Stimulated initiation of mitogen-activated protein kinase phosphatase-1 (MKP-1) gene transcription involves the synergistic action of multiple *cis*-acting elements in the proximal promoter

Stephan RYSER, Abbas MASSIHA, Isabelle PIUZ and Werner SCHLEGEL[1]

Fondation pour Recherche Médicales, University of Geneva, 64 av. de la Roseraie, 1211 Geneva, Switzerland

Mitogen-activated protein kinases (MAPKs) are inactivated by a dual specificity phosphatase, MAPK phosphatase-1 (MKP-1). MKP-1 is transcribed as an immediate early response gene (IEG) following various stimuli. In the pituitary cell line GH4C1, MKP-1 gene transcription is strongly induced by thyrotropin-releasing hormone (TRH) as well as by epidermal growth factor (EGF) as a consequence of activated MAPK/extracellular-signal-regulated kinase (ERK) signalling. Intriguingly, reporter gene analysis with the MKP-1 promoter showed strong basal transcription, but only limited induction by TRH and EGF. Site-directed mutagenesis of the reporter construct combined with band-shift and *in vivo* studies revealed that part of the constitutive activity of the MKP-1 promoter resides in two GC boxes bound by Sp1 and Sp3 transcription factors in the minimal promoter. Basal transcription of transiently transfected luciferase reporter can be initiated by either of the two GC boxes or also by either of the two cAMP/Ca^{2+} responsive elements or by the E-box present in the proximal promoter. On the other hand, when analysed by stable transfection, the five responsive elements are acting in synergy to transactivate the MKP-1 proximal promoter. We show in this study that the MKP-1 promoter can function as a constitutive promoter or as a rapid and transient sensor for the activation state of MAPKs/ERKs. This dual mode of transcription initiation may have different consequences for the control of a block to elongation situated in the first exon of the MKP-1 gene, as described previously [Ryser, Tortola, van Haasteren, Muda, Li and Schlegel (2001) J. Biol. Chem. **276**, 33319–33327].

Key words: cAMP/Ca^{2+}-responsive-element-binding protein (CREB), CREB-binding protein (CBP), elongation, extracellular-signal-regulated kinase (ERK), initiation, synergy, Sp3, transcription.

INTRODUCTION

Signal transduction networks allow the cell to relay, amplify and integrate several extracellular stimuli to induce an appropriate biological response. In eukaryotes, the mitogen-activated protein kinase (MAPK) pathways regulate gene expression and control many cytoplasmic activities. The duration, magnitude and cellular localization of MAPK activation are important for the selective control of long-term adaptive processes. Fine-tuning of MAPK signalling results from regulatory input at many points along its activation cascade, as well as from selective inactivation mechanisms. Activation of MAPK may be achieved by triggering membrane receptors, recruitment of specific 'adaptor/scaffold' proteins and/or cross-talk with other signalling pathways [1,2]. MAPKs may be inactivated by several classes of protein phosphatases which are either serine/threonine and/or tyrosine specific [3]. The dual specificity (threonine/tyrosine) MAPK phosphatases (MKPs) play a major role in regulating the activity of MAPKs [4,5]. MKPs are proposed to regulate MAPKs acutely following stimulation, whereas other phosphatases, mainly cytoplasmic and often constitutive, control the basal activity of MAPK [3].

The family of mammalian MKPs comprises eleven members, some cytoplasmic, others nuclear, with different kinetics of expression [5]. The first enzyme identified, MKP-1, is encoded for by an immediate early response gene (IEG) that can be induced by multiple stimuli involving different signalling pathways [6–13]. MKP-1 is regulated at the gene transcription level but also at the protein level. MAPKs can increase MKP-1 protein activity in two distinct ways. First, by subtype-specific enhancement of its catalytic activity triggered by the direct interaction with MAPK [14,15] and, secondly, by phosphorylation by MAPK which increases the half-life of the protein [16]. These sophisticated mechanisms, combined with different mechanisms of transcriptional activation, result in tight regulation of MKP-1 levels and activity, which may contribute importantly to controlling MAPK activities in a dose- and time-dependent manner.

A report by Bhalla et al. [17] confirms the importance of MKP protein levels for the thermodynamic behaviour of a MAPK signalling network. Computational simulations, based on a set of experimentally validated parameters, indeed predict that at a low level of MKP protein the MAPK network is bistable, displaying an active positive feedback loop, whereas at high levels of MKP the signalling network is monostable. In the latter situation, MAPK activity may oscillate, whereas in the former case, the signalling cascade is either on or off. This suggests that a well-controlled MKP-1 gene expression may determine the behaviour of a MAPK signalling network and further downstream responses depending upon monostable or bistable MAPK signalling.

Such quantitatively precise and timed MKP-1 gene expression could be coupled directly to the strength of the upstream MAPK signalling pathway in a mechanism similar to that proposed by Hazzalin and Mahadevan [18] in their model for quantitative gene

Abbreviations used: CBP, CREB-binding protein; ChIP, chromatin immunoprecipitation; CRE, cAMP/Ca^{2+}-responsive element; CREB, CRE-binding protein; CREM, cAMP responsive element modulator; CTD, C-terminal domain; DMS, dimethyl sulphate; EGF, epidermal growth factor; EMSA, electrophoretic mobility shift assay; ERK, extracellular-signal-regulated kinase; FAM, 6-carboxyfluorescein; IEG; immediate early response gene; MEK, MAPK/ERK kinase; LM-PCR, ligation-mediated PCR; PCAF, p300/CBP-associated factor; pol II, polymerase II; TAF_{II}s, TATA box-associated factors; TRH, thyrotropin-releasing hormone; USF, upstream stimulatory factor; SFM, Ham's F10 serum-free medium; MAPK, mitogen-activated protein kinase; MKP, MAPK phosphatase; PK, protein kinase; RT, reverse transcriptase; TAMRA, 6-carboxytetramethylrhodamine; wt, wild-type.

[1] To whom correspondence should be addressed (e-mail werner.schlegel@medecine.unige.ch).

Table 1 Sequence of the oligonucleotides used for site-directed mutagenesis of −203Luc and −79Luc constructs and EMSA experiments

The sequences of the upper strands from 5′ and 3′ only are shown here for the oligonucleotides used. Site-directed mutagenesis of the E-box element in −203Luc construct, previously mutated at position −126 CRE, was performed with the oligos Δ −126CRE/−108E-box. Mutated oligonucleotides are underlined. wt, wild type; M, mutant; GC1, GC box 1; GC3, GC box 3.

Oligonucleotides (sense)	Promoter region	Sequence
Δ −173CRE	−193 to −149	CGACCCGCTCCCAGGCGGACTGCAGCCACGCTTTTGGCTTTGGTG
Δ −126CRE	−147 to −101	CGCCAAGCCCGGCAGGATCGGCTGCAGCCTCACCCGGTCACGTGTCC
Δ −108Ebox	−128 to −86	GGTGACGTCACCACCCCTCAGGCCTTTCGCCATTCAAACAAAC
Δ −26TATA	−44 to −7	GCCCCCAAGAGGCCAGAATTCTCCGCGCTCCTTCGAAC
Sp1 consensus wt*		ATTCGATCGGGGCGGGGCGAGC
GC1 wt*	−61 to −29	GTGCCGCGCCCGGGACCGCCCCCAAGAGGCCAC
ΔGC1 = M1	−61 to −29	GTGCCGCGCCCGTAACAACACCCAAGAGGCCAC
GC3 wt*	−90 to −55	CAAACAAACCGTTCCCCCCCCCTCCTCCTGTGCCGC
ΔGC3 = M3	−90 to −55	CAAACAAACCGTTCACCAACACTCCTCCTGTGCCGC
AP1		GTCATAGTAAGACTTAGTGACCCTTCCCG
AP2		CTCCGGAGCAGCGCCTGCGTCAGCGCAGAC
Δ −126CRE/−108USF	−127 to −82	GCTGCAGCCTCACCCCTCAGGCCTTTCGCCATTCAAACAAACAAAC

* End-labelled oligonucleotide probes used in EMSA.

regulation of IEGs by MAPKs. Indeed, tight control of MKP-1 gene expression involves multiple regulated events directly associated with initiation and elongation of MKP-1 transcription, as well as the remodelling of chromatin [7,19–23]. These events have not so far been fully characterized at the molecular level, leaving open important questions, notably with regard to the link between initiation and elongation of transcription, and the signalling components which may link these processes.

We have shown previously [19,20] strong induction of the MKP-1 gene in neuroendocrine GH4C1 cells by either thyrotropin-releasing hormone (TRH) or epidermal growth factor (EGF). Both of these stimuli lead to a marked increase of MKP-1 mRNA within minutes. We showed that calcium signals triggered by TRH were important to release a block to transcriptional elongation found in the first exon of the MKP-1 gene. It appeared that MKP-1 expression was indeed controlled during elongation, whereas initiation of transcription was high already under basal conditions.

To understand the relationship between initiation and elongation of MKP-1 transcription, we now characterize further elements in the MKP-1 promoter required to initiate MPK-1 transcription in resting and stimulated conditions. We show in this study, that in nonstimulated conditions transcriptional initiation is controlled by multiple *cis*-acting elements involving two cAMP/Ca^{2+}-responsive elements (CREs), one E box and two GC boxes, but not the TATA-box-like sequence. We confirmed by *in vitro* and *in vivo* studies that one GC box is associate with Sp1/Sp3 transcription factors in basal and TRH-stimulated cells. The proximal promoter drives strong basal MKP-1 reporter luciferase gene expression in transient transfection. In these experiments, any of the five *cis*-acting elements can drive transcription, even when all the others are inactivated by mutation. In cells stably transfected with MKP-1 reporter and stimulated by TRH, synergistic modulation of transcription by the same elements can be observed. Based on these results and on previous results [19,20], we propose a model of transcriptional regulation for the MKP-1 gene, suggesting mechanisms by which MAPKs/extracellular-signal-regulated kinases (ERKs) may couple constitutive initiation to elongation of transcription.

MATERIALS AND METHODS

Materials, cell culture and stimulation

TRH (Roche) and EGF (Sigma) were diluted in water at 27.6 mM and stored in aliquots at −80 °C. Geneticin (G418; Invitrogen, Groningen, The Netherlands) was diluted in water at 100 mg/ml (stock solution). U0126 MAPK/ERK kinase (MEK) inhibitor (Promega, Catalys AG, Wallisellen, Switzerland) and nifedipine (Sigma) stock solution were prepared in DMSO at a concentration of 10 mM and 1 mM respectively. GH4C1 pituitary cells were maintained in Ham's F10 medium (Life Technologies, Basel, Switzerland) supplemented with 2.5 % fetal bovine serum and 15 % horse serum at 37 °C in a humidified atmosphere with 5 % CO_2. Confluent GH4C1 cells were incubated in Ham's F10 serum-free medium (SFM) containing 5 μg/litre transferrin for 24 h and then stimulated for the indicated times with either 100 nM TRH or 10 nM EGF.

Reporter gene assays

GH4C1 cells maintained in culture medium were detached from Petri dishes with trypsin, transiently transfected using FuGENE 6 transfection reagent (Roche) and seeded in 24-well multi-dishes (Falcon, Lincoln Park, NJ, U.S.A.) at a density of 10^5 cells/well. After 24 h the medium was replaced with SFM, 24 h before exposure to the various stimuli. Cells were stimulated with TRH and EGF prediluted into SFM. Stimulation was performed for 3 h at 37 °C and stopped by removal of the medium. Firefly luciferase activity was determined using a luciferase reporter assay system and following the manufacturer's instructions (Promega). Cells were cotransfected with a *Renilla* luciferase expression plasmid to verify uniformity of transfection from experiment-to-experiment. MKP-1 −631Luc, pSV40 and c-*fos*/intron constructions have been described previously [19,24]. Plasmids −434Luc, −203Luc and −79Luc were generated by cloning the region of the rat MKP-1 gene extending from −434, −203 or −79 to +19 between the *Kpn*I and *Nhe*I restriction sites of the pGL3 enhancer vector (Promega). Site-directed mutagenesis of the −203Luc and −79Luc constructs was performed with the Quick-Change mutagenesis kit (Stratagene, Amsterdam, The Netherlands). The list of oligonucleotides used for the mutagenesis and the corresponding sequence are shown in Table 1. The sequences of the mutated clones were checked using the SBI dye terminator method (PerkinElmer, Courtaboeuf, France).

In vivo genomic footprinting

Genomic DNA methylation by dimethyl sulphate (DMS; Fluka, Buchs, Switzerland) as well as piperidine (Fluka) cleavage was performed as described previously [25]. Guanine ladders were

obtained from 200 μg *in vivo* methylated, genomic DNA isolated from basal or TRH-stimulated GH4C1. *In vivo* genomic footprinting was done by ligation-mediated PCR (LM-PCR) as described previously [26] using Vent DNA polymerase (New England Biolabs, Schwalbach, Germany) for all steps. The rat MKP-1 promoter was analysed between the region -1 to -130 using primer set A for the noncoding strand and primer set B for the coding stand (see Figure 3A).

Electrophoretic mobility shift assay (EMSA) and supershift

Nuclear extracts were prepared from GH4C1 pituitary cells according to the method of Andrews and Faller [27]. For the EMSA two double-stranded MKP-1 promoter oligonucleotides comprising sequences between -61 to -29 (GC box 1) and -90 to -55 (GC box 3) and the consensus Sp1 sequence (Promega) were used as a probes. Sequences and regions of the corresponding wild-type (wt), mutant and nonspecific oligonucleotides are indicated in Table 1. Oligonucleotide end-labelling, binding reaction, gel preparation and migration was performed based on Promega guidelines for gel-shift assays. Briefly, EMSA analysis was performed with 10 μg of GH4C1 nuclear extract from unstimulated cells. In the competition assays, a 100-fold excess of unlabelled probes were added in the nuclear extract prior to the addition of the [γ-^{32}P] end-labelled consensus Sp1, GC boxes 1 and 3 probes. Binding reaction were performed at 37 °C for 20 min. The DNA–protein interaction was resolved on a 5 % native polyacrylamide gel, and the dried gel was exposed to X-Omat film (Kodak, Rochester, NY, U.S.A.). Film exposure for the consensus Sp1 and GC box 1 EMSA gels was 16 h at -80 °C, whereas the GC box 3 EMSA gel was exposed for 3 days at -80 °C. Unlabelled double-stranded competitor DNA was added 5 min prior to the probe. For supershift EMSA, the [γ-^{32}P] end-labelled probe was incubated with 10 μg of nuclear extract at 37 °C for 20 min then antibodies against Sp1 and/or Sp3 (1 μg/sample) were added and incubated for another 15 min on ice. Rabbit anti-Sp1 and anti-Sp3 polyclonal antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). Horseradish peroxidase-conjugated goat anti-rabbit IgG (Transduction Laboratories, Lexington, KY, U.S.A.) was included as a negative control.

RNA preparation and quantitative reverse transcriptase (RT)-PCR

Total RNA was extracted from cells with an acid phenol/guanidinium reagent (TRI-Reagent; Molecular Research Center, Cincinnati, OH, U.S.A.) according to the manufacturer's instructions. Quantification of MKP-1 mRNA levels shown in Figure 5 was analysed by real time RT-PCR (PerkinElmer) from three series of GH4C1 cells stimulated with either TRH or EGF in the presence of inhibitors. Three independent replica RNA samples (diluted to 10 ng/μl) were analysed and a standard curve was included for each plate. MKP-1 and 18 S RT-PCR, primers, Taqman probes, standard curves and quantification procedures have been described previously [19]. Quantification of the MKP-1 and luciferase mRNA levels in Figure 6(B) was performed by real time RT-PCR (PerkinElmer) from the total RNAs extracted from three series of the cellular clone 631C9 (originating from GH4C1 cells) and cultured in TRH- or EGF-stimulated conditions. The luciferase amplification was performed for each RT sample with 200 nM Luc forward primer 5′-TGACCGCCTGAAGTCTCTGA-3′, 200 nM Luc reverse primer 5′-TGGAGCAAGATGGATTCCAAT-3′, and 250 nM Luc TaqMan oligonucleotide probe, with 6-carboxyfluorescein (FAM) as a label and 6-carboxytetramethylrhodamine (TAMRA) as a quencher at the 5′ and 3′ end respectively, 5′-(FAM)-CAGCGGGAGCCACCTGATAGCCT-(TAMRA)-3′.

Western-blot analysis

GH4C1 cells were washed with PBS, pelleted and lysed in a buffer containing 20 mM Tris/HCl, pH 8, 137 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 1 % Triton X, 10 % glycerol, 50 mM NaF, 1 mM Na_3V0_4, 100 μM PMSF, 20 μM leupeptin, 1 mM aprotinin. Protein extracts (20 μg) were solubilized in sample buffer (125 mM Tris/HCl, pH 6.8, 4 % SDS, 0.2 % Bromophenol Blue, 10 % 2-mercaptoethanol), boiled for 5 min and resolved by SDS/PAGE (10 % gel). Transfer and immunoblotting of the membranes were performed as described previously [20] and according to the instructions supplied by Cell Signaling Technology (Beverly, MA, U.S.A.). The blots were first analysed with the phospho-p44/42 MAPK antibody (Cell Signaling Technology) then stripped and reprobed with the p44/42 MAPK antibody (Cell Signalling Technology). The primary antibodies were detected with a horseradish peroxidase-conjugated goat anti-rabbit IgG (Transduction Laboratories) and the enhanced chemoluminescence (ECL®) kit (Amersham Biosciences Europe, Sarclay, France).

Chromatin immunoprecipitation (ChIP)

ChIP assays were performed as described previously [28,29] and adapted for our purposes. Briefly, formaldehyde-crosslinked chromatin extract was prepared from 5×10^7 cells and fragmented with a Branson sonifier 250. With an output control of 50 % and 20 s hold/cycle, 10 cycles of sonication/sample were performed to generate small chromatin fragments (DNA < 1 kb). The amount of chromatin was estimated based on the DNA concentration measured by absorbance at 260 nm. Chromatin extracts (50 μg) were precleared with Protein A–Sepharose beads (Pharmacia) for 1 h and then incubated with 10 μg of antibody at 4 °C overnight. Anti-CREB1 (CRE-binding protein; ab7540) was purchased from Abcam Limited (Cambridge, U.K.), anti-Sp1 (sc $-$ 59), anti-Sp3 (sc $-$ 644) and anti-CBP (CREB-binding protein; sc $-$ 369) were purchased from Santa Cruz Biotechnologies, and anti-pol II (polymerase II; 8WG16) from Convance, Berkeley, CA, U.S.A. Protein A–Sepharose beads were then added to the mixture for 3 h at 4 °C and the immunoprecipitated DNA–protein complexes were isolated from the beads after several washing steps. Reversal of the cross-linking of chromatin was performed overnight at 65 °C in the presence of proteinase K and 10 % SDS. After phenol extraction the immunoprecipitated DNA was precipitated, dissolved in water and used as a template for semiquantitative PCR. PCR (31 cycles) was performed in 25 μl with 2.5 μl of immunoprecipitated material, 25 pmol of each primer set, and a *Taq* DNA polymerase PCR master mix (Promega). The following primer sets were used for the analysis of the MKP-1 proximal promoter, the coding strand primer was 5′-CGGAGCCAGCGCTCAAAG-3′ and primer A3 was used for the non-coding strand (see Figure 3A). For the analysis of a distal region from the promoter, primers were selected for the exon 4 sequences within the gene. The coding strand primer 5′-GAGCCAGTTTCTTCCCTTGC-3′ and the non-coding strand primer 5′-CAACACTGGCTTCGTCCATC-3′. PCR products were electrophoresed on 1.5 % agarose gels with a SYBR® Green loading dye (Molecular Probes, Eugene, OR, U.S.A.) and analysed with a fluorescent trans-illuminator adapted for DNA quantification (Raytest, Fujifilm). Each experiment was performed at least three times, and representative data are shown.

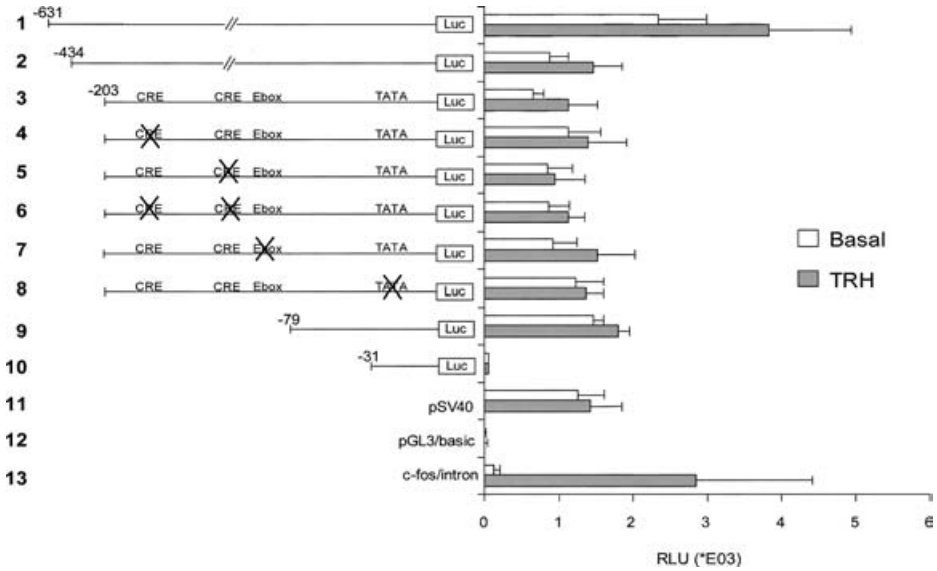

Figure 1 Identification of a GC-rich region in the minimal promoter of MKP-1 required for basal MKP-1 promoter activity

Transient-transfection reporter-gene assays were performed with different MKP-1 promoter–luciferase constructs in GH4C1 cells, resting (open bars) or stimulated for 3 h with 100 nM TRH (closed bars). The different constructs based on various lengths of the MKP-1 promoter, containing either wt or mutated (×) CRE, E-box and TATA-like sites are schematically represented on the left-hand side. Chemiluminescence analysis was performed for firefly and *Renilla* luciferase activities and the normalized luciferase activity was calculated. Shown are the relative light unit (RLUs) obtained for the different constructs in basal and TRH-stimulated cells (means ± S.E., $n = 3$). Similar results were obtained with EGF (results not shown). Generation and site-directed mutagenesis of these constructs are described in the Materials and methods section. The list of oligonucleotides used for the mutagenesis is presented in Table 1. Control vectors shown here were described previously in [19,24].

RESULTS

A GC-rich region in the promoter of MKP-1 drives basal MKP-1 reporter gene expression

GH4C1 cells transiently transfected with a rat MKP-1 (−631 to +19) luciferase reporter plasmid, display (under basal conditions) a strong luciferase activity, similar to levels obtained with a constitutive reporter gene driven by a viral promoter [19]. To determine which *cis*-acting elements are controlling basal and TRH-stimulated MKP-1 gene transcription we shortened and/or mutated the MKP-1 promoter. Although deletion of distal parts of the promoter reduced its activity, a much shorter promoter (−203 to +19) maintained a strong basal luciferase expression (Figure 1, lines 1 to 3). Hence, promoter-proximal elements are sufficient in isolation to initiate MKP-1 gene transcription. Within this proximal part, two CREs and one E box element, bound respectively by the factors CREB and USF (upstream stimulatory factor), were suggested to control the transcriptional activity [21,23]. Single inactivation by site-directed mutagenesis of any one of these three elements resulted in little or no effect on transcription. Even when the two CREs were mutated simultaneously, transcription of the reporter was maintained. The results demonstrate that neither the CREs at position −173/−126 nor the E-box at position −108 are necessary or sufficient to maintain the activity of the reporter gene (Figure 1, lines 4 to 7). Moreover, mutation of the TATA-box-like sequence did not abolish the transcription of the reporter gene, indicating that the rat MKP-1 promoter functions like a TATA-less promoter (Figure 1, line 8). To map the minimal promoter region of the MKP-1 gene required for initiating its gene transcription, we made deletions in the short promoter, −203. A construct starting from −79 to +19 was still able to maintain strong basal and TRH-stimulated activity (Figure 1, line 9). When the region between −79 to −31 was deleted, transcription of the reporter gene fell to the background level observed with the basic pGL3 vector (Figure 1, line 10). An SV40 promoter reporter gene (pSV40) and a c-*fos*/intron construct were used as a control for constitutive and TRH-stimulated gene transcription [19,24]. The c-*fos* construct was strongly induced by TRH, whereas for the MKP-1 reporter gene, TRH induction was limited to a maximal 1.2–1.5-fold increase at best. Similar results were obtained when stimulating cells with EGF (results not shown). Taken together, these data suggest that important *cis*-regulatory elements responsible for the elevated basal promoter activity are located in the region between −79 and −31, just upstream of the site of transcription initiation. This region contains three GC boxes, which are known to be potent binding sites for Sp1/3 transcription factors (Figure 2A). On the other hand, the distal region of the promoter (−631 to −434) contributes significantly to the high basal level of luciferase activity. This region contains several putative *cis*-acting elements for Sp1/Sp3, SAP-1, RREB-1, E2F and AP1 transcription factors that may further cooperate with the proximal-promoter elements defined here between −203 to +19.

In vivo genomic footprinting of the proximal MKP-1 promoter

To determine whether the three putative GC boxes in the region between −79 and −31 are recognized by specific transcription factors *in vivo*, we analysed this genomic region by methylation interference assays using LM-PCR (Figure 2). Resting and TRH-stimulated (15 min or 60 min) cells were exposed to DMS, which methylates guanosine residues and to a lesser extent adenosine residues. Genomic DNA was then extracted, cleaved by treatment with piperidine, and amplified by LM-PCR using specific primers complementary to sequences flanking the short rat MKP-1 promoter, as detailed in Figure 2(A). A G-specific sequence ladder was generated as a reference using essentially the same approach. Footprinting primers were initially designed to analyse *in vivo* protein–DNA interactions occurring in the −1 to −130 region of the MKP-1 promoter. Primers A1, A2 and A3 were able to detect

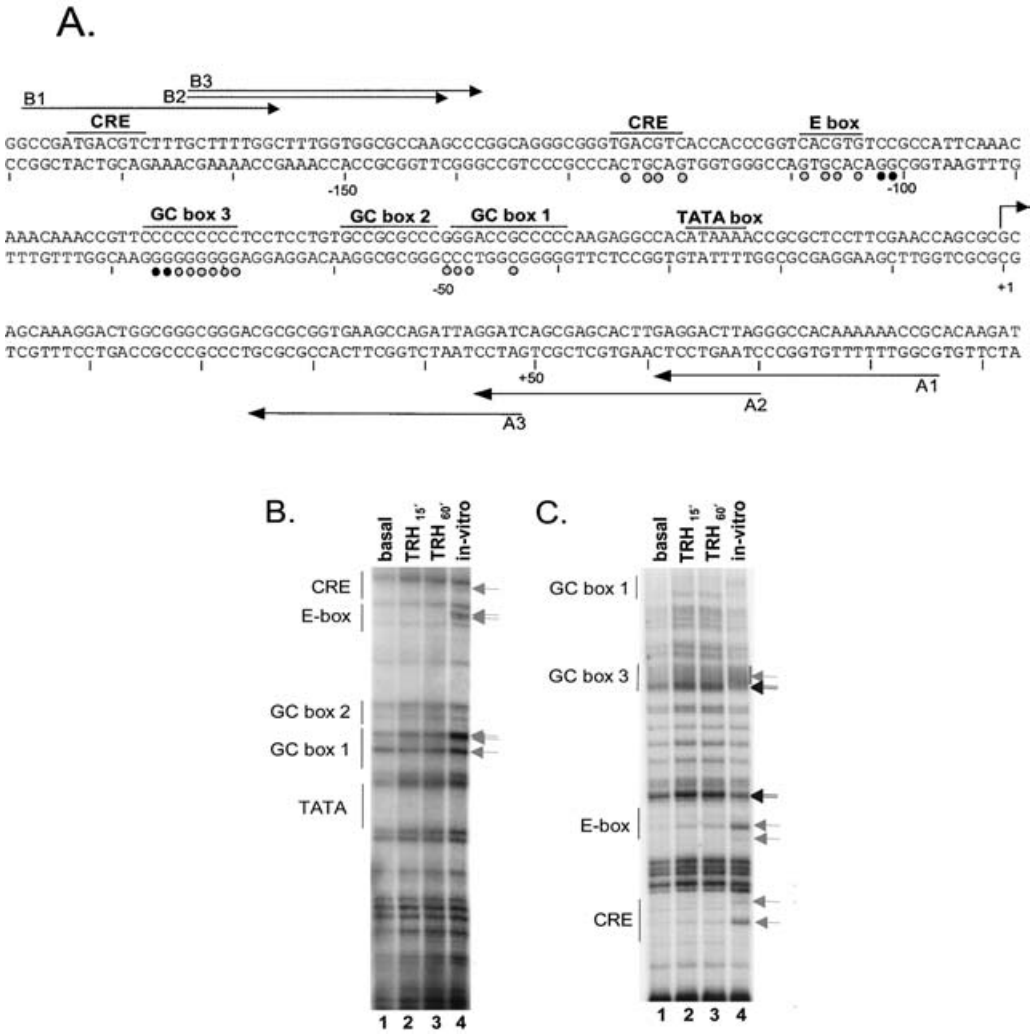

Figure 2 ***In vivo*** **genomic footprinting of the proximal MKP-1 promoter in GH4C1 cells**

(**A**) Sequence of the minimal MKP-1 promoter. Consensus DNA binding motifs CREs, E-box, GC boxes and the TATA box are shown in the sequence. Positions along the sequences are indicated based on the transcription start site (angled arrow; position +1). Arrows correspond to primers used in genomic footprinting. Primers A1, A2 and A3 were designed to characterize the coding strand; primers B1, B2 and B3 were used for the non-coding strand. DMS-protected guanines observed in *in vivo* footprinting are indicated by grey circles, whereas black circles represent DMS-hypersensitive guanines. (**B**) Coding-strand analysis and, (**C**) non-coding-strand analysis. Cells were either in resting condition (lanes 1) or stimulated with TRH for 15 min (lanes 2) or 60 min (lanes 3) and then treated with DMS. Naked DNA was treated *in vitro* with DMS (lanes 4). Genomic DNA was extracted and treated with piperidine. All DNA samples were amplified by LM-PCR and visualized on a sequencing gel. DMS-protected sites are indicated with grey arrows and DMS-hypersensitive sites with black arrows.

occupancy on GC boxes 1 and 2 in the coding strand whereas the primers B1, B2 and B3 were probing for occupancy of the GC box 3 where all the guanines are present in the non-coding strand.

In basal and TRH-stimulated GH4C1 cells (Figure 2B, lines 1, 2 and 3), cleavage of G residues −44 to −50 in GC box 1 was reduced compared with the G ladder (Figure 2B, lines 4), indicating the presence of a DNA-binding protein on this site. No such effect was observed in the sequence corresponding to GC box 2. Interestingly, the E-box and the −126 CRE in the proximal promoter were strongly protected by protein–DNA interactions both in basal and TRH-stimulated conditions (Figures 2B and 2C). Cleavage was efficiently reduced in the G residues −120 to −125 for the CRE and the G residues −104 to −109 in the E-box. The two guanosines at −101 and −102 flanking the E-box were hypersensitive to DMS. Finally, analysis of the GC box 3 in the non-coding strand revealed two major hypersensitive Gs (−75 and −76) but also reduced cleavage at the G residues −69 to −74, indicating the presence of a DNA binding protein on this site. Quantification by densitometry and normalizing each lanes to G residues −81, −82 and −86, which are presumably cleaved at the same efficiency, confirmed that the G residues −69 to −74 in GC box 3 were both protected in non-stimulated and TRH-stimulated conditions (results not shown).

Taken together, these experiments suggest that two major GC boxes in the proximal promoter of MKP-1 are bound *in vivo* by specific DNA binding proteins. Candidates for this interaction are Sp1/3 transcription factors, which are relatively specific for GC-rich sequences; basal MKP-1 gene transcription might be controlled by the Sp1/3 class of transcription factors.

Sp1 and Sp3 transcription factors bind the GC box 1 but not the GC box 3 in the minimal promoter of MKP-1

To identify nuclear proteins interacting with the GC box motifs in the promoter of the rat MKP-1 gene, EMSAs were performed using two probes spanning the sequences of GC box 1 and 3,

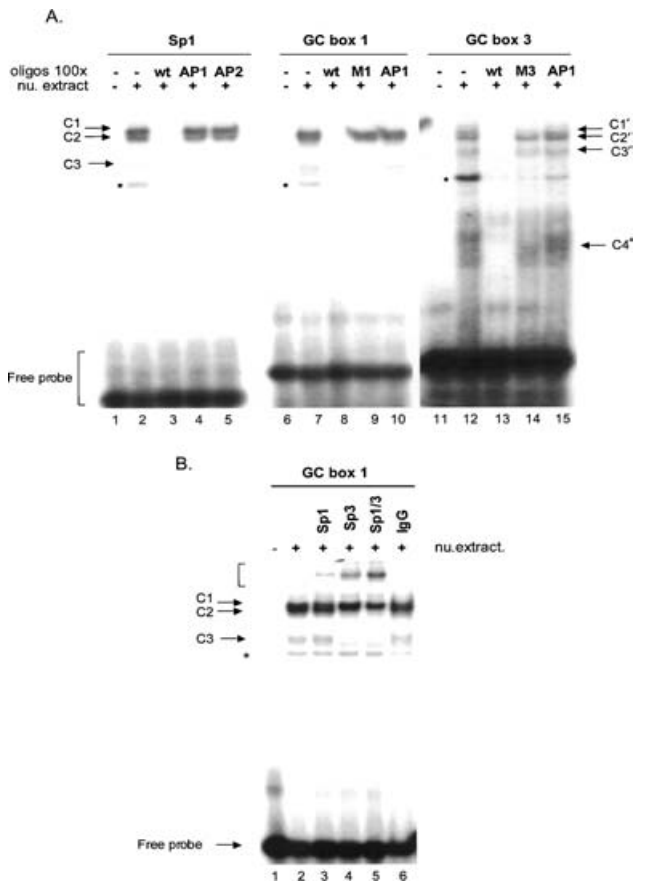

Figure 3 Sp1 and Sp3 transcription factors bind the GC box 1 but not the GC box 3 in the minimal promoter of MKP-1

(**A**) Binding and competition EMSA assays carried out with double stranded end-labelled [γ-^{32}P] oligonucleotides for the GC boxes 1 and 3 of the rat MKP-1 promoter. A consensus Sp1 probe was included as a positive control. Sequence of the wt, mutated and non-specific oligonucleotides used in this experiment are shown in Table 1. The mutations M1 and M3 destroy the Sp1-like site found respectively in the GC box 1 and 3 sequence. The two non-specific unlabelled probes AP1 and AP2, used here, are homologous to AP1-like transcription factor elements found in the sequence of the mouse c-*fos* oncogene [24]. DNA-binding complexes formed in the EMSA are indicated with the prefix C1–3 for consensus Sp1 and GC box 1 probes and C1′–4′ for the GC box 3. The asterisks denote non-specific interaction bands, which were not consistently seen with different nuclear extract preparations. Nuclear extract, nu. extract; competition assay with 100-fold excess of unlabelled probes, oligos 100×. (**B**) Supershift EMSA assays carried out with double-stranded end-labelled [γ-P^{32}] oligonucleotides for the GC box 1 of the rat MKP-1 promoter. Binding reactions were performed as in (**A**), then an antibody against Sp1 (αSp1), Sp3 or a normal goat IgG were added for a further 15 min on ice. In lane 5 both antibodies αSp1 and αSp3 (Sp1/3) were added at the same time. Supershift complexes are marked with a square bracket.

respectively (see Table 1). A consensus Sp1 probe was included as a positive control. As shown in Figure 3(A), nuclear extracts from basal GH4C1 cells formed three complexes (C1, C2 and C3) with the consensus Sp1 and GC box 1 probes (Figure 3A, lanes 2 and 7). GC box 3 probe generated four different complexes (C1′, C2′, C3′, and C4′) with different molecular masses and DNA-binding affinities compared with the two previous probes (Figure 3A, lane 12). Formation of these different complexes were all efficiently inhibited by a 100-fold molar excess of the corresponding wt nucleotides (Figure 3A, lanes 3, 8 and 13) whereas non-specific unlabelled oligonucleotides had no effect (Figure 3A, lanes 4, 5, 10 and 15). To define the specific base-pairs critical for complex formation, competition was performed using two double-stranded mutant oligonucleotides, each containing 4 bp mutations (see Table 1). Both mutants M1 and M3 of the GC boxes 1 and 3 respectively, were unable to effectively compete for the DNA–protein interaction (Figure 3A, lanes 9 and 14). This EMSA analysis showing the specific binding potential of the GC boxes 1 and 3 was consistent with the presence of DNA-binding proteins in the region −79 to −31 of the MKP-1 promoter observed by *in vivo* footprinting (Figure 2), a region that includes these critical GC-rich motifs.

In order to identify the protein factors that form these complexes, we used EMSA supershift experiments with antibodies to Sp1 and Sp3 transcription factors known to bind GC-rich sequences (Figure 3B), and anti-IgG antibodies as negative control. In the EMSA performed with the GC box 1 probe, addition of the anti-Sp1 and/or anti-Sp3 shifted the C1, C2, and C3 complexes to a higher molecular mass (Figure 3B, lanes 3 and 4), whereas the anti-IgG had no effect (Figure 3B, lane 6). A combination of anti-Sp1 and anti-Sp3 antibodies increased the level of the slowest migrating complex when compared with either anti-Sp1 or anti-Sp3 antibodies alone (Figure 3B, lane 5). Analysis with the GC box 3 probe did not show supershifted complexes (results not shown). These data indicate that the GGACCGCC motif in the GC box 1 region is important for the binding of Sp1 and Sp3 transcription factors. The same transcription factors have a low binding affinity to the G stretch found in the region from −69 to −76; it is most likely that different transcription factors bind the GC box 3.

Several responsive elements in the MKP-1 promoter (CREs, E-box and GC boxes) mediate basal MKP-1 reporter gene transcription in transient transfection

To determine whether GC boxes 1 and 3 are essential and sufficient to maintain MKP-1 transcriptional activity, these response elements in the MKP-1 promoter −79 and −203 luciferase reporter genes were inactivated by site-directed mutagenesis and the resulting vectors analysed by transient transfection (Figure 4). Individual mutation of either the GC box 1 or the GC box 3 reduces, but does not fully abolish, the activity of the −79 promoter (Figure 4, line 3 and 4). However, when both GC boxes were mutated the luciferase activity almost returned to the level of the basic pGL3 vector (Figure 4, line 5). This result indicates that the GGACCGCC motifs, important for the binding of Sp1/3 transcription factors and the G-stretch region from −69 to −76, are essential for transcriptional initiation on the MKP-1 promoter −79 in basal and stimulated conditions.

We then analysed whether these GC-rich elements were also sufficient to maintain the strong basal activity in the promoter construct −203. Surprisingly, neither single nor double mutants of the GC boxes 1 and 3 fully reduced the luciferase activity in basal and TRH-stimulated cells (Figure 4, line 7 to 9). In the context of the −203 promoter, the GC box 1 (and to a lesser degree GC box 3) attenuate strong transcription initiation mediated by the CREs and E-box. Experiments with a control construct with the mutated CREs and E-box motifs (Figure 4, line 10) confirmed that the GC-rich elements in the −203 promoter produced a level of the reporter gene transcription similar to the level reached with the MKP-1 promoter −79 construct. Further site-directed mutagenesis with the double mutant GC boxes 1 and 3 promoter showed that both the CREs and the E-box could maintain a strong luciferase activity independently (Figure 4, lines 11 and 12). The reporter-gene activity was completely abolished in the MKP-1 promoter −203 construct only when the five responsive elements (the CREs, E-box and the two GC boxes) were simultaneously inactivated by mutation (Figure 4, line 13).

Taken together these data suggest that each of the five responsive elements described here for the promoter −203 is able,

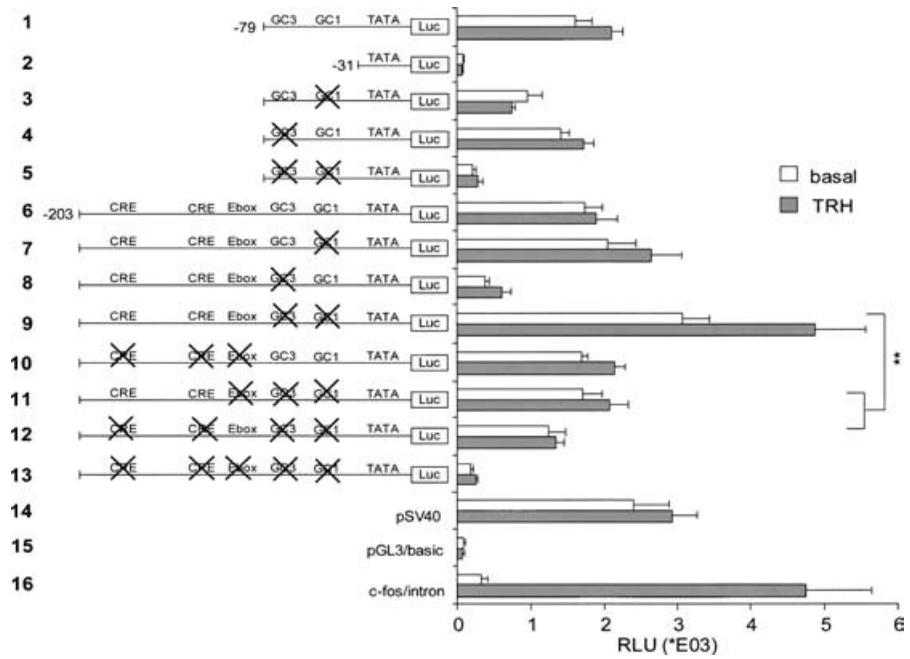

Figure 4 Several responsive elements in the MKP-1 promoter (CREs, E-box and GC boxes) mediate basal MKP-1 reporter gene transcription in transient transfection

Reporter gene assays were performed with different MKP-1 promoter constructs, transiently transfected in GH4C1 cells, stimulated or not with TRH, as in Figure1 (see the Materials and methods section for details). A schematic representation of the luciferase reporter genes (Luc) driven by the MKP-1 promoters − 79 and − 203 containing either wt or mutated (×) GC boxes, CREs and E-box sites is shown on the left-hand side. Shown are the average relative light units (RLU) obtained for the different constructs in basal and TRH-stimulated cells (means ± S.E., $n = 3$). Similar results were obtained with EGF (results not shown). Generation and site-directed mutagenesis of these constructs are described in the Materials and methods section. The list of oligonucleotides used for the mutagenesis is presented in Table 1.

on its own, to initiate MKP-1 reporter gene transcription in transiently transfected cells. Small changes in the average luciferase expression, driven by the different mutant promoters, may reflect differences in the efficiency of transcriptional initiation for the CREs, E-box and GC boxes. For instance, the CRE elements combined with the E-box seemed to be more potent (Figure 4, line 9) than the CREs (Figure 4, line 11) or the E-box (Figure 4, line 12) alone. This is consistent with a previous study performed by Sommer et al. [21] suggesting that the E-box can co-operate with the CRE elements to stimulate MKP-1 gene transcription.

In the minimal − 79 promoter, the GC boxes 1 and 3 appear to operate as activators (Figure 4, lines 1 to 5), whereas in the − 203 construct rather as repressors for CRE- and E-box mediated gene transcription (Figure 4, line 6 versus line 9). This may be explained mostly by the Sp3 transcription factor bound at GC box 1 (Figures 3B and 7). Sp3 can function either as an activator or a repressor depending on the promoter context [30–32]. Furthermore, the GC3-binding protein may attenuate the repressive effect mediate by Sp3 at the GC box 1 (Figure 4, lines 6 and 7 versus line 8), and may also attenuate strong transactivation mediated by the co-operation of CREB and USF (Figure 4, lines 6 and 7 versus line 9).

Endogenous MKP-1 gene expression is strongly induced by TRH and EGF via the ERKs signalling pathway

The data obtained by transient transfection of the MKP-1 reporter gene (Figure 4) indicate that proximal-promoter elements in the MKP-1 gene are sufficient to initiate transcription in basal condition, but may be not explain stimulation of MKP-1 transcription by TRH or EGF. Indeed endogenous MKP-1 mRNA is strongly induced by TRH and EGF 30 min after stimulation (Figure 5A). We analysed the effect of several inhibitors of protein kinases and protein phosphatases in the presumptive signalling pathways for EGF- and TRH-induced expression of MKP-1. Interestingly we found that the most potent inhibitor of MKP-1 gene expression in TRH and EGF conditions was the MEK inhibitor U0126 (Figure 5A). In GH4C1 cells, TRH induces an increase in intracellular free calcium ($[Ca^{2+}]_i$) via the mobilization of intracellular calcium stores but also via a sustained calcium influx at the membrane (cf. [19] and references therein). Inhibitors of Ca^{2+}-sensitive protein kinases PKA, PKC, CaMK (Ca^{2+}/calmodulin-dependent protein kinase) or the phosphatase calcineurin could not reduce significantly the level of the MKP-1 mRNA in TRH-stimulated cells (results not shown). The L-type calcium channel blocker nifedipine significantly reduced MKP-1 gene transcription stimulated by TRH, albeit less efficiently than the Ca^{2+} chelator EGTA applied during TRH stimulation [19]. Combination of nifedipine and U0126 showed that the MKP-1 mRNA level was not further reduced (Figure 5A), suggesting that activation by TRH of the MAPKs/ERKs cascade may be downstream to an increase in $[Ca^{2+}]_i$. Indeed only part of the active ERKs is reduced by nifedipide in TRH-stimulated cells compared with the treatment with U0126 (Figure 5B, lane 5 versus lane 8). Other mechanisms directly associated with the G-protein-coupled receptor for TRH may also be important to stimulate the MAPKs/ERKs in parallel with the calcium signalling pathway [33]. An important role for the MAPKs/ERKs for stimulated MKP-1 expression is consistent with the function of the MKP-1 protein as a negative regulator of activated MAPK, induction of MKP-1 is counterbalancing ERK activation by the same stimuli in a time- and cell-compartment-dependent manner.

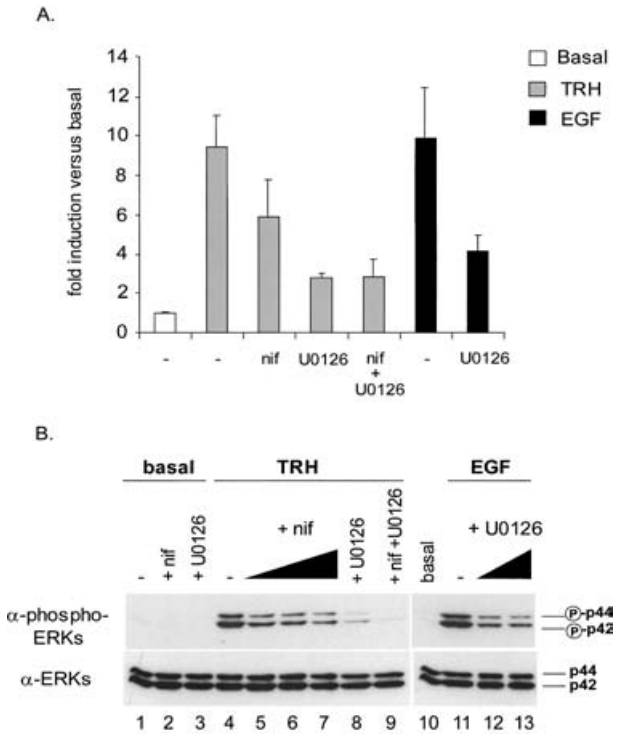

Figure 5 Endogenous MKP-1 gene expression is strongly induced by the ERK signalling pathway in TRH- and EGF-stimulated cells

(**A**) Effect of the MEK inhibitors U0126 (10 μM) and the L-type calcium channel blocker nifedipine (nif; 1 μM) on the levels of MKP-1 mRNA in GH4C1 cells stimulated with either TRH or EGF. The inhibitors were added 60 min prior to TRH and EGF treatment. Stimulation was performed for 30 min and total RNA was isolated. MKP-1 mRNA and 18 S RNA levels were analysed by real-time quantitative PCR using sequence-specific primer pairs and probes design for the rat MKP-1 cDNA and the 18 S ribosomal RNA, as described in the Materials and methods section. Shown are the average of MKP-1 mRNA levels normalized to the 18 S RNA levels and expressed as fold induction over basal values (means $\pm$ S.E., $n = 3$). (**B**) ERK/MAPK activation shown by Western blotting using anti-ERK and anti-phospho-ERK antibodies. Cells were stimulated for 15 min by TRH or EGF in the presence or absence of the MEK inhibitor U0126 (10 μM in lanes 3, 8, 9, 12; 20 μM in lane 13), or the Ca^{2+} channel blocker nifedipine (nif; 1 μM in lanes 2, 5, and 9; 2 μM in lane 6 and, 5 μM in lane 7).

CREs, E-box and GC 1/3 boxes in the proximal promoter of MKP-1 act in synergy to mediate TRH activation of transcription via the signalling pathway of ERK in stable transfection studies

A striking discrepancy has been described between the expression of MKP-1 reporter genes and transcription of endogenous MKP-1: reporter genes are strongly expressed under basal conditions, but poorly inducible, whereas endogenous MKP-1 is well induced by several stimuli (Figures 1 and 4 versus Figure 5). This difference could be due to a regulated block to elongation in exon 1 of the gene, which is not present in the reporter construct (see [19]). Furthermore, monitoring of reporter enzyme activity in transiently transfected cells after 3 h (the time needed to express luciferase protein) might poorly reflect a rapid induction of MKP-1 reporter gene transcription. Therefore we based further reporter gene studies on the measurement of the luciferase mRNA expression level. To this end, reporter genes driven by wt or mutant MKP-1 promoter were stably transfected into GH4C1 cells. Then, one cellular clone for each construct was selected and analysed for luciferase mRNA levels in basal conditions and after TRH stimulation. Figure 6(A) shows the luciferase data from a series of clones expressing the wt long MKP-1 promoter (-631Luc) or the short MKP-1 promoter (-203Luc). Stably transfected into neuro-endocrine cells, both MKP-1 promoters (-631 to $+19$ and -203 to $+19$) still behave very much like constitutive promoters, driving strong expression of the reporter with little induction observed 3 h after TRH stimulation (approx. 1.25-fold on average). For further analysis, a representative clone for each construct was chosen (e.g. Figure 6A, open triangle; the selection of clones expressing the mutant MKP-1 promoter is not shown here). Timed analysis of luciferase mRNA with one cellular clone (in this case, 631C9), revealed that induction levels were best at 30 min after either TRH or EGF stimulation rather than at later times (Figure 6B).

We then wanted to determine which combinations of *cis*-acting elements in the -203 promoter were involved in the induction by TRH, and sensitive to the activated MAPKs/ERKs signalling pathways. Stable cellular clones corresponding to the wt and mutant MKP-1 promoters were stimulated with TRH for 30 min and analysed for their luciferase mRNA level by real-time RT-PCR (Figure 6C). Maximal induction of luciferase mRNA was detected only for the wt MKP-1 promoter and not for the mutant promoters. The grouped double mutant GC box 1/3 promoter with or without the mutant E-box or the grouped double CRE mutants could not respond to TRH in the first 30 min of stimulation. As for the SV40 promoter clone, no significant induction was observed for these mutants. This result indicates that the transcription factors bound to the CREs, E-box and GC box 1/3 are acting in a synergistic manner in TRH-stimulated conditions. Moreover, addition of the MEK inhibitor U0126 in these conditions abolished transactivation of the wt promoter. The MAPKs/ERKs signalling pathway can therefore strongly stimulate MKP-1 transcription through responsive elements found in the proximal promoter. Similar preliminary data were observed for wt and mutant promoters of MKP-1 in EGF-stimulated conditions (results not shown).

The RNA pol II, CREB, Sp3 activators and the co-activator CBP are continuously present *in vivo* on the proximal promoter of MKP-1

The previous *in vitro* and *in vivo* experiments (Figures 2, 4 and 6) have confirmed that multiple *cis*-acting elements in the proximal promoter are important to control basal and TRH-stimulated MKP-1 gene transcription. To determine the DNA-binding proteins associated with the proximal promoter of MKP-1 *in vivo*, we performed ChIP assays with different antibodies. Cells were harvested either at quiescence or 15 min following TRH stimulation and then exposed to formaldehyde, which crosslinks protein and DNA in chromatin. After isolation and fragmentation, the chromatin was immunoprecipitated with antibodies specific for the RNA pol II, CREB, Sp1, Sp3 and the co-activator CBP. DNA was released from the immunoprecipitates and then amplified by PCR with primers selected to detect the presence of MKP-1 promoter sequences and distal sequences corresponding to the exon 4 of the gene.

As shown in Figure 7, RNA pol II is strongly recruited to the promoter of MKP-1 in resting cells, consistent with the fact that the promoter behaves like a constitutive promoter in this cell line. Furthermore, CBP, CREB and Sp3 are immunoprecipitated with MKP-1 promoter sequences in resting cells. Initiation of MKP-1 gene transcription in quiescent GH4C1 cells may be mediated by the constitutive presence of these different factors on the proximal promoter. In TRH-stimulated conditions, only small changes in signal intensity could be observed for any of the CREB, Sp3, CBP and pol II immunoprecipitated DNA compared with non-stimulated conditions (Figure 7). Transactivation of the MKP-1 promoter in stimulated conditions (as observed in Figure 6C) is apparently not correlated with a significant further

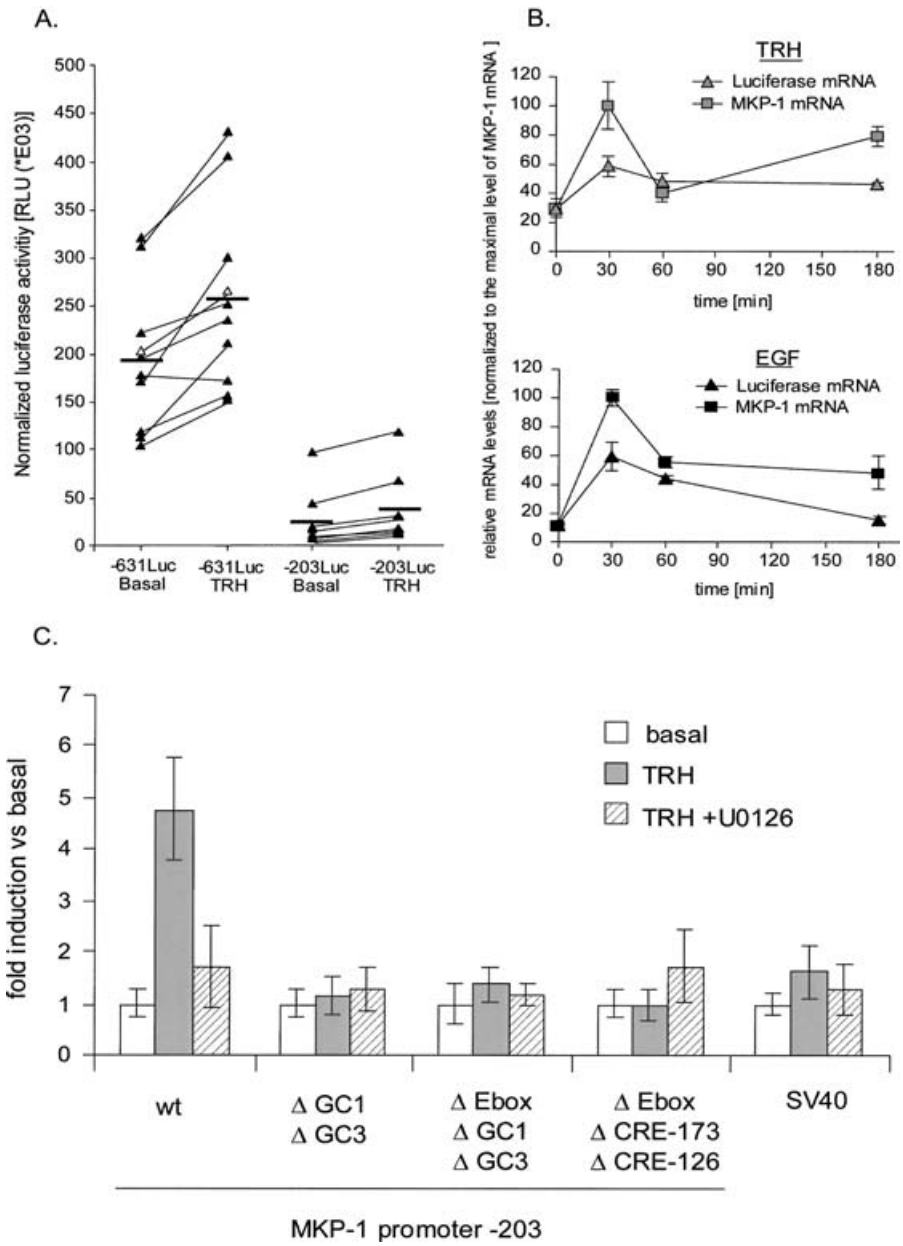

Figure 6 CREs, E-box and GC1/3 boxes in the proximal promoter of MKP-1 act in synergy to mediate TRH activation of transcription via the MAPKs/ERKs signalling pathway in stable transfection studies

(**A**) Reporter genes driven by MKP-1 promoters stably transfected into GH4C1 cells maintain a high level of basal expression but only limited induction by TRH and EGF. MKP-1 reporter gene constructs were co-transfected with pCDNA3 in GH4C1 cells and selected with the drug geneticin (0.2 mg/ml). Stably transfected cellular clones were incubated for 24 h in SFM prior to the analysis and stimulated, when indicated, by TRH for 3 h. Cell extracts were analysed for luciferase activity normalized to the total protein contents. Normalized luciferase activity of basal and TRH-stimulated cells are indicated for 10 clones of the MKP-1 promoter -631 and 8 clones of the promoter -203 reporter gene (-631Luc, -203Luc respectively). For each clone, values of luciferase activity in basal and stimulated (TRH) conditions are shown linked together with a thin line. Mean luciferase basal and TRH-stimulated activities for the clones are indicated with a bar. Clone 9 of the MKP-1 promoter -631 reporter gene (631C9) is shown with a open triangle. Clonal selection of the mutant MKP-1 promoter and SV40 promoter are not shown. (**B**) Time course of expression of the luciferase mRNA and the endogenous MKP-1 mRNA in the cellular clone 631C9 stimulated with TRH or EGF. The mRNA levels were derived by real-time quantitative PCR using sequence-specific primer pairs and probes designed for the luciferase cDNA, the rat MKP-1 cDNA and the 18 S ribosomal RNA as described in the Materials and methods section. Shown are the average relative mRNA levels (means $\pm$ S.E., $n = 3$), normalized to the maximal level of MKP-1 mRNA observed 30 min after stimulation. (**C**) Stable cellular clones transfected with luciferase reporter constructs were analysed for their relative luciferase mRNA level at basal and at 30 min after TRH stimulation in the presence or absence of the MEK inhibitor U0126. Stimulations were performed as in Figure 5. Luciferase reporter constructs chosen for this experiment are represented in Figure 4 as follows: wt (line 6), ΔGC1 ΔGC3 (line 9), ΔE-box ΔGC1 ΔGC3 (line 11) and ΔE-box ΔCRE $-$ 173 ΔCRE $-$ 126 (line 10) $-$ 203 MKP-1 promoter constructs. Quantification was performed as in (**B**) with the luciferase and the 18 S TaqMan probes. Shown are the average of luciferase mRNA levels normalized to the 18 S RNA levels and expressed as fold induction over basal values (means $\pm$ S.E., $n = 5$).

recruitment of CREB, Sp3, CBP and the RNA pol II to promoter-proximal sequences. Other mechanisms may be involved to activate complete transcription of the MKP1 gene that are mediated by ERK MAPK.

DISCUSSION

Transcription of the MKP-1 gene can be activated by multiple intracellular signals, including active mitogenic or stress-activated

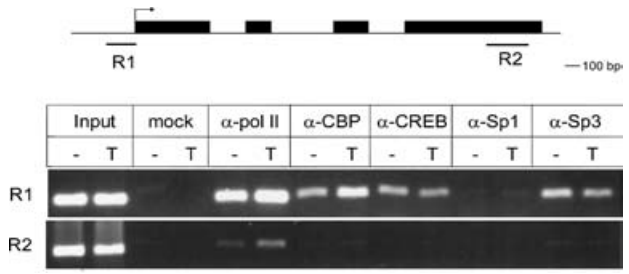

Figure 7 The RNA pol II, CREB, Sp3 activators and the co-activator CBP are continuously present *in vivo* on the proximal promoter of MKP-1

ChIP assays were used to examine the *in vivo* interaction of RNA pol II, transcription factors and co-activator with the MKP-1 proximal promoter in intact cells. GH4C1 cells were harvested either at quiescence (−) or 15 min following TRH stimulation (T) and cross-linked with the addition of formaldehyde. Chromatin was immunoprecipitated with antibodies to either RNA pol II (α-pol II), CBP (α-CBP), CREB (α-CREB), Sp1 (α-Sp1) or Sp3 (α-Sp3). After reversing the cross-link, DNA released from the immunoprecipitates was used for PCR analysis to measure the presence of MKP-1 promoter sequences. Shown are the MKP-1 promoter (R1) and exon 4 (R2) PCR products run on an agarose gel and stained with SYBR-green. Input, fragmented and reverse cross-linked genomic DNA (10 %); mock, no antibody. For details see the Materials and methods section.

MAPKs cascades, PKC, cAMP, Ca^{2+}, glucocorticoids and retinoic acids [6–9,11–13,21]. What mechanisms ensure a specific transcriptional response of MKP-1 following activation by such diverse intracellular signals? What are the decisive responsive elements involved in the regulation the gene? Answering these questions is important to our understanding of the physiological and pathophysiological consequences of the expression of MKP-1, already shown to be of fundamental importance, e.g. in cardiac hypertrophy, monocyte/macrophage chemotaxis and tumorigenesis [10,34,35].

In the present study we characterize the rat MKP-1 promoter and identify two GC boxes bound by Sp1 and Sp3 transcription factors *in vitro* plus two CREs and one E-box that are important for MKP-1 transcription initiation. In transient transfection, each of these five responsive elements in the minimal MKP1 promoter can initiate transcription of the reporter gene, even when all the other elements are mutated (Figure 4). This indicates that each transcription factor alone (CREB, USF, Sp3 and the GC3-binding protein) has the intrinsic ability to recruit the basal transcription machinery in the proximal-promoter of MKP-1 when the other transcription factors are absent. In contrast, when integrated into the genome after stable transfection of MKP-1 reporter constructs, the five responsive elements function in synergy to mediate stimulation of transcription by either TRH or EGF (Figure 6C). Combination of the five *cis*-acting elements in the promoter of MKP-1 seem to be more important in the context of the chromatin compared with transient transfection studies. This striking discrepancy may be explained in part by the methodology used to study MKP-1 transcriptional initiation. Transient transfection of reporter genes is generally used to identify essential *cis*-acting elements in gene promoters, but this method has some limitations in the understanding of transcriptional processes related to chromatin context. Based on this argument, we believe that the results obtained by stable transfection are more in accordance with a model of regulation for endogenous MKP-1 transcriptional initiation compared with transient transfection studies. In stimulated conditions, transcription factors bound to the proximal promoter are acting in synergy to stimulate MKP-1 gene transcription.

Transient transfection luciferase data, showing a redundancy in the action of *cis*-acting elements (Figure 4), are possibly reflecting association of the transcription factors with the same co-activator(s). We showed by ChIP that CBP is a candidate for the organization of the MKP-1 promoter, since this co-activator is recruited both in resting and TRH-stimulated cells. CBP/p300 is known to be tightly associated with CREB/CREM (cAMP responsive element modulator) and recently the Sp/Krüppel-like factor (KLF) family of transcription factors [36,37]. On the other hand, differences in basal transcription efficacy obtained with various combinations of responsive elements (Figure 4) could also result from the differential recruitment of specific TAF_{II}s (TATA box-associated factors) by the different transcription factors bound on the proximal promoter. Previous reports have demonstrated that Sp3 can interact with $TAF_{II}110$, $TAF_{II}40$, $TAF_{II}55$ and TATA-box-binding protein (TBP) [30–32], CREB with $TAF_{II}110$, $TAF_{II}135$ [38] and USF with $TAF_{II}55$ [39].

As mentioned previously, we have shown that the MKP-1 gene is mainly controlled at the level of transcriptional elongation in GH4C1 cells [19]. In this context, how does the MKP-1 promoter work? What is the link between the continuous presence of Sp3, CREB and CBP on the promoter and the tightly regulated block to elongation in the first exon? Studies performed in mammalian systems on the regulation of transcriptional elongation of IEGs such as hsp70, c-*fos* or c-*myc* have shown that there is a strong correlation between the activation of transcription factors at the level of initiation and the competency of pol II to enter into a processive mode of transcriptional elongation [40–44]. Analogous to these observations we propose that, in basal conditions, MKP-1 gene transcription is initiated by constitutive transcription factors (Sp3, CREB and USF) or the co-activator (CBP); however, RNA pol II is not competent for elongation as it pauses or arrests in the promoter-proximal region of the gene. In TRH- (or EGF-) stimulated conditions, possible phosphorylation of CREB, Sp3 and the co-activator CBP by PKs/ERKs signalling pathways may stimulate the basal transcription machinery and the assembly of an elongation-competent pol II, which can overcome the block to elongation imposed by intragenic elements and/or chromatin structure.

In agreement with this hypothetical model, previous reports have shown that transcription factors may stimulate either initiation, elongation or both [45]. Sp1 transcription factors are more potent in stimulating initiation than elongation. Activation of transcription factors can stimulate transcriptional elongation by the phosphorylation of the C-terminal domain (CTD) of pol II and/or recruit specific cofactors, elongation factors to generate an elongation-competent pol II complex [46,47]. Note that in contrast with other transcription factors, gene transcription driven by Sp1 promoters alone is not affected by the deletion of the CTD of the RNA pol II [48]. MKP1 gene transcription, initiated via Sp3 may be abortive because the CTD is under-phosphorylated in basal condition. Phosphorylation of the CTD by activated CTD kinase in stimulated conditions may recruit positive or relieve negative elongation factors to overcome the block to elongation [47]. Alternatively, activated transcription factors may cause a major conformational change recruiting further co-activator(s) and a different set of general transcription factors, resulting in an elongation-competent pol II. For instance it has been shown that RNA pol II can be present in a complex containing CPB/p300 and the PCAF (p300/CBP-associated factor) [49,50]. Interestingly, p300 interacts specifically with the initiation-competent form of pol II (non-phosphorylated) whereas PCAF interacts with the elongation-competent phosphorylated form of pol II [49].

Structure and use of a given promoter, like the proximal-promoter of MKP-1, will most likely strongly influence the efficiency of transcriptional elongation. Studies realized on the hsp70, c-*fos* or c-*myc* IEGs have indicated causal relationships between elements in the promoter and the processivity of the RNA

polymerase [40–44]. Further studies are needed to elucidate the link between elements in the MKP-1 promoter and the regulation of transcriptional elongation in its first exon. Successful approaches for such studies are difficult to predict; however, such work will certainly be helpful in the understanding of mechanisms of transcriptional elongation in the expression of IEG, and may provide new insight into how the processivity of the polymerase can influence quantitative gene regulation of IEGs, as proposed by Hazzalin and Mahadevan [18].

In summary, our present and previous studies together suggest that the control of MKP-1 gene transcription involves multiple elements located in the promoter and exon 1 that play different roles depending upon the conditions of stimulation. The MKP-1 gene provides an interesting model in which to study further the molecular mechanisms which provide for a co-ordinated regulation of transcription initiation and elongation by intracellular signalling pathways.

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