

Molecular cloning of the Matrix Gla Protein gene from *Xenopus laevis*

Functional analysis of the promoter identifies a calcium sensitive region required for basal activity

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To analyze the regulation of Matrix Gla Protein (MGP) gene expression in *Xenopus laevis*, we cloned the xMGP gene and its 5' region, determined their molecular organization, and characterized the transcriptional properties of the core promoter. The *Xenopus* MGP (xMGP) gene is organized into five exons, one more as its mammalian counterparts. The first two exons in the *Xenopus* gene encode the DNA sequence that corresponds to the first exon in mammals whereas the last three exons show homologous organization in the *Xenopus* MGP gene and in the mammalian orthologs. We characterized the transcriptional regulation of the xMGP gene in transient transfections using *Xenopus* A6 cells. In our assay system the identified promoter was shown to be transcriptionally active, resulting in a 12-fold induction of reporter gene expression. Deletional analysis of the 5' end of the xMGP promoter revealed a minimal activating element in the sequence from -70 to -36 bp. Synthetic reporter constructs containing three copies of the defined regulatory

element delivered 400-fold superactivation, demonstrating its potential for the recruitment of transcriptional activators. In gel mobility shift assays we demonstrate binding of *X. laevis* nuclear factors to an extended regulatory element from -180 to -36, the specificity of the interaction was proven in competition experiments using different fragments of the xMGP promoter. By this approach the major site of factor binding was demonstrated to be included in the minimal activating promoter fragment from -70 to -36 bp. In addition, in transient transfection experiments we could show that this element mediates calcium dependent transcription and increasing concentrations of extracellular calcium lead to a significant dose dependent activation of reporter gene expression.

Keywords: Matrix Gla protein; gene expression; *Xenopus*; DNA-binding, calcium.

Matrix Gla protein (MGP) is an 84-residue secreted protein originally isolated from bovine bone [1] and was later shown to accumulate in bone in different mammals [2,3] as well as in amphibians [4] and in shark vertebra [5]. Its mRNA has been detected in bone, cartilage and in soft tissues such as heart, kidney, and lung in a variety of species [4,6,7]. MGP is also secreted *in vitro* by a number of cell lines of different origins including human MG63, MCF7, several smooth muscle-derived cell lines and rodent cell lines such as NRK, UMR106 and Ros17/2.8 [8–13]. The primary structure of MGP includes a signal peptide, a phosphory-

lation domain, and a γ -carboxylase recognition site. Additionally, MGP contains five residues of gamma-carboxylated glutamic acid (Gla), through which MGP and all other members of this vitamin K-dependent protein family can bind to mineral and, in particular, calcium-containing-mineral such as hydroxyapatite [2].

Although the exact mode of action of MGP at the molecular level is currently unknown, the spontaneous calcification of arteries and cartilage in mice lacking MGP indicates that it functions as an inhibitor of mineralization [7]. There is evidence from mouse models showing that ectopic calcification progresses unless actively inhibited, and that MGP is absolutely required to actively prevent this process (reviewed in [14]). The available data also show that MGP is involved in protecting tissues from ectopic calcification in humans [15,16]. In chicken, on the other hand, MGP functions as a developmental inhibitor of cartilage mineralization, playing a role in the regulation of ossification and chondrocyte maturation during early limb development [17]. Therefore, MGP must be expressed in areas where progression of calcification takes place in order to counteract ectopic calcification, suggesting the presence of a calcium sensing mechanism in specific target cells that are capable of modulating MGP gene transcription. This signal could be extracellularly monitored as osmotic stress or

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Abbreviations: MGP, Matrix Gla Protein.

Note: the complete *Xenopus laevis* MGP gene sequence was submitted to the GenBank under the accession number AF234631.

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might be mediated by a transmembrane protein acting as a calcium sensing receptor as previously suggested by the work of Farzaneh-Far *et al.* [18]. However, nothing is known about how this signal is conveyed to the nucleus, and few data on the regulation of MGP transcription are available.

Cell culture experiments have shown that MGP can be regulated *in vitro* by 1,25-(OH)₂ vitamin D₃ and retinoic acid as well as by growth factors and cell proliferation events [8–12], but to date only a regulatory element for retinoic acid has been identified in the human MGP promoter [12]. Furthermore, it has been shown that point mutations within the human MGP promoter alter binding of an AP1 complex. This has been demonstrated to influence MGP transcription rates and, in turn, to result in changes in MGP serum levels [19], but the mechanisms responsible for the transcriptional regulation of MGP still remain largely unknown.

The purification of MGP from lower vertebrates such as amphibians and sharks [4,5] has provided clear evidence that the protein motifs required for adequate cellular processing and calcium binding through specific gamma carboxylated glutamic acid residues have been conserved throughout the last 400 million years of vertebrate evolution. In addition, as already described for mammalian and bird development [7,17], MGP in amphibians was detected early in development prior to the onset of calcification [4]. Taken together, these data suggest that the function of MGP is evolutionarily conserved and thus make animals such as *Xenopus laevis* a suitable model system to further analyze MGP gene expression. In this report we present the cloning and organization of the MGP gene from *X. laevis* and the functional characterization of its 5' promoter region. In transient transfection experiments using different deletion mutants of the *X. laevis* MGP gene promoter (xMGP) we have identified a 35-bp DNA sequence located between -70 and -36 that is capable of mediating basal transcription of xMGP. Furthermore, we demonstrate specific binding of *Xenopus* nuclear factors to the characterized minimal activating promoter and show that this element is responsible for the mediation of transcriptional calcium sensitivity.

MATERIALS AND METHODS

Cloning of the *Xenopus* MGP gene

Full length xMGP cDNA (AF055588.1) was used to screen a genomic library derived from partially digested *Xenopus* DNA cloned into the EMBL-3 bacteriophage (obtained from I. Dawid, NIH, Bethesda, MD, USA). Altogether, 1.8 × 10⁶ phage plaques were screened, one positive clone was obtained and plaque-purified following standard procedures [20]. Selected genomic restriction fragments were subcloned into pBSSK (Stratagene). The structure of the gene including the 5' and 3' flanking regions was determined by double-stranded DNA sequencing, exons were identified according to the sequence of the *Xenopus* MGP cDNA.

Primer extension analysis

Total RNA was prepared from *X. laevis* bone extracts (previously shown to express the MGP gene, Cancela *et al.* 2001) by the acid guanidium isothiocyanate procedure [21]. Fifteen micrograms of RNA were coprecipitated with

10 pmol of ³²P-labeled reverse primer (5'-GATGTCTTT TCAATGGTAGCTTCTTCAG-3'), dissolved in 15 µL hybridization buffer (10 mM Tris/HCl pH 8.3, 150 mM KCl, 1 mM EDTA) and denatured at 90 °C. Primers were annealed at 65 °C for 90 min, extension was performed using 10 U of MMLV reverse transcriptase (GibcoBRL) in 10 mM Tris/HCl (pH 8.3), 5 mM MgCl₂, 50 mM KCl, 0.15 mg·mL⁻¹ actinomycin D, 10 mM dithiothreitol and 1 mM dNTP at 37 °C for 60 min. Reactions were stopped by addition of 105 µL of RNase reaction mix (100 µg·mL⁻¹ calf thymus DNA and 20 µg·mL⁻¹ RNase A). The extended products were ethanol precipitated, washed with 70% ethanol and analyzed on 6% denaturing polyacrylamide gels in 1 × Tris/borate/EDTA at room temperature. Gels were dried and subjected to autoradiography.

Cell culture and transfection

The *X. laevis* cell line A6 (derived from kidney epithelial cells, ATCC# CCL102) was cultured at 24 °C in 0.6 × L15 medium supplemented with 5% fetal bovine serum and 1% antibiotics (all GibcoBRL). Cells were seeded at 60% confluence in 12-well plates and transient transfections were carried out using the standard calcium phosphate coprecipitation technique [22]. To evaluate dose-dependent effects of extracellular calcium on MGP transcription cells were grown in medium supplemented with either calcium chloride (Sigma) or water 24 h after transfection. Luciferase activity was assayed as recommended by the manufacturer (Promega) in a ML3000 luminometer (Dynatech). Relative light units were normalized to β-galactosidase activity and protein concentration using the Bradford dye-assay (Bio-Rad). All experiments were repeated at least five times.

Isolation of *X. laevis* genomic DNA and genomic Southern blot analysis

A6 cells were harvested upon confluence, genomic DNA was prepared following the established protocol (Sambrook *et al.* [20]). DNA was digested with selected restriction endonucleases and separated on 0.8% agarose gels, then transferred to 0.45 µm Nytran nylon membranes (Schleicher & Schuell). The *X. laevis* MGP probe was radio-labeled with [α-³²P]dCTP (Amersham) using the Prime-it-II labeling kit (Stratagene). Membranes were prehybridized 3 h at 42 °C and probes were hybridized at 42 °C for 18 h in the buffers recommended by the manufacturer. Unspecific radioactivity was removed by two washing steps (15 min) at room temperature in 6 × SSC (1 × SSC: 150 mM NaCl, 15 mM Na citrate, pH 7.0) containing 0.1% SDS followed by two washing steps (15 min) at 65 °C in 1 × SSC 0.1% SDS. Membranes were exposed to X-ray films and hybridization was visualized by autoradiography.

Reporter plasmids

xMGP luciferase reporter plasmids -949LUC, -783LUC and -54LUC were generated by PCR amplification with the common reverse oligonucleotide (5'-CACGCAAGCTTCT CTTGAGTCTCTATGAAGG-3') and the 5' specific oligonucleotides (5'-CCGGAGCTCGAGACTCTTAGTAA ATGTGCC-3') for amplification of the fragment from -949 to +33 (5'-CCGGAGCTCGAGCCGCTAAAGA

GGAAAC-3') for amplification of the region from -783 to +33, and (5'-CCGGGAGCTCGAGGGAGATGAGGAGGTGTGG-3') for amplification of the region from -54 to +33, respectively. Newly introduced restriction sites are underlined. All DNA fragments were *Xba*I and *Hind*III digested and inserted into pGL2LUC (Promega). All numbers indicated are in relation to the transcriptional start site. The constructs -648LUC, -464LUC, -185LUC, -949/-326LUC and -949/-708LUC were generated by restriction digestion and the fragments of interest (spanning the regions -648 to +44, -464 to +44, -185 to +44, -949 to -326, and -949 to -708, respectively) were blunt ended and inserted at the *Sma*I site of pGL2LUC. The constructs -180/-36TATALUC and -180/-72TATALUC were generated by PCR amplification with a common, sense oligonucleotide (5'-CGGGGATCCAACTCTGGCTAAATTAGG-3') and the 3' specific oligonucleotides (5'-GAAGATCTACCACACCTCCTCATCTCC-3') for amplification of the region from -180 to -36 and (5'-GAAGATCTAACTAGATTTCACCATTGG-3') for amplification of the region from -180 to -72, respectively. The -134/-36TATALUC construct was PCR amplified with the oligonucleotides (5'-CGGGGATCCCATGTGGGTTTCCATTCTCC-3') and (5'-GAAGATCTACCACACCTCCTCATCTCC-3'), spanning the region from -134 to -36. Newly introduced restriction sites are underlined. All DNA fragments were *Bam*HI and *Bgl*II digested and inserted into pTATALUC [23]. The construction of the -70/-36TATA LUC and 3x(-70/-36)TATALUC involved the cloning of one or three copies of double stranded oligonucleotides spanning the region from -70 to -36 of the xMGP promoter (5'-GATCCAGGGGAGGGAAAACAAGGAGATGAGGAGGTGTGGT-3', and 5'-GATCTACCACACCTCCATCTCCTGT~~TT~~CCCTCCCTCG-3') as *Bam*HI/*Bgl*II fragments into pTATALUC. All constructs were verified by double stranded DNA sequencing. Transfection efficiencies were monitored using the control plasmid pTk-LUC [24].

DNA binding studies

Whole cell extracts were prepared exactly as described by Buettner *et al.* (1993) [25]. Six micrograms of extract were mixed with 1 µg poly(dI/dC) as nonspecific DNA competitor in sample buffer (10 mM Tris/HCl pH 8.0, 40 mM KCl, 0.05% Nonidet P-40, 6% (v/v) glycerol, 1 mM dithiothreitol). The -180/-36 bp DNA fragment was labeled by Klenow polymerase (New England Biolabs) fill in reaction using [α -³²P]dATP (Amersham Pharmacia). ³²P-labeled oligonucleotide probe (0.5 ng) were added to the reaction mixture. Complexes were allowed to form on ice for 30 min. Samples were separated on 5% nondenaturing polyacrylamide gels at 4 °C in 0.5 × Tris/borate/EDTA. Gels were dried and subjected to autoradiography.

RESULTS

X. laevis MGP gene structure and organization

Screening of the *X. laevis* genomic library using the ³²P-labeled xMGP-cDNA identified one positive clone (spanning \approx 12 kb of chromosomal DNA) which was further analyzed by restriction mapping and Southern

blotting. The nucleotide sequence of the entire structural gene and its adjacent 5' and 3' flanking regions was determined (submitted as GenBank accession number AF234631). The sequence spanning from -981 to +69 is present in Fig. 1. The xMGP gene spans 8071 bp and is organized into five exons, identified according to the sequence of the full length xMGP cDNA [4] and by comparison with the corresponding mouse [26] and human [27] genes. The sequence on either side of each exon-intron junction (Table 1) is conform to the GT/AG rule for splice donor and acceptor sites as described by Breathnach & Chambon [28]. Exon I in the mammalian genes (mouse and human, Table 2) is represented by two exons in the *X. laevis* genome (exons IA and IB) because an additional intron (intron 1) is localized within the 5' untranslated region (UTR) of the *X. laevis* MGP gene. A comparison between the xMGP gene and other known MGP genes (mouse and human) indicates that all other introns (2, 3 and 4) are located at conserved sites within the MGP coding sequence (Fig. 2). Analysis of the phase of each of the xMGP introns located within the coding region revealed that introns 2 and 3 are of phase I while intron 4 is of phase II [29]. The same phases are found in the corresponding introns of the mouse and human genes. The consensus polyadenylation signal AATAAA is located in the 3' UTR at nucleotide +8049. Genomic Southern analysis using *Eco*RI restriction digestion is consistent with the presence of a single copy gene for xMGP (Fig. 3). However, Southern analysis with *Bam*HI (Fig. 3A) shows additional fragments that cannot be accounted from the known *Bam*HI restriction pattern within the xMGP gene (Fig. 3B).

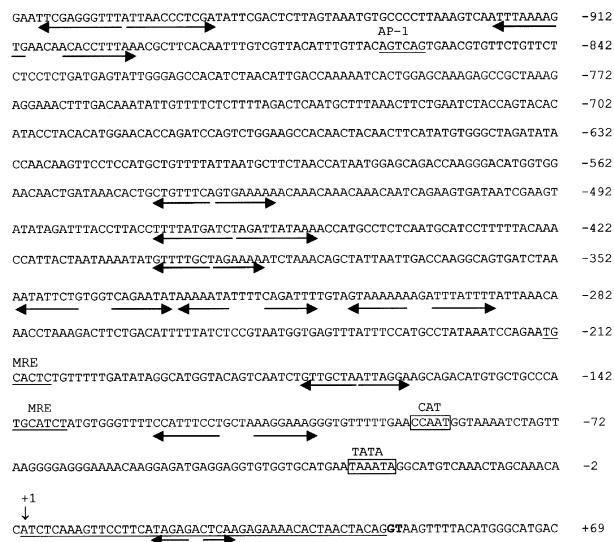


Fig. 1. Sequence of the *X. laevis* MGP gene promoter. Nucleotide sequence of the 5' end of *X. laevis* MGP gene and its promoter region, from -981 to +69. Nucleotide positions are numbered according to the transcription start site indicated as +1 (vertical arrowhead). Sequence of the first exon is underlined and the conserved 5' intron boundary is indicated by bold letters. Perfect and imperfect inverted repeats are shown by horizontal arrows. TATA like and CCAAT-motifs are boxed. Putative AP-1 and metal responsive elements (MRE) are underlined. Accession number for the complete xMGP gene and flanking DNA: AF234631.

Table 1. Exon-intron structure of the *Xenopus* MGP gene. Exon-intron junctions and flanking sequences are indicated. The consensus 5'-gt and ag-3' donor/acceptor sites (according to Breathnach & Chambon [28]) of each intron, are shown in bold. Phase of intron is shown according to Patthy [29].

Splice donor	Intron no. (length; bp)	Splice acceptor	Phase of intron
acag gtaa	1 (2929)	g(t) ₅ aacag aagaa	Not in coding region
tatg gtaa	2 (986)	c(t) ₄ gtatacag actc	I
tatg gtaa	3 (1985)	a(t) ₄ cag atcc	I
agag gtaa	4 (1490)	c(t) ₄ ag aatc	II

Table 2. Comparison between exon structures in *Xenopus* and mammalian MGP genes. Numbering of each exon is indicated on top of each column. Exon IA has no counterpart in the mammalian genes. Numbers represent size in base pairs. UTR, untranslated region. Numbers in parenthesis indicate size of the 5' or 3' UTR regions in each exon. Numbers in bold indicate size of the coding region in each exon. References for MGP genes are: human [27]; mouse [40]; *Xenopus*, this study.

Source/exon no.	IA	IB	II	III	IV
Human	None	5' UTR(55) + 61	33	76	139 + 3' UTR(248)
Mouse	None	5' UTR(76) + 61	33	76	142 + 3' UTR(222)
<i>Xenopus</i>	5' UTR(47)	5' UTR(61) + 61	33	77	142 + 3' UTR(260)

Mapping the transcription start site of the xMGP gene

To identify the site of transcription initiation, a reverse primer located in exon IB (corresponding to the region from nucleotides 79 to 108 of the xMGP mRNA) was used for primer extension experiments. The initiation site identified for the xMGP gene (Fig. 4, site 'A') corresponds to the previously identified 5' end of the xMGP cDNA [4]. The lower group of bands, identified as site 'B' in Fig. 4, probably corresponds to a premature arrest of the reverse transcriptase due to the presence of an inverted repeat capable of forming a hairpin loop (+18 to +28, Fig. 1).

Identification of putative regulatory elements within the xMGP gene promoter

The 5' flanking sequence of the xMGP gene is typical for a RNA polymerase II transcribed gene. Immediately upstream from the transcription initiation site a TATA-like sequence (TAAATA) is located between base pairs -28 and -23. A CCAAT-consensus box is located at -86 bp (CCAAT), a reverse CCAAT motif lies at -825 bp (ATTGG) (Fig. 1). In addition, the xMGP gene promoter

contains sequence elements that show homology to regulatory motifs bound by well characterized nuclear factors including a putative binding site for the transcription factor AP-1 (AGTCAG [30]; and putative metal responsive elements (MRE) (TGCA/GCT/CC) [31]) (Fig. 1). Because treatments with 1,25-dihydroxyvitamin D₃ and retinoic acid have been shown to modulate MGP gene expression *in vitro* and *in vivo* [8-10,12,32], the xMGP promoter was analyzed for the presence of response elements for the vitamin D₃ and retinoic acid receptor. However, no regulatory elements for steroid hormone receptors or growth factors could be identified based on sequence similarities.

The xMGP promoter directs transcription of a luciferase reporter gene *in vitro*

In order to test the ability of the xMGP promoter to direct transcription, a reporter plasmid (-949LUC) was constructed that contains the xMGP sequence spanning from -949 to +33 upstream of a luciferase reporter gene. The levels of luciferase gene expression after transfection of -949LUC, promoter-less pTATALUC plasmid (negative control), and Tk-LUC (positive control) demonstrated that

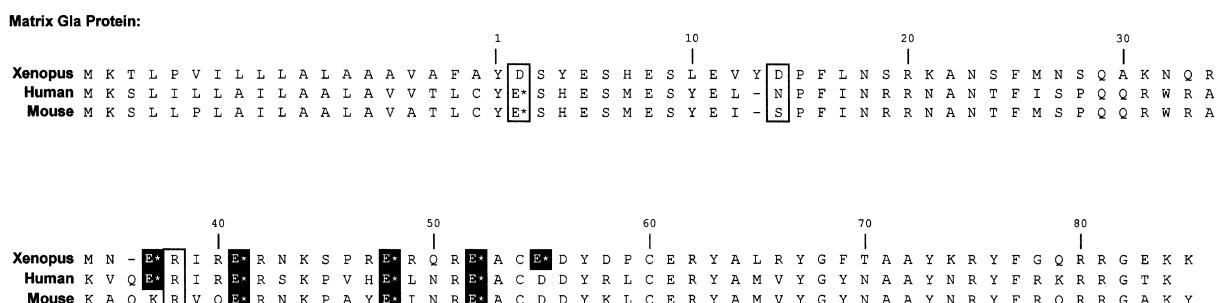


Fig. 2. Sites of intron insertions within the amino-acid sequence of *Xenopus*, human and mouse MGPs. Conserved sites of intron insertions in mammalian and *X. laevis* MGPs are boxed. The gamma-carboxyglutamate residues are shown in black boxes. Amino acids are numbered according to the *X. laevis* sequence, starting at the first residue of the mature protein. xMGP, described in this study; human MGP [24]; mouse MGP [37].

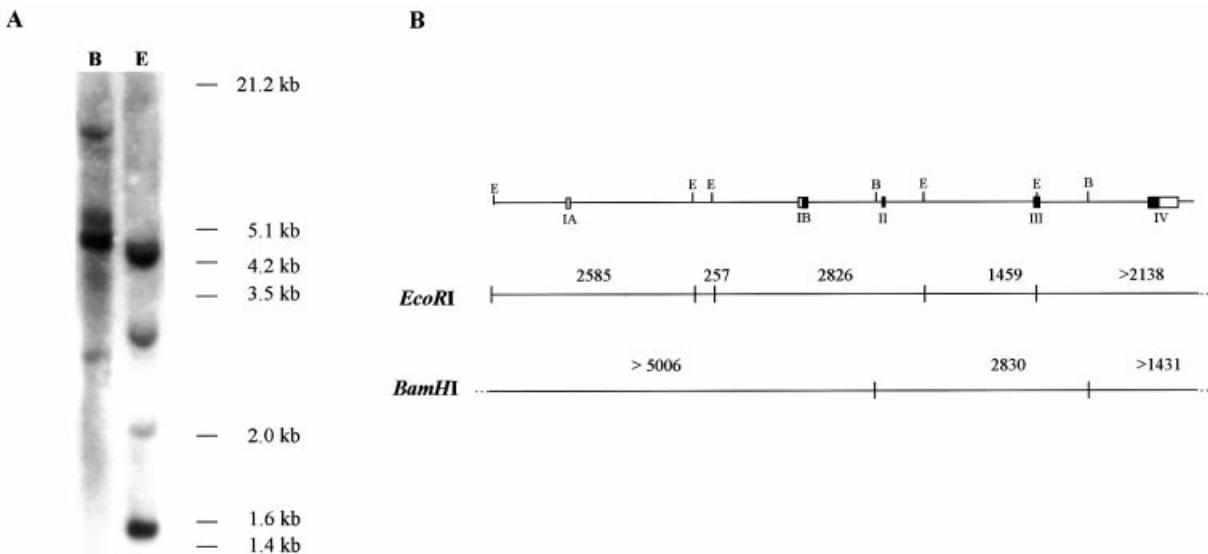


Fig. 3. Analysis of the *Xenopus* MGP gene chromosomal DNA by Southern hybridization. (A) Genomic Southern hybridization with full length xMGP cDNA. Restriction digestion was performed using either *Eco*RI (E) or *Bam*HI (B). DNA size standards are indicated. (B) Localization of the MGP gene within the genomic DNA fragment analysed. Exons (IA–IV) are indicated by boxes. Protein coding and noncoding sequences are marked by closed and open boxes, respectively. Restriction sites for *Eco*RI and *Bam*HI as determined by DNA sequence analysis are shown. Distances in base pairs are indicated.

the xMGP promoter region was capable of promoting transcription in the A6 cell culture system to levels similar to those obtained with the positive control (Fig. 5 and data not shown). Cotransfection experiments using the xMGP promoter constructs in combination with expression plasmids for mammalian nuclear receptors (including the vitamin D, retinoic acid and thyroid hormone receptors) did not modulate the activity of the -949LUC reporter significantly, either in presence or absence of the cognate ligands (N. Conceição, M. L. Cancela & R. Schule, unpublished results).

Identification of regulatory motifs within the xMGP gene promoter

Different deletion mutants of the xMGP promoter were fused to the luciferase reporter gene and assayed for transcriptional activation in A6 cells. All results were analyzed in direct comparison with the expression levels obtained with the full length -949LUC reporter. Deletion of 5' flanking sequences up to -185 only moderately change the promoter activity (Fig. 5). A reporter construct containing only the promoter region from position -54 to +33 bp, including the TATA box (-54LUC), showed a drastic drop in luciferase activity. Internal deletions of DNA sequences from -326 to +33 or from -708 to +33, deleting the TATA box, completely abolished luciferase activity (Fig. 5). To examine more closely the sequences within the proximal MGP promoter, DNA fragments spanning the regions from -180/-36, -180/-72, -134/-36 and -70/-36 (Fig. 6) were fused upstream of a TATA minimal promoter. Plasmids -180/-36TATALUC and -134/-36TATALUC showed significant activity (12-fold induction) in comparison to the control plasmid (pTATALUC). In contrast, the reporter construct -180/-72TATALUC is inactive (Fig. 6), suggesting that the promoter region spanning -72 to -36

contains *cis*-acting elements necessary for transcriptional activation. To further analyze this region, one copy of a double stranded oligonucleotide spanning the region from -70 to -36 was fused upstream of pTATALUC. Evaluation of reporter activity following transfection of A6 cells revealed strong luciferase activity (Fig. 6). Further increase was observed with a reporter plasmid containing three copies of this sequence element. The effect on transcriptional activity obtained with the -70/-36TATALUC was approximately sevenfold higher than the one obtained with the -134/-36TATALUC, suggesting the presence of negative regulatory elements located in the region between -134 and -70 (Fig. 6).

Nuclear factor(s) from *X. laevis* A6 cells bind within the -70 to -36 bp region of the xMGP promoter

Presence of nuclear factors from A6 cells that are capable of interacting with the xMGP promoter were determined using electrophoretic mobility shift assays. The regulatory region of the xMGP promoter from -180 to -36 bp that has been identified in the deletion experiments (Fig. 6) was ³²P-labeled and incubated with A6 cell nuclear extracts. As indicated by the arrows in Fig. 7, one major and two minor DNA-protein complexes were observed. Competition assays (100- or 50-fold molar excess, respectively) with the unlabeled -180/-36 bp (lanes 1 and 7) and the -134/-36 bp (lanes 3 and 9) fragments from the xMGP gene promoter almost completely prevented the formation of the DNA-protein complexes (Fig. 7). In contrast, addition of an excess of DNA fragment spanning the sequence from -180/-72 (lanes 2 and 8) or from -54/+33 (lanes 4 and 10) both failed to displace binding. Specific competition by the -70/-36 bp oligonucleotide (lanes 5 and 11) was clearly detectable even when lowest levels of unlabeled competitor were used (Fig. 7, compare lanes 2 with 5, and lanes 8 with 11).

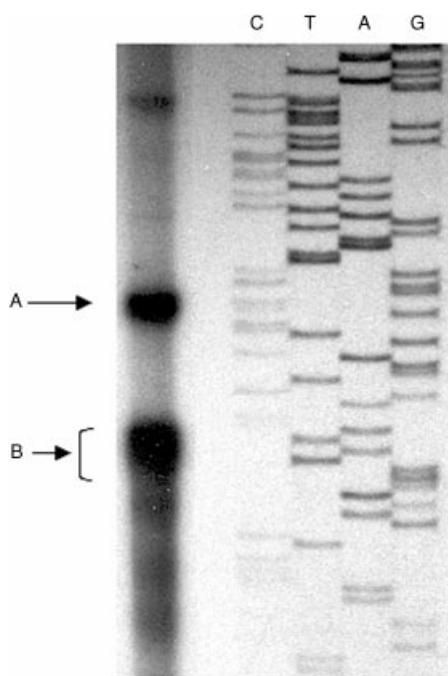


Fig. 4. Determination of the transcription start site of the xMGP gene. Primer extension experiments were performed with an oligonucleotide complementary to nucleotides 79–108 of exon IB. The extension products are separated in lane 1, the sequencing reaction (lanes G, A, T, and C) serves as a 1-bp size standard. 'A' represents the major site of transcription initiation, 'B' corresponds to a region of premature transcriptional arrest.

xMGP gene transcription is stimulated by extracellular Ca^{2+} concentration

To investigate whether changes in calcium concentration affect the levels of xMGP gene transcription through the identified regulatory site (−70 to −36 bp), we examined the effects of extracellular Ca^{2+} concentrations (1.8, 3.0 and 6.0 mM) on the transcriptional activation of the 3x(−70/−36)TATA-LUC reporter plasmid in A6 cells. Increasing extracellular calcium concentrations resulted in a significant ($P \leq 0.05$) dose-dependent stimulation of MGP transcription compared to mock treated cells (Fig. 8). In total, expression of luciferase under control of the 3x(−70/−36)TATA-LUC construct increased approximately three-fold with the highest Ca^{2+} concentration used (Fig. 8).

DISCUSSION

In this study, we present the molecular organization of the first nonmammalian MGP gene and the functional analysis of its promoter. We identified a region within the first 70 bp of the xMGP promoter that mediates transcriptional activation in response to changing extracellular calcium concentrations.

The xMGP gene spans ≈ 8 kb of chromosomal DNA and is organized in five exons, one more than present in the two mammalian MGP genes that have been previously identified (human and mouse [26,27]). In direct comparison, the sequence encoding exon I in the human and mouse MGP genes is split into two exons (IA and IB) in the *X. laevis* gene, with the site of the intron insertion localized within the 5' UTR region of the xMGP gene (Fig. 1 and Table 2). The other introns (2, 3 and 4) are inserted at

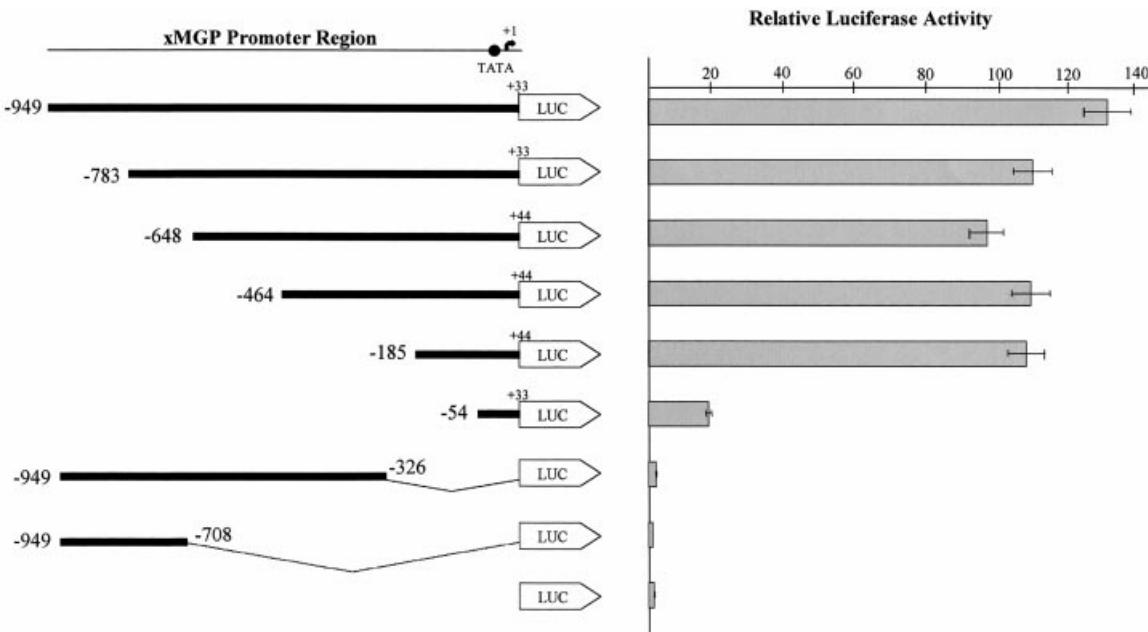
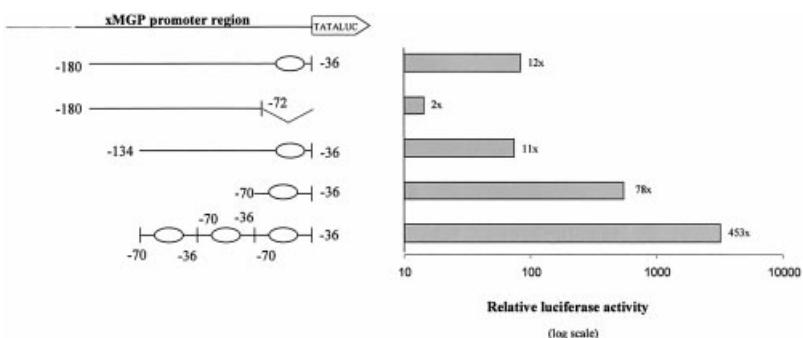


Fig. 5. Relative transcriptional activity of xMGP gene promoter constructs in A6 cells. A schematic representation of the xMGP promoter constructs used for transient transfections of A6 cells is shown to the left. The nomenclature of the promoter deletions is based on the transcription start of the xMGP gene (compare Fig. 1). The xMGP-TATA box is represented by a filled circle. Each transfection was carried out at least five times and standard deviations were less than 10%.

Fig. 6. Identification of a promoter sequence between -70 and -36 bp essential for basal transcriptional activity in A6 cells. A6 cells were transfected with reporter plasmids containing the indicated xMGP promoter fragments. The transcriptional read-out is presented using a logarithmic scale. Fold induction of luciferase expression over the control plasmid (TATALUC) is indicated to the right of each column. The data show a representation of five independent experiments.



conserved positions within the protein coding region compared to the human and mouse sequences (Fig. 2). The 5' transcription initiation site as determined by primer extension analysis is in full agreement with the previously identified 5' end of the xMGP cDNA (determined by 5' RACE in Cancela *et al.* 2001 [4]) and is located 23 bp downstream of a TATA-like motif. The *Xenopus* MGP gene

is approximately twice as long as its known mammalian counterparts due to the presence of the additional intron 1. Interestingly, this intron contains a sequence motif homologous to a regular TATA box (TATAAA) near its 3' border. This sequence element could be used as an internal alternative promoter, a situation that has been previously identified in other genes containing an intron

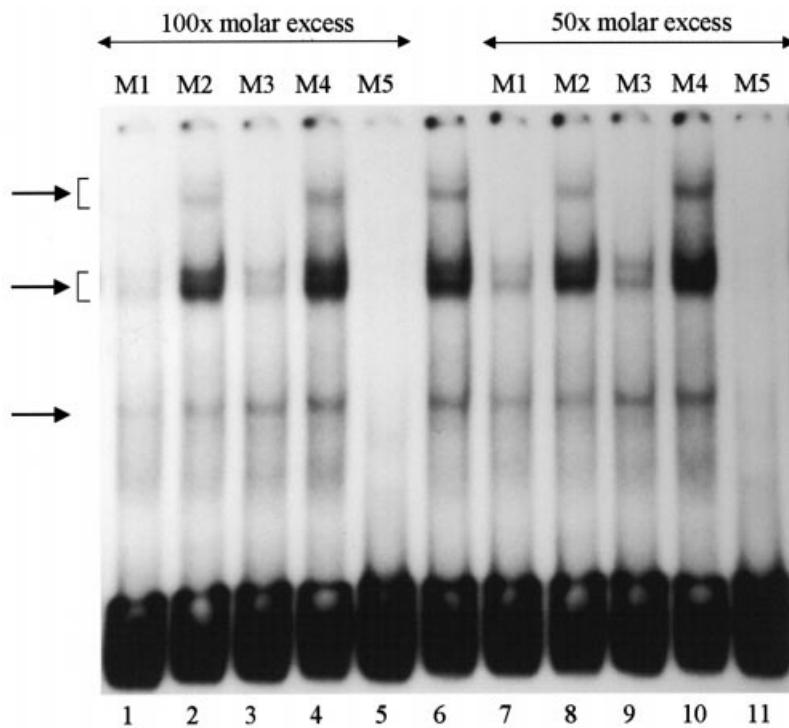
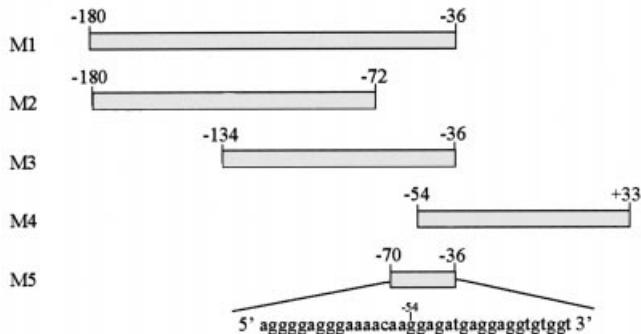


Fig. 7. Binding of a nuclear factor from A6 cells to the -70/-36 region of the xMGP promoter. The electrophoretic mobility-shift assays were performed by using the -180/-36 bp DNA fragment of the xMGP promoter and A6 cell nuclear extracts. No competitor was used in lane 6, whereas in lanes 1–5 a 100-fold, and in lanes 7–12 a 50-fold molar excess of the indicated competitors were used. The positions of the three major DNA–protein complexes are marked by arrows.



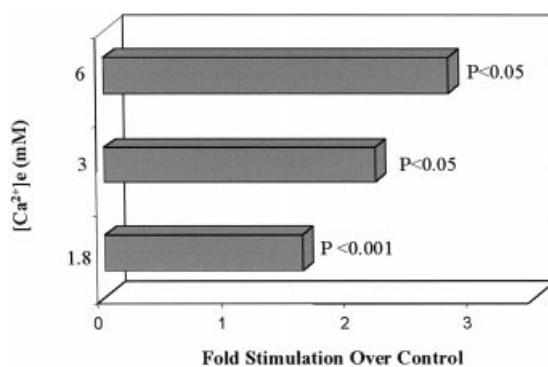


Fig. 8. Dose-dependent transcriptional activation by the -70/-36 TATALUC reporter by extracellular Ca²⁺. Transcription of the 3x(-70/-36) TATALUC reporter plasmid is significantly enhanced by exposure to extracellular calcium at 1.8 mM ($P \leq 0.001$), at 3 mM ($P \leq 0.05$), and at 6 mM Ca²⁺ ($P \leq 0.05$) in comparison to A6 cells cultured in growth medium lacking Ca²⁺.

within their 5' UTR [33]. Alternative splicing and/or use of alternate promoters could contribute to explain previously reported size differences in MGP mRNAs [34,35].

The presence of additional genomic *Bam*HI fragments in genomic Southern analyses could possibly result from mutations at related sites in one or several of the MGP alleles in the tetraploid *X. laevis* (Fig. 3). Alternatively, this phenomenon could reflect the presence of more than one MGP gene, although this finding is not supported by results obtained with the *Eco*RI digestion. All genomic DNA fragments obtained were localized based on the known restriction map of the xMGP cDNA, rather suggesting that MGP is the product of a single-copy gene. Our results are in agreement with previous published data for mammalian MGP [27,36] as well as with the currently available data from the human genome sequence (<http://www.ncbi.nlm.nih.gov>).

We have shown that a 949-bp fragment of the xMGP promoter was able to activate transcription of a luciferase reporter gene in *X. laevis* A6 cells (Fig. 5). The relative activity is comparable with the read-out obtained from a luciferase reporter construct under control of the *Herpes simplex* thymidine kinase promoter (pTkLUC). Cotransfection experiments with expression vectors for mammalian steroid hormone receptors (glucocorticoid receptor, vitamin D₃ receptor, retinoic acid receptors, estrogen receptors α and β , and thyroid hormone receptor β) in concert with -949LUC did not influence luciferase activity significantly, though the receptors were able to mediate ligand dependent transactivation of their cognate reporter genes in A6 cells (N. Conceição, M. L. Cancela & R. Schule, unpublished results). Our results demonstrate that the mammalian steroid hormone receptor orthologs do not influence transcription of the xMGP gene, which does not exclude *Xenopus* nuclear receptors requiring different regulatory elements for proper DNA-binding.

In order to delineate the *cis*-regulatory sequences involved in mediating transcriptional activation of the xMGP gene, we engineered several promoter constructs involving 5' and internal deletions. We identified a core regulatory region located at -70 to -36. Removal of this sequence (i.e. -180/-72TATALUC) completely abolished transcription activa-

tion, emphasizing the need for this sequence for proper MGP gene expression. One copy of this putative regulatory sequence cloned upstream of a TATA box resulted in a 78-fold increase in relative luciferase activity when transfected in A6 cells. In contrast, the use of a slightly longer fragment (-134/-36) in similar experiments led to only 12-fold induction of reporter gene expression (Fig. 6), suggesting that the region located between -134 and -70 might contain negative regulatory elements. A pTATALUC reporter plasmid containing three copies of the -70/-36 regulatory sequence led to a nearly 400-fold induction of reporter gene expression, further confirming the importance of the regulatory element for xMGP gene expression.

These data suggested the presence of specific binding sites for nuclear factors involved in the regulation of MGP gene transcription in the -70/-36 region. Binding of A6 nuclear protein(s) to this region was clearly demonstrated by electrophoretic mobility shift assays, confirming its importance for MGP gene transcription (Fig. 7). The specificity of the DNA/protein complexes was demonstrated by competition experiments (lane 5 and 11), further indicating that binding of nuclear factors from A6 cells are required for efficient transcriptional activation.

The level of transcriptional activation could be further induced (up to threefold) in the presence of increasing calcium concentrations in the extracellular medium (ranging from 1.8 to 6 mM Ca²⁺), thus providing evidence that binding within the -70/-36 region is associated with a calcium sensitive regulatory mechanism. The amplitude of the observed transactivation and the effective range of calcium concentrations are similar to the data presented for the human MGP promoter. Expression of reporter genes driven by the human MGP promoter was found to be moderately induced by calcium (approximately twofold) in transient transfections of human F9 cells [18]. The mechanism was described as being functionally related to a calcium-sensing receptor but different from those previously identified; the region(s) of the human MGP promoter that mediate this effect have not been identified so far.

Interestingly, sequence analysis of the 35-bp region identified a DNA motif identical to the consensus DNA binding site (GGAAAA [37]), for a family of calcium regulated nuclear factors (nuclear factor of activated T-cells, NFAT) which control cellular responses to osmotic stress [38]. The NFAT response element in the xMGP promoter is located in the sequence between -70/-54, the region shown to be responsible for the specific competition observed in the electrophoretic mobility shift assay (Fig. 7). Although these factors were originally identified as T-cell specific transcription factors, recent evidence suggested that tissue distribution and mode of action might vary among the five NFAT isoforms described [38,39]. Recently, a region within the proximal human MGP promoter was identified that mediates binding of the AP1 transcription factor [19]. Although this region shows no homology with regulatory sequences in the xMGP promoter identified in this work, it is interesting to note that AP1 was previously shown to interact with members of the NFAT gene family to specifically induce transcription of target genes (reviewed in [38]). Whether members of the AP1 and NFAT transcription factor family could function as calcium sensitive regulators of xMGP transcription is the topic of ongoing investigations.

The understanding of the fine tuning of MGP gene expression requires further investigation and the use of different vertebrate systems may be useful in bringing new insights into the matter of MGP gene regulation. Given the complexity of the mammalian system and because studies in mammals and birds have clearly linked MGP to the regulation of calcification [7,14,16,17], in particular during early limb development [17,26,34], the use of *X. laevis* as an established model for early vertebrate development can be clearly advantageous. Furthermore, the absence of interference of maternal environment during the free swimming stages of development provides a unique system to directly analyze gene expression in response to changes in external calcium concentration and environmental osmotic stress.

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