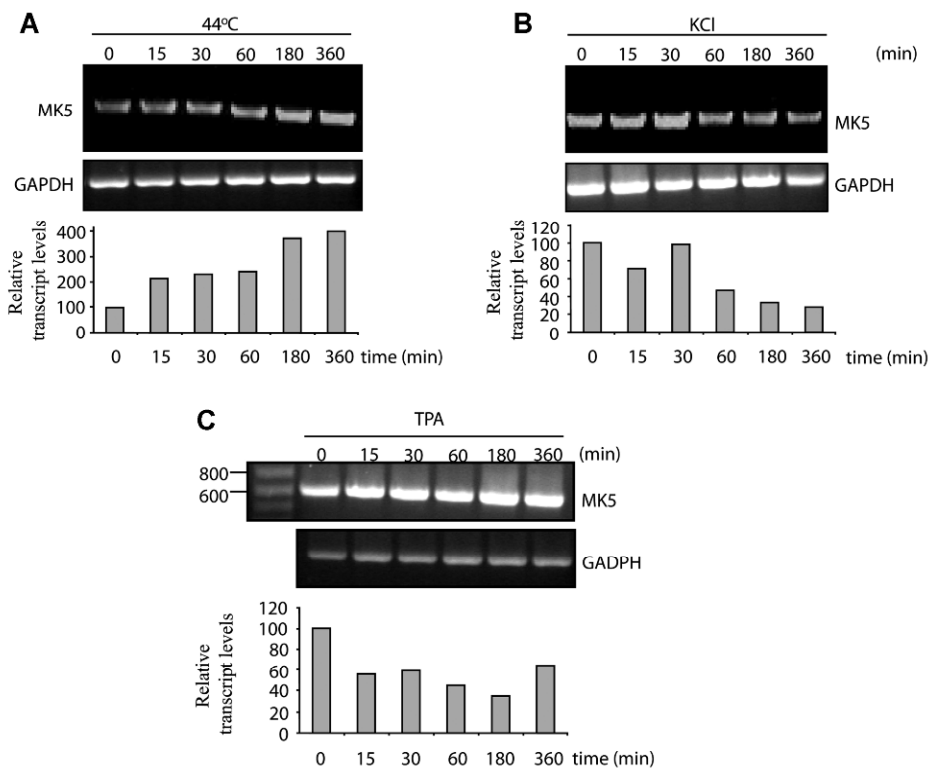

Fig. 11. Temperature shock, but not KCl or TPA, increases MK5 transcript levels. PC12 cells were either exposed to a temperature of 44ºC for 20 min and then moved to 37ºC or treated with KCl (60 mM) or TPA (16 nM). Poly(A)$^+$ RNA was isolated at the time points (in min) indicated and equal amounts of mRNA were converted into cDNA by reverse transcriptase. MK5 and GADPH transcripts were amplified by PCR using specific primers. The PCR products were analyzed by agarose gel electrophoresis and the products were visualized by ethidium bromide staining. Densitometry was performed and the ratio of the densitometry values of MK5:GADPH were calculated. The ratio of the MK5:GADPH value for untreated cells was arbitrarily set as 100% and the other values were correlated to this.

DISCUSSION

The protein levels of most MAPKs seem to be unaffected by extracellular stimuli, but these signals can provoke activation of MAPKs through post-translational phosphorylation [3]. Another mechanism that can control the availability of active MAPKs is protein stability. For example, the atypical MAPK ERK3 appears to be constitutively phosphorylated and active, but it is very unstable and almost undetectable in all the cells examined. During differentiation, ERK3 protein accumulates due to increased stabilization (possibly by MK5), which enables ERK3 to exert its functions. The signals and mechanism responsible for enhanced ERK3 expression during differentiation remain elusive [40, 41]. Finally, cell type-specific and stimulus-induced transcription can also control the availability of certain MAPKs [4-13, 15, 16]. Here, we report that MK5 expression occurs in a spatio-temporal manner. There was incomplete consistency in the expression pattern of MK5 mRNA in the examined tissues as also reported by other researchers. While we failed to observe MK5 mRNA in the liver with our two assays (Clontech and Biochain), Ni *et al.* and New *et al.* detected MK5 expression in this organ [21, 22]. The quality of the RNA, the species investigated, and the detection method may explain the differences. To unequivocally resolve the expression pattern of MK5, other detection methods such as western blotting, *in situ* hybridization, immunohistochemistry and reverse transcriptase-PCR should be used.

Since the MK5 promoter contains a CRE motif, CREB may mediate forskolin-induced stimulation of MK5 transcription. Despite clear increases in the MK5 transcript levels in forskolin-treated cells, we were unable to observe augmented MK5 protein levels. The reason for this remains unknown, but may be attributed to the lack of nuclear export of MK5 mRNA or the prevention of translation of cytoplasmic MK5 mRNA. In immature *Xenopus laevis* oocytes, the mRNA of the MAPK MOS is not translated into protein until testosterone is added. This hormone induces the interaction of the proteins EG2 and cytoplasmic polyadenylation element-binding protein with the 3'-untranslated region of MOS mRNA, which leads to increased polyadenylation of MOS mRNA and a subsequent augmentation in MOS protein levels [10 and references therein]. Whether a similar polyadenylation-dependent mechanism is operational for MK5 transcripts is not known, and we have not yet examined the 3'-region of MK5 transcripts in untreated and forskolin-treated cells.

A microRNA-mediated mechanism may also explain the discrepancy between enhanced MK5 transcript levels and unchanged protein levels after forskolin treatment. Indeed, a CREB-induced microRNA, miRNA132, was recently isolated from PC12 cells. The transcription of the miR132 gene occurred rapidly after neurotrophin-induced CREB activation and persisted for at least 24 h. There exist several additional predicted targets for this miRNA, one being the transcript for the GTPase-activating protein p250GAP [42]. The involvement of such a mechanism may explain our results as well: forskolin induces

transcription of the *mk5* gene and miRNA (e.g. miR132 or another CREB-inducible miRNA). This miRNA will prevent translation of the MK5 mRNA so that no obvious change in the total MK5 protein levels is detected between the untreated and forskolin-treated cells. It is not known whether MK5 may be a putative target of miR132, but stimulation with forskolin for up to 48 h did not induce MK5 protein level changes in PC12 cells. This corresponds well with the reported long-lasting expression of miR132 in PC12 cells after CREB activation [42].

In order to find out whether MK5 transcripts can leave the nucleus, we could monitor the subcellular movements of its mRNA. One method to visualize mRNA movement within a cell relies on the binding of MS2-GFP fusion protein to a specific transcript. This fusion protein consists of the RNA-binding protein of the bacteriophage MS2 and the green fluorescent protein. A reporter plasmid expressing the mRNA of interest has been engineered with several MS2 binding motifs fused to the 3' untranslated region of the mRNA. The MS2-GFP fusion protein will bind to the modified mRNA and the GFP moiety will enable visualization of the mRNA in the cell [43]. Thus, by transfecting cells with an expression plasmid for MS2-GFP and a plasmid encoding MK5 mRNA with MS2 binding motifs, the trafficking of MK5 mRNA can be compared between untreated and stimulus-treated cells.

Another method that allows the detection of transcripts of a specific endogenous gene uses a pair of molecular beacons, one with a donor and the other with an acceptor fluorophore. They hybridize to adjacent regions on the same mRNA target, resulting in fluorescence resonance energy transfer (FRET). Detection of the FRET signal allows the localization of specific endogenous RNAs in living cells [44].

Our studies showed that CREB interacted with the MK5 CRE motif *in vitro*. Chromatin immunoprecipitation studies with PC12, HEK293T and HepG2 cell extracts revealed that CREB is bound to the MK5 promoter *in vivo* [45, 46]. However, forskolin treatment in HEK293T cells did not seem to induce changes in the MK5 transcript levels [46]. A lack of a cAMP response of the MK5 promoter was also found in our transient transfection studies with a reporter plasmid containing an 850-bp fragment of the hMK5 promoter in PC12, SK-N-DZ, COS-1 and A549 cells. One explanation could be that the 850-bp fragment lacks additional elements that are required for forskolin-induced gene transcription. For example, the hMK5 promoter possesses an additional distal CRE motif and an AP2 site. The latter can mediate cAMP-induced transcription [32]. Three copies of the proximal hMK5 CRE (-758/-751, see Fig. 3B) were also unable to make a heterologous promoter cAMP responsive, while three consensus CRE motifs rendered the same promoter cAMP-responsive (e.g. Fig. 9A and 9B). One explanation may be that the sequences adjacent the hMK5 CRE affect the functionality of the CRE, as has been shown for other CRE motifs [29].

A previous study in PC12 cells demonstrated that MK2 mRNA levels were increased approximately 10-fold after KCl treatment, while forskolin triggered a 20-fold increase in MK2 mRNA [16]. The kinetics of forskolin induction was

different from that of the MK5 that we observed. While the MK2 levels increased only 1.5 h after stimulation, peaked after 4 h, and remained elevated for at least 24 h, we found that MK5 mRNA levels were highest after 30 min, and had returned to control levels after 3 h. This difference may be explained by the distinct regulation of the genes and/or by the use of differing concentrations of forskolin. We used 10 μM forskolin, which is sufficient to provoke maximum PKA activation [47], while Vician *et al.* used 50 μM. While our work was under revision, the group of Gaestel reported that 50 μM forskolin and 50 mM KCl increased MK2 transcript and protein levels in PC12 cells in a time-dependent manner (respectively 3- and 6-fold at the protein level). This induction of MK2 expression could be abrogated by the inhibition of PKA. The authors did not examine the cAMP/PKA responsiveness of the MK2 promoter, but they suggested that the presence of a half CRE motif in the MK2 promoter (5'-ACGT-3') could mediate the cAMP response [48].

Finally, the MK5 promoters of the different species contain numerous other regulatory elements (Fig. 3). This led us to test relevant stimuli such as heat shock, TPA, arsenite, spermine NONOate, LPS, vanadate, KCl, arsenite, RA, LPS and serum. None of the stimuli tested had an effect on the MK5 protein levels or RNA levels, except heat shock, which increased the MK5 mRNA levels. The presence of multiple HSF binding sites in the rat promoter is in accordance with the heat shock-inducible transcription of this gene. HSF sites are lacking in the human promoter, so it would be interesting to test the effect of this stimulus on the transcription of the human *mk5* gene.

In conclusion, our studies failed to detect stimulus-induced regulation of MK5 expression at the protein level, despite the plethora of transcription factor binding sites in the promoter and the cell- and developmental-specific expression pattern of the gene. The regulation of MK5 remains unsolved in many aspects because the physiological stimuli that activate this protein kinase remain to be identified. We recently showed that activation of the PKA signalling pathway stimulated the phosphorylation and kinase activity of MK5, and provoked nuclear export of MK5 [49]. Whether other signal pathways control these activities is not known.

REFERENCES

1. Manning, G., Whyte, D.B., Martinez, R. and Hunter, T. The protein kinase complement of the human genome. **Science** <u>298</u> (2002) 1912-1934.
2. Imajo, M., Tsuchiya, Y. and Nishida, E. Regulatory mechanisms and functions of MAP kinase signalling pathways. **IUBMB Life** <u>58</u> (2006) 312-317.
3. Raman, M., Chen, W. and Cobb, M.H. Differential regulation and properties of MAPKs. **Oncogene** <u>26</u> (2007) 3100-3112.
4. Song, F. and Goodman, R.M. OsBIMK1, a rice MAP kinase gene involved in disease resistance responses. **Planta** <u>215</u> (2002) 997-1005.
5. Hong, W.F., He, C., Wang, L., Wang, D.J., Joseph, L.M., Jantasuriyarat, C., Dai, L. and Wang, G.L. *BWMK1* Responds to Multiple Environmental Stresses and Plant Hormones. **J. Integ. Plant Biol.** <u>49</u> (2007) 843-851.
6. Fu, S.F., Chou, W.C., Huang, D.D. and Huang, H.J. Transcriptional regulation of a rice mitogen-activated protein kinase gene, *OsMAPK4*, in response to environmental stresses. **Plant Cell Phys.** <u>43</u> (2002) 958-963.
7. Huang, H.J., Fu, S.F., Tai, Y.H., Chou, W.C. and Huang, D.D. Expression of *Oryza sativa* MAP kinase gene is developmentally regulated and stress-responsive. **Physiol. Plant.** <u>114</u> (2002) 572-580.
8. Ma, S. and Bohnert H.J. Integration of Arabidopsis thaliana stress-related transcript profiles, promoter structures, and cell-specific expression. **Genome Biol.** <u>8</u> (2007) R49.
9. Mizoguchi, Y., Irie, K., Hirayama, T., Hayashida, N., Yamaguchi-Shinozaki, K., Matsumoto, K. and Shinozaki, K. A gene encoding a mitogen-activated protein kinase kinase kinase is induced simultaneously with genes for a mitogen-activated protein kinase and an S6 ribosomal protein kinase by touch, cold, and water stress in *Arabidopsis thaliana*. **Proc. Nat. Acad. Sci. USA** <u>93</u> (1996) 765-769.
10. Rasar, M., DeFranco, D.B. and Hammes, S. R. Paxillin regulates steroid-triggered meiotic resumption in oocytes by enhancing an all-or-none positive feedback kinase loop. **J. Biol. Chem.** <u>281</u> (2006) 39455-39464.
11. Schneider, E.M., Weiss, M., Du, W., Leder, G., Buttenschön, K, Liener, U.C. and Brückner, U.B. MAPkinase gene expression, as determined by microarray analysis, distinguishes uncomplicated from complicated reconstitution after major surgical trauma. **Ann. N.Y. Acad. Sci.** <u>1090</u> (2006) 429-444.
12. Fambrough, D., McClure, K., Kazlauskas, A. and Lander, E.S. Diverse signaling pathways activated by growth factor receptors induce broadly overlapping, rather than independent, sets of genes. **Cell** <u>97</u> (1999) 727-741.
13. Reynolds, L.J. and Richards, R.J. Can toxicogenomics provide information on the bioreactivity of diesel exhaust particles? **Toxicology** <u>165</u> (2001) 145-152.
14. Gaestel, M. MAPKAP kinases - MKs - two's company, three's a crowd. **Nat. Rev. Mol. Cell. Biol.** <u>7</u> (2006) 120-130.

15. Maizels, E.T., Mukherjee, A., Sithanandam, G., Peters, C.A., Cottom, J., Mayo, K.E. and Hunzicker-Dunn, M. Developmental regulation of mitogen-activated protein kinase-activated kinases-2 and -3 (MAPKAPK-2/-3) in vivo during corpus luteum formation in the rat. **Mol. Endocrinol.** 15 (2001) 716-733.

16. Vician, L.J., Xu, G., Liu, W., Feldman, J.D., Machado, H.B. and Herschman, H.R. MAPKAP kinase-2 is a primary response gene induced by depolarization in PC12 cells and in brain. **J. Neurosci. Res.** 78 (2004) 315-328.

17. Travnickova-Bendova, Z., Cermakian, N., Reppert, S.M. and Sassone-Corsi, P. Bimodal regulation of mPeriod promoters by CREB-dependent signaling and CLOCK/BMAL1 activity. **Proc. Nat. Acad. Sci. USA** 99 (2002) 7728-7733.

18. Johannessen, M., Delghandi, M.P., Seternes, O.M., Johansen, B. and Moens, U. Synergistic activation of CREB-mediated transcription by forskolin and phorbol ester requires PKC and depends on the glutamine-rich Q2 transactivation domain. **Cell. Signal.** 16 (2004) 1187-1199.

19. Moens, U., Subramaniam, N., Johansen, B., Johansen, T. and Traavik, T. A steroid hormone response unit in the late leader of the noncoding control region of the human polyomavirus BK confers enhanced host cell permissivity. **J. Virol.** 68 (1994) 2398-2408.

20. Mikalsen, T., Johannessen, M. and Moens, U. Sequence- and position-dependent tagging protects extracellular-regulated kinase 3 protein from 26S proteasome-mediated degradation. **Int. J. Biochem. Cell Biol.** 37 (2005) 2513-2520.

21. New, L., Jiang, Y., Zhao, M., Liu, K., Zhu, W., Flood, L.J., Kato, Y., Parry, G.C.N. and Han, J. PRAK, a novel protein kinase regulated by the p38 MAP kinase. **EMBO J.** 17 (1998) 3372-3384.

22. Ni, H., Wang, X.S., Diener, K. and Yao, Z. MAPKAPK5, a novel mitogen-activated protein kinase (MAPK)-activated protein kinase, is a substrate of the extracellular-regulated kinase (ERK) and p38 kinase. **Biochem. Biophys. Res. Commun.** 243 (1998) 492-496.

23. Natale, D.R., Paliga, A.J.M., Beier, F., D'Souza, S.J.A. and Watson, A.J. p38 MAPK signaling during murine preimplantation development. **Dev. Biol.** 268 (2004) 76-88.

24. Paliga, A.J., Natale, D.R. and Watson, A.J. p38 mitogen-activated protein kinase (MAPK) first regulates filamentous actin at the 8-16-cell stage during preimplantation development. **Biol. Cell** 97 (2005) 629-640.

25. Wingender, E., Chen, X., Hehl, R., Karas, H., Liebich, I., Matys, V., Meinhardt, T., Pruss, M., Reuter, I. and Schacherer, F. TRANSFAC: an integrated system for gene expression regulation. **Nucleic Acids Res.** 28 (2000) 316-319.

26. Ryseck, R.P. and Bravo, R. c-JUN, JUN B, and JUN D differ in their binding affinities to AP-1 and CRE consensus sequences: effect of FOS proteins. **Oncogene** 6 (1991) 533-542.

27. Johannessen, M. and Moens, U. Multisite phosphorylation of the cAMP response element-binding protein (CREB) by a diversity of protein kinases. **Front. Biosci.** 12 (2007) 1814-1832.

28. Fass, D.M., Butler, J.E.F. and Goodman, R.H. Deacetylase activity is required for cAMP activation of a subset of CREB target genes. **J. Biol. Chem.** 278 (2003) 43014-43019.

29. Johannessen, M. and Moens, U. Transcription of genes in response to activated cAMP/protein kinase A signalling pathway: There is more to it than CREB. In: **Trends in Cellular Signalling** (Caplin, D., Ed.). Nova Science Publishers. New York, 2005, 41-78.

30. Ravnskjaer, K., Kester, H., Liu, Y., Zhang, X., Lee, D., Yates, J.R. 3rd and Montminy, M. Cooperative interactions between CBP and TORC2 confer selectivity to CREB target gene expression. **EMBO J.** 26 (2007) 2880-2889.

31. Lee, W., Mitchell, P. and Tjian, R. Purified transcription factor AP-1 interacts with TPA-inducible enhancer elements. **Cell** 49 (1987) 741-752.

32. Imagawa, M., Chiu, R., and Karin, M. Transcription factor AP-2 mediates induction by two different signal-transduction pathways: protein kinase C and cAMP. **Cell** 51 (1987) 251-260.

33. Luscher, B., Mitchell, P.J., Williams, T. and Tjian, R. (1989). Regulation of transcription factor AP-2 by the morphogen retinoic acid and by second messengers. **Genes Dev.** 3 (1989) 1507-1517.

34. Crowe, D.L., Kim, R. and Chandraratna, R.A.S. Retinoic acid differentially regulates cancer cell proliferation via dose-dependent modulation of the mitogen-activated protein kinase pathway. **Mol. Cancer Res.** 1 (2003) 532-540.

35. Kim, S.W., Hong, J.S., Ryu, S.H., Chung, W.C., Yoon, J.H. and Koo, J.S. Regulation of mucin gene expression by CREB via a nonclassical retinoic acid signalling pathway. **Mol. Cell. Biol.** 27 (2007) 6933-6947.

36. De Ruiter, N.D., Wolthuis, R.M.F., van Dam, H., Burgering, B.M.T. and Bos, J.L. Ras-dependent regulation of c-Jun phosphorylation is mediated by the Ral guanine nucleotide exchange factor-Ral pathway. **Mol. Cell. Biol.** 20 (2000) 8480-8488.

37. Kitta, K., Day, R.M., Kim, Y., Torregroza, I., Evans, T. and Suzuki, Y.J. (2002). Hepatocyte growth factor induces GATA-4 phosphorylation and cell survival in cardiac muscle cells. **J. Biol. Chem.** 278 (2002) 4705-4712.

38. Johannessen, M., Delghandi, M.P., and Moens, U. What turns CREB on? **Cell. Signal.** 16 (2004) 1211-1227.

39. Imagawa, S., Fujii, S., Dong, J., Furumoto, T., Kaneko, T., Zaman, T., Satoh, Y., Tsutsui, H. and Sobel, B.E. Hepatocyte growth factor regulates E box-dependent plasminogen activator inhibitor type 1 gene expression in HepG2 liver cells. **Arterioscler. Thromb. Vasc. Biol.** 26 (2006) 2407-2413.

40. Coulombe, P., Rodier, G., Pelletier, S., Pellerin, J. and Meloche, S. Rapid turnover of extracellular signal-regulated kinase 3 by the ubiquitin-proteasome pathway defines a novel paradigm of mitogen-activated protein

kinase regulation during cellular differentiation. **Mol. Cell. Biol.** 23 (2003) 4542-4558.

41. Seternes, O.M., Mikalsen, T., Johansen, B., Michaelsen, E., Armstrong, C.G., Morrice, N.A., Turgeon, B., Meloche, S., Moens, U. and Keyse S.M. Activation of MK5/PRAK by the atypical MAP kinase ERK3 defines a novel signal transduction pathway. **EMBO J.** 23 (2004) 4780-4791.

42. Vo, N., Klein, M.E., Varlamova, O., Keller, D.M., Yamamoto, T., Goodman, R.H. and Impey, S. A cAMP-response element binding protein-induced microRNA regulates neuronal morphogenesis. **Proc. Nat. Acad. Sci. USA** 102 (2005) 16426-16431.

43. Fusco, D., Accornero, N., Lavole, B., Shenoy, S.M., Blanchard, J.M., Singer, R.H. and Bertrand, E. Single mRNA molecules demonstrate probabilistic movement in living mammalian cells. **Curr. Biol.** 13 (2003) 161-167.

44. Santangelo, P.J., Nix, B., Tsourkas, A. and Bao, G. Dual FRET molecular beacons for mRNA detection in living cells. **Nucleic Acids Res.** 32 (2004) e57.

45. Impey, S., McCorkle, S.R., Cha-Molstad, H., Dwyer, J.M., Yochum, G.S., Boss, J.M., McWeeney, S., Dunn, J.J., Mandel, G., and Goodman, R.H. Defining the CREB regulon: a genome-wide analysis of transcription factor regulatory regions. **Cell** 119 (2004) 1041-1054.

46. Zhang, X., Odom, D.T., Koo, S.H., Conkright, M.D., Canettieri, G., Best, J., Chen, H., Jenner, R., Herbolsheimer, E., Jacobsen, E., Kadam, S., Ecker, J.R., Emerson, B., Hogenesch, J.B., Unterman, T., Young, R.A. and Montminy, M. Genome-wide analysis of cAMP-response element binding protein occupancy, phosphorylation, and target gene activation in human tissues. **Proc. Nat. Acad. Sci. USA** 102 (2005) 4459-4464.

47. Montminy, M. Transcriptional regulation by cyclic AMP. **Annu. Rev. Biochem.** 66 (1997) 807-822.

48. Thomas, T., Hitti, E., Kotlyarov, A., Potschka, H., and Gaestel, M. (2008). **Eur. J. Neurosci.** 28 (2008) 642-654.

49. Gerits, N., Mikalsen, T., Kostenko, S., Shiryaev, A., Johannessen, M. and Moens, U. Modulation of F-actin rearrangement by the cyclic AMP/PKA pathway is mediated by MAPKAP Kinase 5 and requires PKA-induced nuclear export of MK5. **J. Biol. Chem.** 282 (2007) 37232-37243.

50. Benson, D.A., Karsch-Mizrachi, I., Lipman, D.J., Ostell, J. and Wheeler, D.L. GenBank. **Nucleic Acids Res.** 35 (2007) D21-25.