Transcriptional elongation of many eukaryotic, prokaryotic, and viral genes is tightly controlled, which contributes to gene regulation. Here we describe this phenomenon for the MAP kinase phosphatase 1 (MKP-1) immediate early gene. In rat GH4C1 pituitary cells, MKP-1 mRNA is rapidly and transiently induced by the thyrotropin-releasing hormone (TRH) and the epidermal growth factor (EGF) via transcriptional activation of the gene. Ca2+ signals are necessary for the induction of MKP-1 in response to TRH but not to EGF. Reporter gene analysis with the newly cloned rat promoter sequence shows only limited induction in response to various stimuli, including TRH or EGF. By nuclear run-on assays we demonstrate that in basal conditions, a strong block to elongation in the first exon regulates the MKP-1 gene and that stimulation with either TRH or EGF overcomes the block. Ca2+ signals are important to release the MKP-1 elongation block in a manner similar to the c-fos oncogene. These results suggest that a common mechanism of intragenic regulation may be conserved between MKP-1 and c-fos in mammalian cells.

Long term cellular processes such as proliferation, differentiation, and neuronal plasticity are controlled by extracellular stimuli and require the synthesis of new gene products. Following stimulation, expression of immediate early genes (IEGs) precedes the expression of late response genes, the latter encoding for proteins implicated in specific functions. The best known IEG products are transcription factors such as c-Fos, c-Jun, and c-Myc, which control the expression of late response genes (1, 2). Not all IEGs encode for transcription factors. For instance, structural proteins like actin and tropomyosin, cytokines, and other regulatory proteins show a rapid and transient induction by growth factors (1).

Recently, a group of dual specificity phosphatases have been identified as being IEG products induced by various stimuli (growth factors, stress, neurotransmitters, etc.; reviewed in Ref. 3). These dual specificity (threonine/tyrosine) phosphatases have been named MAP kinase phosphatases (DSPs or MKPs), since they are effective in the inactivation of MAP kinases by dual dephosphorylation (4). MAP kinase phosphatase-1 (MKP-1/CL100/3CH134) is one example of this group of nuclear enzymes encoded by an IEG. Although MKP-1 gene transcription is activated by multiple signals, such as mitogens (5, 6), cytokines (7), oxidative stress (8), heat shock (8), or hypoxia (9), the precise mode of gene regulation of this immediate early gene by such stimuli remains unclear. A comparison of the 5′-flanking sequence of the murine and the human genes revealed two conserved Ca2+/cAMP-responsive elements (CREs) and one E box motif in the promoter region of MKP-1. Recently, the upstream stimulatory factor, a member of the basic/helix-loop-helix/leucine zipper family has been shown to bind to the E box motif and transactivate MKP-1 expression in synergy with protein kinase A (10). Since multiple intracellular signals can target MKP-1 gene expression, it is likely that regulatory elements other than the CREs and the E box motifs may influence its transcription. Alternatively, additional regulation at the level of transcriptional elongation, termination, and/or mRNA stability may be important to control MKP-1 gene expression.

Regulation of gene expression at the level of transcriptional elongation is well established in prokaryotes and in an increasing number of eukaryotic genes (11, 12). Earlier studies in mammalian cells have reported that the IEGs c-fos, c-myc, hsp70, and tumor necrosis factor-α display a strong block to transcriptional elongation in the promoter-proximal region (13–16). Characterization of the block in vivo, by either nuclear run-on or KMnO4 footprinting, showed that promoter-proximal pause sites in c-fos, c-myc, and hsp70 are important for controlling RNA polymerase II processivity (15, 17–19). In the case of c-fos, an additional transcriptional pause site is located in the first intron of this oncogene that is sensitive to Ca2+ stimulation (17, 20–23). The precise mechanism of intronic regulation of c-fos by Ca2+ signals is not known.

Here we show a rapid and massive increase in MKP-1 mRNA triggered by either thyrotropin-releasing hormone (TRH) or epidermal growth factor (EGF) in GH4C1 neuroendocrine cells. Ca2+ signals are necessary for the induction of MKP-1 gene expression in response to TRH but not to EGF. After cloning
the rat MKP-1 genomic fragment, we demonstrate by a reporter gene assay that activity of the MKP-1 promoter alone cannot explain induction of MKP-1 expression in GH4C1 cells. Using nuclear run-on experiments, we show that control of MKP-1 transcription involves a block to elongation in the first exon. This block represents a decisive element in the regulation of MKP-1 gene expression.

**EXPERIMENTAL PROCEDURES**

**Materials**—The thyrotropin-releasing hormone TRH (Roche Molecular Biochemicals) and the epidermal growth factor EGF (Sigma) were diluted in H₂O at 27.6 μg/ml and stored at −80 °C. EGTA (Fluka Chemie) was diluted in H₂O and stored at room temperature as 100 mM stock solution (pH 7.5). Actinomycin D (Sigma) and cycloheximide (Sigma) were diluted respectively in EtOH and Me₂SO at 5 and 10 mg/ml and stored at 4 °C. CPT-cAMP (Roche Molecular Biochemicals) was diluted in H₂O, stored in aliquots at −20 °C at a concentration of 10 mM. KCl (Fluka Chemie) was diluted in H₂O and stored 4 °C at a concentration of 3 M.

**Cell Culture and Stimulation**—GH4C1 pituitary cells were maintained in Ham's F-10 medium (Life Technologies, Inc.) supplemented with 2.5% fetal bovine serum and 15% horse serum at 37 °C in a humidified atmosphere with 5% CO₂. Confluent GH4C1 cells were incubated with 10 serum-free medium (SMF) containing 5 μg/liter transferrin for 24 h and then stimulated for the indicated time either 100 nM TRH or 10 nM EGF. When indicated, 0.6 mM EGTA was added to the medium 5 min before TRH or EGF stimulation to chelate free extracellular calcium ([Ca²⁺]ᵢ < 0.1 μM). Actinomycin D (5 μg/ml) and cycloheximide (10 μg/ml) were added in SMF 30 min before stimulation with either TRH or EGF; total RNA was isolated 30 min after TRH and EGF stimulation.

**RNA Preparation, Northern Blot Analysis, and RNase Protection Assay**—Total RNA was extracted from cells with an acid phenol-guanidinium reagent (TRI-Reagent, Molecular Research Center, Inc.) according to the manufacturer's instructions. RNA samples (10 μg) were denatured by incubation in glycoxal, subjected to electrophoresis in a 0.7% agarose gel and stained with ethidium bromide. RNA samples (100 ng/liter transferrin for 24 h and then stimulated for the indicated time) were added to the medium 5 min before TRH or EGF stimulation to chelate free extracellular calcium ([Ca²⁺]ᵢ < 0.1 μM). Actinomycin D (5 μg/ml) and cycloheximide (10 μg/ml) were added in SMF 30 min before stimulation with either TRH or EGF; total RNA was isolated 30 min after TRH and EGF stimulation.

**Primer Express 1.0 software from PerkinElmer Life Sciences.**

**Quantification of the MKP-1 mRNA** was performed with a riboprobe prepared from the rat MKP-1 gene using primer Express 1.0 software from PerkinElmer Life Sciences.

**Cloning of the Rat Gene**—The rat MKP-1 gene was amplified by PCR from rat genomic DNA of GH4C1 cells using primers directed toward two conserved regions in the promoter and exon 4 of the mouse and the human gene: 5'-TCT TGC AAC CCT CCT CCC TTG G-3' (sense) and 5'-GTT GAA CTC GGG AGG TGT TTG-3' (antisense). The resulting 2892-bp PCR fragment was cloned, sequenced, and aligned to the mouse and human MKP-1 gene. The sequence of the rat MKP-1 gene in GH4C1 was compared and corrected according to sequences amplified in rat pancreatic β INS-1 and rat vascular smooth muscle cells. The cloned GH4C1 sequence is available in the GenBank™ data base with the accession number AF357203.

**Nuclear Run-on Transcription Assays**—GH4C1 cells maintained in culture medium were detached from Petri dishes with trypsin, transiently transfected using FuGENE 6 transfection reagent (Roche Molecular Biochemicals), and seeded in 24-well multidishes (Falcon) at a density of 6 × 10⁴ cells/well. After 1 day, the medium was replaced by SFM 24 h before exposure to the various stimuli. Cells were stimulated with the addition of KCl, CPT-cAMP, 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 according to Promega’s Luciferase Reporter assay system instructions. Cells were co-transfected with Renilla luciferase expression plasmid to verify uniformity of transcription from experimentation to experimentation. Plasmid pM1 was generated by cloning the region of the rat MKP-1 gene extending from −631 to +19 between the KpnI and Nhel restriction sites of the pGL3 enhancer vector (Promega). Plasmid pSV40 was obtained by subcloning the SV40 promoter from the pGL3-control vector (+49 to +244) between the KpnI and Nhel restriction sites of the pGL3 enhancer vector. The “33320” construction was previously described (17, 21).}

**Quantification of MKP-1 mRNA** was performed by TaqMan RT-PCR (PerkinElmer Life Sciences) from the total RNAs extracted from 10 series of GH4C1 cells cultured in nonstimulated or stimulated conditions. Each RNA sample (diluted to 10 ng/μl) was analyzed six times independently (replicates), and a standard curve was included in each plate. The standard curve was prepared from the total RNA of a TRH-treated sample with a high level of expression of MKP-1, diluted from 100 ng/μl to 1 pg/μl. Each dilution of the standard sample was amplified in triplicate. 18 S rRNA was simultaneously amplified in each tube for normalization.

Briefly, 5 μl of each total RNA (corresponding to 50 ng of the unknown samples), primed with random hexamers, were retrotranscribed into cDNA in a final volume of 50 μl using the TaqMan Gold RT-PCR Kit of PerkinElmer Life Sciences (PE 8500-234). The RT-PCR manufacturer’s instructions. MKP-1 and 18 S rRNA were simultaneously amplified from each sample using 4 μl of the previous cDNA, 1× PerkinElmer's Universal PCR Master Mix (PE 4304437), 0.3× 18 S rRNA Predeveloped Assay Reagent (PE 4310893E), 200 nM MKP-1 forward primer 5'-CCGCCTCCATCTCAAGTCTC-3', 200 nM MKP-1 reverse primer 5'-GTTGACAGTGTGCCACA-3', and 200 nM MKP-1 TaqMan probe 5'-FAM (6-carboxyfluoresein)-AGCCGAAAAGCCTTG-ATACCTCTTTGTTGAMAR (6-carboxytetramethylrhodamine)-3'. The primers and probe for the quantification of MKP-1 were designed using Primer Express 1.0 software from PerkinElmer Life Sciences. These primers amplified an 87-bp fragment of the rat's MKP-1 gene comprising the exon 1–2 boundary, to avoid amplification of genomic DNA. The amplification and quantification of MKP-1 and 18 S rRNA were performed using the ABI PRISM 7700 Sequence Detection System of PerkinElmer Life Sciences, using standard conditions. After fixing the base line in each experiment and the threshold for each amplification, two different values of threshold cycle were obtained for each replica: one for the amplification of MKP-1 and another for the amplification of 18 S rRNA. The corresponding standard curve. MKP-1 was then normalized to 18 S rRNA, and the mean relative quantity and S.D. for each set of replicates in each series were obtained. The mean relative quantity and S.D. of the 10 series were calculated, and values were expressed relative to basal level.

**Southern Blotting**—Plasmid DNA was loaded on a 0.7% agarose gel and electrophoresed at 1 V/cm. After transfer to a nitrocellulose membrane, the membrane was hybridized with 1 μg of a nick-translated 33P-labeled probe complementary to the rat MKP-1 gene cloned in pB.SK− (27) and probed with either ShuI or PstII restriction enzymes. The resulting 2892-bp fragment was cloned, sequenced, and aligned to the mouse and human MKP-1 gene. The sequence of the rat MKP-1 gene in GH4C1 was compared and corrected according to sequences amplified in rat pancreatic β INS-1 and rat vascular smooth muscle cells. The cloned GH4C1 sequence is available in the GenBank™ data base with the accession number AF357203.

**Reporter Gene Assays**—GH4C1 cells maintained in culture medium were detached from Petri dishes with trypsin, transiently transfected using FuGENE 6 transfection reagent (Roche Molecular Biochemicals), and seeded in 24-well multidishes (Falcon) at a density of 6 × 10⁴ cells/well. After 1 day, the medium was replaced by SFM 24 h before exposure to the various stimuli. Cells were stimulated with the addition of KCl, CPT-cAMP, 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 according to Promega’s Luciferase Reporter assay system instructions. Cells were co-transfected with Renilla luciferase expression plasmid to verify uniformity of transcription from experimentation to experimentation. Plasmid pM1 was generated by cloning the region of the rat MKP-1 gene extending from −631 to +19 between the KpnI and Nhel restriction sites of the pGL3 enhancer vector (Promega). Plasmid pSV40 was obtained by subcloning the SV40 promoter from the pGL3-control vector (+49 to +244) between the KpnI and Nhel restriction sites of the pGL3 enhancer vector. The “33320” construction was previously described (17, 21).
buffer. Isolated nuclei were resuspended in 100 μl of storage buffer (50 mM Tris-HCl, pH 8.3, 40% glycerol, 5 mM MgCl2, 0.1 mM EDTA), quantified to obtain 5 × 107 transcription-competent nuclei per aliquot and stored at −80 °C until use. Nuclear run-on was carried out as described by Collart et al. (27). After in vitro elongation, the same amount of labeled RNA from each reaction was hybridized to the pBSK(−) plasmid containing different DNA inserts from the rat MKP-1 locus: A position +25 to +464), B (SacII(+464) to SpeI(+824)), C (840 to +1469), D (1454 to +1903), E (StuI(+1854) to +2237), which had been immobilized on a N+ nylon membrane. Mouse c-fos oncogene fragments F (+53 to +764) and G (+418 to +1130), cloned in pBSK(−), were also blotted on the same membrane. pBSK(−) and GAPDH cDNA (+1 to +1220), subcloned in pBSK(−), were spotted and used, respectively, as a control for hybridization and an invariant internal control. Blots were developed by autoradiography and quantified with a Molecular Dynamics PhosphorImager.

RESULTS

Rapid Induction of MKP-1 mRNA by TRH and EGF in Pituitary GH4C1 Cells Depends on Transcriptional Activation—

FIG. 1. Rapid induction of MKP-1 mRNA by TRH and EGF in pituitary GH4C1 cells depends on transcriptional activation. A, serum-depleted (24 h) GH4C1 cells were stimulated with TRH (100 nM) or EGF (10 nM) for the indicated times. 10 μg of total RNA were analyzed by Northern blotting using [α-32P]CTP-labeled cDNA probes for MKP-1 and GAPDH, as described under “Experimental Procedures.” B, time course of MKP-1 mRNA induction by TRH and EGF; Phospho- 

FIG. 2. Induction by TRH of MKP-1 mRNA depends upon Ca2+ signaling; induction by EGF does not. After stimulation of GH4C1 cells with TRH and EGF under experimental conditions that do or do not allow Ca2+ signaling, MKP-1 mRNA levels were quantitatively assessed as follows. A, RNase protection assay of total RNA from GH4C1 cells isolated after 30 min of stimulation with either TRH or EGF. Where indicated, 0.6 mM EGTA was added 5 min before stimulation. 20 μg of total RNA were co-hybridized to two [α-32P]UTP RNA probes for MKP-1 and GAPDH, as described under “Experimental Procedures” and digested with RNase A and T1. Free probes, protected fragments, and the RNA CenturyTM size marker (Ambion) were resolved on a 6% polyacrylamide sequencing gel. B, quantification by PhosphorImager of the RNA levels detected by RNase protection in A (±S.E., n = 6). C, example of quantitative RT-PCR amplification records obtained for MKP-1 (upper panel) and 18 S mRNA (lower panel) under basal and TRH-stimulated conditions. Multiplex quantitative PCR amplification (TaqMan; PerkinElmer Life Sciences) was performed using sequence-specific primer pairs and probes corresponding to the junction of exons 1–2 of MKP-1 and to the transcribed region of the ribosomal 18 S RNA as described under “Experimental Procedures.” Threshold cycle values are indicated by circles. D, average (± S.E., n = 10) MKP-1 mRNA levels derived from threshold cycle values obtained in sextuplicate and plotted standard curves for MKP-1 were normalized for 18 S mRNA levels and then expressed as fold induction over basal values.

TRH elicits both acute and long term responses in rat pituitary clonal growth hormone (GH) cell types, leading to enhanced secretion of both prolactin and GH (28, 29). Activation of prolactin gene transcription and long term hormone secretion are preceded by the synthesis of the immediate early gene products, c-fos, junB, and c-jun, postulated to be involved in the regulation of the prolactin gene. TRH stimulates the transient induction of these immediate early genes via Ca2+-dependent mechanisms (30–32).
MKP-1 originally identified as an immediate early gene product, is induced by mitogens (5, 6), heat shock, or oxidative stress (8) and also by Ca\(^{2+}\) ionophore in cultured cells (33). As we show here by Northern blot analysis with a rat MKP-1 cDNA probe, TRH also stimulates the expression of MKP-1 (Fig. 1). TRH induction is as strong and rapid as with the mitogenic stimulus EGF, which was used as a positive control for MKP-1 induction. GH4C1 cells exposed to 100 nM TRH (Fig. 1) showed maximally enhanced levels of MKP-1 mRNA as early as 30 min, followed by a rapid decline to lower but still significantly elevated levels at 60 min which were sustained up to 6 h. Slightly slower induction kinetics were observed for EGF, with MKP-1 mRNA levels peaking at 45 min (Fig. 1, A and B). After more than 6 h of treatment, the level of MKP-1 mRNA was higher for TRH-stimulated cells compared with EGF-treated cells, where the MKP-1 message returned almost to the basal level (Fig. 1B). GAPDH mRNA levels were not changed by either TRH or EGF stimulation and can thus be used as an invariant internal control. Based on the kinetics shown in Fig. 1, MKP-1 expression was assessed in all subsequent experiments at 30 min after TRH or EGF stimulation.

To show that TRH- or EGF-induced MKP-1 mRNA accumulation is linked to transcriptional activation of the gene, we analyzed the effect of the transcriptional inhibitor actinomycin D. Actinomycin D (Act D) completely suppressed MKP-1 mRNA induction by TRH and EGF (Fig. 1C, lanes 6 and 9). In addition, the protein synthesis inhibitor cycloheximide (CHX) did not prevent TRH or EGF induction of MKP-1, confirming that MKP-1 is an immediate early gene. In contrast to what was observed for other immediate early genes in GH cells (31), cycloheximide, per se, did not induce MKP-1. Nor did cycloheximide potentiate TRH or EGF induction of MKP-1 mRNA (Fig. 1C, lanes 3, 4, 7, and 10). Instead, cycloheximide treatment lowered MKP-1 mRNA levels under stimulated conditions (compare lanes 5 and 8 with lanes 7 and 10, respectively).

Induction of MKP-1 mRNA by TRH Depends upon Ca\(^{2+}\) Signaling, and Induction by EGF Does Not—In GH4C1 cells, the increase of [Ca\(^{2+}\)]\(_i\) stimulated by TRH consists of two phases: an initial spike, due to intracellular Ca\(^{2+}\) store mobilization, followed by a “plateau” phase of enhanced action potential firing, causing spikes of [Ca\(^{2+}\)]\(_i\), due to Ca\(^{2+}\) influx during action potentials (34, 35). Previous reports have shown that the prolonged phase of [Ca\(^{2+}\)]\(_i\) spiking was essential to enhance and maintain proto-oncogene mRNA levels over time (31, 32, 36). To examine the role of Ca\(^{2+}\) influx in MKP-1 expression induced by TRH, GH4C1 cells were preincubated for 5 min in serum-free medium containing 0.6 mm EGTA (free [Ca\(^{2+}\)]\(_i\) < 0.1 μM). This procedure has little effect on the initial spike of [Ca\(^{2+}\)]\(_i\), but prevents Ca\(^{2+}\) action potentials and the
involved in Ca\(^{2+}\)-dependent and Ca\(^{2+}\)-independent transcriptional activation of the MKP-1 gene GH4C1 cells, we cloned and sequenced the rat MKP-1 gene. The mouse and the human MKP-1 gene had been cloned previously in other studies (6, 37). PCR was performed with GH4C1 genomic DNA and primers situated in two regions that are conserved in the promoter and the exon 4 of the mouse and the human genes (see “Experimental Procedures”). The sequence of the resulting 2892-bp PCR fragment was 66% identical to the mouse MKP-1 gene. To verify that this fragment corresponds to the rat MKP-1 gene and does not contain any repetitive sequences, Southern blotting was performed with GH4C1 genomic DNA digested with StuI or PstI restriction enzymes (Fig. 3A). DNA probes corresponding to the region of the promoter or the introns in the 2892-bp PCR fragment hybridize to the same band as the exon 1 probe and do not cross-react with other genomic DNA fragments. This indicates that the different probes are specific for a single gene, namely the rat MKP-1 sequence. Alignment of the rat, mouse, and human MKP-1 sequences shows that the critical promoter elements identified in the mouse and human promoters are conserved in the rat promoter (Fig. 3B).

To map the transcriptional start site in the rat gene, an antisense RNA probe overlapping the TATA box and the MKP-1 ORF was designed. RNase protection assays with total RNA from TRH-stimulated GH4C1 cells showed two protected fragments: a major band at 183 nt and a minor band at 185 nt. The antisense DNA sequence starting at the MKP-1 ORF run in parallel with the protected fragments allows us to determine the position of initiation of transcription in the MKP-1 sequence (Fig. 3C). The two transcriptional start sites identified in the rat MKP-1 gene are located at the same positions as those determined for the mouse and the human gene, at 20 and 22 residues, respectively, downstream of the consensus TATA sequence (Fig. 3B).

Limited Stimulation of Reporter Gene Expression Driven by the Rat MKP-1 Promoter—The search for regulatory elements involved in Ca\(^{2+}\)-dependent and Ca\(^{2+}\)-independent activation of MKP-1 transcription was started using a reporter gene approach. The promoter region −631 to +19 of the rat MKP-1 gene was inserted into a luciferase reporter vector. GH4C1 cells were transiently transfected and subsequently stimulated with a variety of agonists. As shown in Fig. 4, luciferase expression obtained with this construct was already high under unstimulated basal conditions and only marginally (2-fold) increased following stimulation of the GH4C1 cells by KCl plus cAMP, TRH, or EGF. The lack of induction was not due to saturation of the system, since we obtained similar results in cells transfected with lower plasmid concentrations, and a linear relation was observed between plasmid concentration and luciferase expression in the range of concentrations used routinely (data not shown).

As a further control, we compared the MKP-1 reporter construct with an SV40 promoter construct and with a “c-fos/intron” construct that contains the sequence from the promoter to exon 2 fused to the ORF of the luciferase gene (25, 26). These reporter constructs were compared in basal and stimulated conditions with KCl plus cAMP, TRH, or EGF (Table I). As reported previously (25), expression of luciferase from the c-fos construct is highly induced in all three stimulated conditions, whereas with the MKP-1 promoter, stimulation is limited to a 1.5–1.7-fold increase. Comparing the reporter data from the MKP-1 promoter with the SV40 promoter constructs suggests that the MKP-1 promoter behaves very much like a constitutive promoter in this cell line. This may indicate that the 650-bp fragment of the rat MKP-1 gene contains sufficient promoter elements to drive the expression of the reporter gene.

**Cloning of the Rat MKP-1 Gene and Identification of the Transcriptional Initiation Site**—To elucidate the mechanisms involved in Ca\(^{2+}\)-dependent and Ca\(^{2+}\)-independent transcriptional activation of the MKP-1 gene GH4C1 cells, we cloned and sequenced the rat MKP-1 gene. The mouse and the human MKP-1 gene had been cloned previously in other studies (6, 37). PCR was performed with GH4C1 genomic DNA and primers situated in two regions that are conserved in the promoter and exon 4 of the mouse and the human genes (see “Experimental Procedures”). The sequence of the resulting 2892-bp PCR fragment was 66% identical to the mouse MKP-1 gene. To verify that this fragment corresponds to the rat MKP-1 gene and does not contain any repetitive sequences, Southern blotting was performed with GH4C1 genomic DNA digested with StuI or PstI restriction enzymes (Fig. 3A). DNA probes corresponding to the region of the promoter or the introns in the 2892-bp PCR fragment hybridize to the same band as the exon 1 probe and do not cross-react with other genomic DNA fragments. This indicates that the different probes are specific for a single gene, namely the rat MKP-1 sequence. Alignment of the rat, mouse, and human MKP-1 sequences shows that the critical promoter elements identified in the mouse and human promoters are conserved in the rat promoter (Fig. 3B).
MKP-1 Transcriptional Regulation

**TABLE I**

Quantification of MKP-1 promoter activity in GH4C1: comparison with the SV40 promoter and with the c-fos/intron constructs

| Construct       | Induction over basal condition | RLU luc/ren (basal) |
|-----------------|-------------------------------|---------------------|
|                 | KCl + CPT-cAMP | TRH | EGF |
| pMKP-1          | 1 ± 0.1          | 1.5 ± 0.1 | 1.6 ± 0.1 | 1.27 ± 0.1 |
| pSV40           | 1 ± 0.1          | 1.1 ± 0.1 | 1.0 ± 0.1 | 0.30 ± 0.03 |
| c-fos/intron    | 1 ± 0.1          | 10.7 ± 0.7 | 11.6 ± 0.9 | 14.2 ± 0.1 | 0.11 ± 0.01 |

Luciferase activities measured on Fig. 4 are plotted as fold induction over basal conditions for each construct (± S.E., n = 4). In the last column, the basal luciferase activity of the three constructs is shown normalized to constitutive renilla luciferase expression in the same extracts (RLU luciferase/renilla ± S.E., n = 4). The same amount of both plasmids was transfected in all cases (see Experimental Procedures). The pMKP-1 construct contains the region −631 to +19 of the rat MKP-1 gene; pSV40 contains the promoter SV40 of the pGL3-control vector (Promega); and the c-fos/intron construct comprises the region of the mouse c-fos oncogene from the promoter to exon 2 fused to the ORF of the luciferase reporter gene. The transcriptional activity of this construct in GH4C1 was previously described elsewhere (25).

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**FIG. 5.** Identification of a calcium-sensitive elongation block in the region exon 1-intron 1 of the MKP-1 gene. GH4C1 cells were stimulated or not (basal) for 25 min with TRH (100 nM) or EGF (10 nM). When indicated, 0.6 mM EGTA was added 5 min prior to stimulation to chelate-free calcium in the medium. After isolation of the nuclei and nuclear run-on transcription, labeled RNAs were hybridized to DNA fragments spotted on a membrane. Fragments A–E correspond to the MKP-1 gene; fragments F and G correspond to the c-fos oncogene, pBSK, and GAPDH cDNA. Extension of the probes A–G is presented on the MKP-1 and c-fos genomic maps; a precise description is given under “Experimental Procedures.” Shown is a typical experiment, repeated five times for basal, TRH, and TRH/EGTA conditions and three times for EGF and EGF/EGTA conditions. Quantitative analysis of these data is presented in Table II.

but lacks control elements that restrict basal expression and thereby permit strong activation both by Ca\(^{2+}\)-dependent (KCl plus cAMP; TRH) and Ca\(^{2+}\)-independent (EGF) stimuli. The discrepancy between the highly controlled endogenous expression of MKP-1 (Fig. 2) and the lack of control by the same stimuli with the reporter gene expression system (Fig. 4) suggests that important regulatory steps may require elements that are not situated in the promoter region, defined here between −631 and +19.

**MKP-1 Transcription Is Controlled by a Calcium-sensitive Elongation Block in the First Exon.—**To analyze in more detail the transcriptional control of MKP-1 and determine if the gene is also regulated at steps different from initiation (e.g. elongation), we performed nuclear run-on experiments with nuclei isolated from GH4C1 cells after stimulation with TRH or EGF (Fig. 5). Nascent RNAs transcribed in nuclei isolated from stimulated and nonstimulated cells were labeled by in vitro elongation with [\(\alpha^{-32}P\)]UTP, purified, and hybridized to unlabelled DNA fragments spotted on a membrane. These fragments include sequences spread out along the MKP-1 gene (Fig. 5, A–E), two fragments (F and G) corresponding to exon 1 and intron 1 of the mouse c-fos oncogene, the GAPDH gene as a control of transcription, and pBSK as a negative control (see “Experimental Procedures”). As shown in Fig. 5, MKP-1 transcription is initiated under basal conditions (signal in A, lane 1) but does not proceed to the end of the gene. The polymerase is blocked at the exon 1-intron 1 junction. When cells are stimulated with TRH or EGF (lanes 2 and 4), the block to elongation is released, and MKP-1 gene transcription is completed (signal in E). To determine the effect of Ca\(^{2+}\) signals on the block of elongation in MKP-1 gene transcription, nuclear run-on assays were performed with cells stimulated with TRH or EGF in low extra-cellular Ca\(^{2+}\). The addition of 0.6 mM of EGTA prior to TRH stimulation decreases transcription efficiency at the level of the first intron (probes B and D) more than in the first exon (probe A); the ratios B/A and D/A are significantly reduced by EGTA in TRH-stimulated conditions (Table II, bottom). This indicates that TRH-induced Ca\(^{2+}\) signals are important to release the block of elongation. Stimulation with EGF in the presence or absence of EGTA both activate MKP-1 transcription at the level of initiation and elongation. A quantitative analysis of these results is shown in Table II. The signals for fragments A–E were corrected for U content and normalized to the signal of GAPDH (Table II, top). By comparing the signal intensities (as fold induction over basal condition) observed in nuclear run-on assays, for the probes A, B, and D, with the level of induction of MKP-1 messenger measured by RNASe protection assays or TaqMan (Fig. 2), we show that for any condition of stimulation, the level of induction for the probes B and D is similar to the one observed for the MKP-1 mRNA (Table II, bottom). The rise of initiation of transcription under stimulated conditions (monitored on signal A) is not sufficient to account for the level of induction of the MKP-1 mRNA. Additional activation of elongation is necessary to maximally stimulate MKP-1 gene expression. These results together with those reported in Fig. 4 suggest that MKP-1 transcription is controlled by a constitutively active promoter and that a major contribution to gene transcription is conferred by a strong block of elongation within the gene.

A similar result was obtained with the c-fos oncogene in nuclear run-on assays performed with GH4C1 nuclei (probes F and G, Fig. 5). The calcium dependent block to elongation in the intron 1 of c-fos was previously described in microendocrine cells (25) and other cell lines (20–23).

To localize more precisely the block of elongation in the MKP-1 gene, we prepared restriction fragments of the exon 1-intron 1 region and used these probes to map the pause site by nuclear run-on assays. Digested products were separated by gel electrophoresis, transferred to a membrane, and hybridized with labeled RNAs isolated from nuclear run-on reactions performed with basal or TRH-stimulated cells. DNA fragment that does not hybridize in basal conditions but does hybridize in the stimulated conditions indicates that the polymerase was blocked before reaching the sequence corresponding to that
DNA fragment. DNA digested by SacII, NheI, and HindIII (Fig. 6A, lane 1) showed, after nuclear run-on in basal conditions, a hybridized fragment SacII-NheI but not SacII-HindIII (Fig. 6B, lane 1), indicating that the polymerase was blocked before reaching the intron 1. DNA digested by NcoI, NheI, and HindIII (Fig. 6A, lane 2) showed, after nuclear run-on, that the three fragments NcoI-NheI, NcoI-NcoI, and NcoI-HindIII are all hybridized by nascent RNA in basal conditions (Fig. 6B, lane 2). The weak signal corresponding to the NcoI-HindIII fragment in basal condition suggests that RNA polymerase II has transcribed part of the sequence between NcoI and SacII but was blocked before entering intron 1. Taken together, these results show that the block of elongation in MKP-1 is located in the exon 1 possibly in the first 300–400 nt of the 5′-end of the gene. Further fine mapping by KMnO₄ footprinting (18) and nuclear run-on performed with antisense oligonucleotide probes (38) will permit us to determine if the block to transcriptional elongation correlates with promoter-proximal pauses sites and/or intrinsic sites of premature termination in the exon 1 of MKP-1.

**DISCUSSION**

This study identified a calcium-sensitive block to elongation within the first exon of the rat MKP-1 gene as an important element for transcription regulation. TRH strongly stimulates MKP-1 transcription by enhancing initiation and elongation by Ca²⁺-sensitive mechanisms. Stimulation by EGF resulted in a similar rise in MKP-1 transcription, based again on enhanced initiation and elongation but (in contrast to TRH) without the need for Ca²⁺ signaling. The involvement of a calcium-sensitive elongation block in MKP-1 gene expression is reminiscent of c-fos expression for which a block to elongation is an essential element (Fig. 6 and Refs. 20–23 and 25). This suggests that common mechanisms of intragenic regulation may be conserved between MKP-1 and c-fos.

Change in MKP-1 and c-fos gene expression level, via potential deregulation of the block to elongation, may have physiological importance in tumorigenesis. For c-fos, it is well known that this oncogene is up-regulated in several tumor cells (e.g. 39–41). For MKP-1, several studies have shown an overexpression of both the MKP-1 mRNA and protein at different stages of breast and prostate carcinoma (42–45). These latter reports have suggested that up-regulation of MKP-1 may contribute to tumor growth by inactivating preferentially stress-activated MAP kinases (stress-activated protein kinase/Jun N-terminal kinases, p38) that can promote apoptosis in tumor cells exposed to toxic stimuli.

The dual specificity MAP kinase phosphatases, DSPs or MKPs, inactivate MAP kinases by dual dephosphorylation. In turn, MKPs can be controlled in at least three ways: first by subtype-specific enhancement of their catalytic activity triggered by direct interaction with MAP kinases (46, 47); second by MAP kinase phosphorylation (in the case of MKP-1), which stabilizes the otherwise very labile protein (48); and third by transcriptional activation of some MAP kinase phosphatases (e.g. MKP-1 and MKP-2) encoding for immediate early genes (4).

Transcription of the MKP-1 gene can be induced rapidly by multiple intracellular signals, including active mitogenic or stress-activated MAP kinase cascades, protein kinase C, cAMP, and Ca²⁺ signals (5–10, 33). Prior to our study, the induction of the human MKP-1 gene in HeLa cells, by the mitogenic stimuli EGF, fibroblast growth factor, or platelet-derived growth factor, was thought to be essentially mediated by activation through the promoter of the gene (6).

In our quantitative study, we show a clear discrepancy between the levels of induction for the MKP-1 mRNA and reporter gene assays with the promoter of MKP-1. We observed an 18-fold stimulation of the MKP-1 mRNA by TRH and EGF (Fig. 2D), which contrasts with the 1.5- and 1.6-fold stimulation, respectively, of MKP-1 reporter gene expression from the promoter (Table I). This difference can have two possible explanations: (i) chromatin structure influences the basal activity of the gene and may tightly be regulated following stimulation, and (ii) further 5′- or 3′-end sequences not present in the promoter construct may be important to control MKP-1 gene transcription. We showed (Fig. 5) that a controlled release of a block to elongation in the exon 1 cells may explain part of this
MKP-1 Transcriptional Regulation

Calcium is a critical modulator of immediate early gene expression at different levels in mammalian cells. In contrast to a number of studies that examine the role of Ca\(^{2+}\) signaling by using Ca\(^{2+}\) ionophores and other nonphysiologic stimuli, we describe here the role of physiological Ca\(^{2+}\) signaling triggered by the releasing factor TRH in MKP-1 gene expression. TRH stimulates the release of Ca\(^{2+}\) from internal stores and provokes a prolonged precisely patterned Ca\(^{2+}\) influx, due to Ca\(^{2+}\) action potentials (34, 35). GH4C1 cells stimulated by TRH in medium with low free extracellular Ca\(^{2+}\) result in the suppression of sustained intracellular Ca\(^{2+}\) signals. (36, 38). We demonstrated marked differences in MKP-1 gene expression in the absence or presence of extracellular Ca\(^{2+}\) after TRH stimulation, which defines a physiological role for calcium in MKP-1 induction associated with the release of the block to elongation. In addition to Ca\(^{2+}\) signaling, TRH activates other pathways, notably through cAMP and MAP kinases. The residual TRH effects seen for MKP-1 transcription (Table II) in the absence of sustained Ca\(^{2+}\) signaling are probably due to such additional pathways.

How does Ca\(^{2+}\) control MKP-1 gene transcription? Since very few reports have studied the regulation of MKP-1 gene expression, we can only speculate on responsive elements in the MKP-1 gene and relate them to known functional determinants in other Ca\(^{2+}\)-regulated genes (e.g. c-fos). First, considering homologies between the rat, mouse, and human MKP-1 promoters, we find two conserved CRE elements and one E box motif. The CRE element and its binding protein CREB are known to mediate calcium signals in the nucleus for several genes including the c-fos oncogene. Ca\(^{2+}\)-activated gene transcription is mediated through phosphorylation of CREB and the CREB-binding protein by CaM kinase IV. CREB phosphorylation is also a target for multiple signal transduction pathways, which involve different activated protein kinases (49). Thus Ca\(^{2+}\)-, mitogenic, or stress-activated MAP kinases may control MKP-1 transcription, as in c-fos, through phosphorylation of CREB transcription factors at the level of initiation. Consistent with this idea, Sommer et al. (10) have reported that the upstream stimulatory factor that binds the E-box motif in the promoter of MKP-1 cooperates with signals that stimulate CRE-dependent transcription.

Other regulatory elements may be involved to control MKP-1 transcription by calcium. Recently, a Ca\(^{2+}\)-regulated transcriptional repressor (DREAM) that binds to the DRE element has been show to control the transcriptional activity of the c-fos oncogene (50). The DRE element in the human c-fos oncogene is located at 20 nt downstream of the transcriptional initiation site. A similar consensus DRE element is located in the first 10 bp of the rat, mouse, and human MKP-1 gene that could be a target for DREAM-mediated transcriptional repression. Alternatively, Ca\(^{2+}\) regulation of MKP-1 gene transcription may

difference between exogenous and endogenous expression of MKP-1. Although quantitative data in reporter gene assays and nuclear run-on are not directly comparable for technical reasons, the abundance of nascent transcripts extending beyond the elongation block is enhanced 10–20-fold, respectively, by TRH and EGF, with a concomitant increase of only 4–6-fold in early transcripts (signal A, Table II); this result is again compatible with an important contribution of elongation control to the transcriptional activation.

The transient transcriptional activation of MKP-1 by TRH and EGF results in a significant enhancement of MKP-1 mRNA at 30 min (Fig. 1) but also at the protein level 1 h after stimulation (data not shown). It becomes thus evident that a tight control of MKP-1 gene transcription at the level of initiation and elongation, combined with its differential binding and catalytic activation by specific MAP kinases, provides a sophisticated mechanism for rapid and targeted inactivation of selected MAP kinase cascades.

Calcium is a critical modulator of immediate early gene expression in macrophages (36), but in cells from other sources, a quantification of the hybridization signals by film densitometry and normalized for U content gave the following arbitrary values: SacII-NheI (1450 versus 2460), SacII-HindIII (260 versus 1320); NcoI-NcoI plus NcoI (720 versus 1067), and NcoI-HindIII (150 versus 1220) for basal versus TRH stimulated nuclei, respectively.

![Fig. 6. The block to elongation is localized in exon 1 of the MKP-1 gene.](image-url)
depend on transcription factors translocated to the nucleus by the calcium-activated phosphatase calcineurin (51, 52) or on an alternative Ca$^{2+}$ signaling pathway sensitive to the calmodulin antagonist W7. Coulon et al. (23) have reported that this latter calcium signaling pathway affects the intragenic regulation of c-fos expression via suppression of a transcriptional pause site.

In summary, we have identified a novel gene, MKP-1, which is regulated at the level of elongation of transcription in a manner similar to the c-fos oncogene. While the mechanisms that control transcriptional elongation remain relatively unclear, MKP-1 gene expression studies conducted in parallel with the analysis of a similar phenomenon in c-fos expression are certainly useful to further advance our knowledge of the regulation of transcriptional elongation in eukaryotes and on immediate early gene expression in mammalian cells.

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