Functional importance of Myc-associated zinc finger protein for the human parathyroid hormone (PTH)/PTH-related peptide receptor-1 P2 promoter constitutive activity

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Abstract

The aim of the present study was to analyze the functional importance for the parathyroid hormone (PTH)/PTH-related peptide (PTHrP) receptor (PTHR1) gene P2 promoter activity of the putative proximal Myc-associated zinc finger protein (MAZ) site localized at position bp −45 to −39 bp, taking advantage of a G/A mutation identified at position −40 in the human sequence. Wild-type ‘full-length’ (1285P2) and truncated (760P2) promoter sequences were inserted upstream to the luciferase basic (pLucB) and enhancer (pLucE) reporter gene expression vectors. Transient transfections in osteoblast-like SaOS-2 cells and renal cells (RC.SV3A2) showed that the −40 G/A mutation significantly impaired transcriptional activity of wild-type 1285P2-pLucB and 760P2-pLucE promoter constructs. Further truncation of the P2 sequence demonstrated that the sequence −109/−37 bp was essential for promoter activity. Co-transfection with a MAZ expression vector did not modify the wild-type 1285P2-pLucB construct reporter activity but significantly increased 2-fold the mutated construction activity (P<0.05). Electrophoretic mobility shift assays using SaOS-2 nuclear extracts and a double-stranded DNA fragment encompassing the −45 to −39 putative MAZ site (ds-MAZ-oligo) disclosed two specific DNA–protein complexes. Complex II (fast moving) had a lower affinity for the mutated MAZ motif than for the wild-type MAZ motif while complex I (slow moving) had the same affinity for both wild-type or mutated MAZ sequences. Competition studies with Sp1 consensus oligonucleotide (ds-Sp1-oligo) markedly reduced complex I intensity, with a concomitant increase in that of complex II. Finally, ribonuclease protection assays showed that P2-specific PTHR1 mRNA transcript expression was significantly decreased in SaOS-2 cells transfected with ds-MAZ-oligo as compared with that for control (P<0.001) and ds-Sp1-oligo (P<0.05). Taken together, our studies suggest that the putative −45 to −39 MAZ-binding site regulates the constitutive activity of human PTHR1 P2 promoter.

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Introduction

Parathyroid hormone (PTH)/PTH-related peptide (PTHrP) receptor (PTHR1) is a G protein coupled receptor which mediates the actions of both amino-terminal PTH and PTHrP fragments (Kronenberg et al. 1998). The most abundant expression of PTHR1 is found in renal tubular cells and in osteoblasts, where the PTH/PTHrP receptor mediates the endocrine actions of PTH, and in prehypertrophic chondrocytes of the metaphyseal growth plate, where it mediates the autocrine/paracrine actions of PTHrP. In addition, mRNA encoding PTHR1 is found in a large variety of fetal and adult tissues, similarly to the widely expressed PTHrP (Strewler 2000).

In humans, transcripts encoding identical PTH/PTHrP receptors are derived from at least three promoters that are located upstream of four promoter-specific untranslated exons.
(Goltzman & White 2000). P3 promoter activity (downstream) has been identified so far only in humans in adult life (Bettoun et al. 1998, Manen et al. 1998, 2000). Transcripts derived from the P1 promoter are described, the genes encoding for PTHr1 in different species are highly homologous (Kong et al. 1994), and the broad PTHr1 expression is mostly attributed to P2 activity (McCuaig et al. 1995, Goltzman & White 2000). Indeed, the P2 promoter sequence presents several properties which are compatible with a broad expression pattern. No TATA box consensus sequence is detected, and it presents a high G+C content, a situation which resembles that found for most housekeeping genes (McCuaig et al. 1995). In addition, potential/putative binding sites for Sp1 and MAZ (Myc-associated zinc finger protein), two broadly active transcription factors known to bind to G+C-rich sequences (Parks & Shenk 1996, Izzo et al. 1999, Song et al. 2001), are present in the human and mouse P2 sequence (Bettoun et al. 1997, Minagawa et al. 2000).

Regulation of PTHr1 mRNA expression by various hormones, growth factors and cytokines both in vitro and in vivo has been well documented, and demonstrated to be cell-specific (Nissenson 2001). As the P2 promoter ubiquitously drives PTHr1 mRNA in most tissues and cell lines expressing the receptor, its involvement in most of these regulations is suspected. Indeed, retinoic acid-induced expression of PTHr1 expression mediated by P2 promoter has been demonstrated in P19 EC mouse cells (Karperien et al. 1999). Similarly, it has been demonstrated that in rodents, vitamin D3 administration down-regulates PTHr1 gene transcription by inhibiting P2 promoter activity in osteoblasts, but not in chondrocytes (Amizuka et al. 1999). Thus these data demonstrated that P2 promoter activity is subjected to regulation, and that this control may be cell-type specific.

Little is known about the regulation of P2 activity at the molecular level whatever the species. Minimal P2 regions comprising the region extending to nucleotide –91 upstream of the human P2 transcription start (Minagawa et al. 2000) and between positions –128 to +103 in the rat P2 promoter (Kawane et al. 2001) were identified. The regulation of this promoter activity by Sp1 or MAZ transcription factors has not been studied.

In a previous study, we identified a decreased expression in one PTHr1 allele in a patient with Blomstrand lethal chondrodysplasia (Jobert et al. 1998). The decreased expression was compatible with a defect in this allele P2 promoter region (CSilver, A-S Jobert unpublished data). While attempting to characterize a putative P2 promoter defect, we identified a PCR-induced mutation in the proximal consensus MAZ site sequence in the P2 promoter sequence. In the present study we demonstrate the functional importance of this proximal putative MAZ site for P2 promoter activity. In addition, we provide indirect evidence supporting the possibility that endogenous MAZ regulates human P2-specific PTHr1 transcript expression in osteoblast-like cells, and the presence of a protein–DNA complex involving the MAZ site arising from osteoblast-like cell nuclear extracts.

Materials and methods

Cell culture

The human osteosarcoma cell line (SaOS-2) was grown in Dulbecco’s modified Eagle’s medium (DMEM) (Invitrogen) supplemented with 10% fetal calf serum (FCS), 2 mM glutamine, 1 mM sodium pyruvate, 50 U/ml penicillin and 50 µg/ml streptomycin as described (Jobert et al. 1996). A rabbit distal tubule cell line (RC.SV3A2), kindly given by Pierre Ronco (Hôpital Tenon, France) was grown in a 1:1 (v/v) mixture of Ham’s F12 and DMEM containing 5% FCS, 12.5 mM NaHCO₃, 50 U/ml penicillin and 50 µg/ml streptomycin. These two cell lines were maintained in a humidified atmosphere at 37 °C containing 5% CO₂ in air. Medium was changed three times a week and cells were subcultured weekly following Versene and trypsin treatment.
PTHR1 promoter constructs and expression vectors (Fig. 1)

To generate full-length P2 promoter sequences (1285P2-pLucB/wt<sub>maz</sub> and 1285P2-pLucB/mu<sub>maz</sub> (wt = wild type; mu = mutated)), 100 ng human genomic DNA were amplified in a final volume of 50 µl using 1 U Taq DNA polymerase (Invitrogen) and 0.06 U Pfu DNA polymerase (Stratagene, La Jolla, CA, USA) in the presence of 1.5 mM MgCl<sub>2</sub>, 500 mM betaine (Sigma), 0.25 mM each deoxynucleotide and 0.5 µM forward and reverse primers. The sequence of the forward (1285P2fo) and reverse (106P2re) primers used are reported in Table 1, and as indicated, contain respectively NheI and BglII restriction sites at their 5’ end. The

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PCR product was inserted into the pGL3 basic luciferase reporter vector (pLucB) (Promega) using Ncol and BglII sites using standard molecular biology techniques. All promoter reporter constructs were controlled by restriction fragment analysis and sequencing. In one clone, a G to A mutation at position −40 in the MAZ site located at bp −45 to −39 was randomly introduced by PCR (1285P2-pLucB/mumaz) (Fig. 1).

760P2-pLucE/wtmax and 760P2-pLucE/mumaz constructs were generated by excision of XhoI-HindIII fragments from 1285P2-pLucB/wtmaz and 1285P2-pLucB/mumaz constructs respectively and subcloning of the inserts into pGL3 enhancer luciferase reporter vector (pLucB) (Promega). Promoter reporter constructs were controlled by restriction fragment analysis. 109P2-pLucE and 37P2-pLucE constructs were generated by PCR amplification from the 760P2-pLucE/wtmax construct using forward 109P2fo and 37P2fo primers localized 5′ and 3′ of the two MAZ sites respectively (described in Table 1) and the commercial reverse primer GL-Pr-2, then subcloning of the PCR fragments into pLucE. Promoter reporter constructs were controlled by sequencing.

Transcript transfections and luciferase reporter assays

Transient transfections were performed as described previously except that Lipofectamine Plus (Invitrogen) was used instead of Lipofectamine (Jobert et al. 1997, 1998, Zhang et al. 1998). SaOS-2 and RC.SV3A2 cells were seeded in six-well tissue culture plates at a density of approximately 35 × 10^3 and 50 × 10^3 cells/cm^2 respectively in 2 ml culture medium. Twenty-four hours later, cells were transfected with 0·05–0·8 µg reporter construct/well using Lipofectamine Plus according to the manufacturer’s protocol. In short, DNA was dissolved with 3 µl each of Lipofectamine Plus solution and Lipofectamine in 1 ml opti-MEM I medium and added to the cells previously washed with serum-free medium. After 3 h of incubation at 37 °C in humidified atmosphere containing 5% CO_2 in air, an equal volume of culture medium supplemented with 20% FCS was added per well. One day later transfection medium was replaced by fresh culture medium until luciferase assay.

Luciferase assays were performed as previously described (Zennaro et al. 1996). Forty-eight hours after transfection, cells were washed twice with ice-cold PBS. Cells were lysed in 300 µl cold lysis buffer containing 25 mM glycyglycine pH 7-8, 1 mM EDTA, 1 mM dithiothreitol (DTT), 8 mM MgSO_4, 1% Triton X-100, 15% glycerol for 5 min at 4 °C. Lysates were transferred to prechilled Eppendorf tubes and centrifuged for 2 min at 12 000 g at 4 °C. The supernatant was used for luciferase assay. A solution of 250 µM d-luciferine was prepared in buffer containing 25 mM Tris–HCl pH 7·8, 8 mM MgCl_2, 1 mM DTT, 1% Triton X-100, 1% BSA, 15% glycerol and 1 mM ATP. Luciferase activity was measured following the addition of 100 µl luciferine solution to 150 µl cellular lysate supernatant for 5 s at room temperature in a Lumat LB 9501 luminometer (Berthold France, Thoiry). Promoter activity was expressed in relative luminescent units (RLUs)/well or as fold induction relative to the control vector, pLucB or pLucE as appropriate, the activity of which was set to 1.

Preliminary experiments were performed using 1285P2-pLucB/wtmax construct or pLucB plasmid alone to determine optimal conditions to measure luciferase activity. It was determined that optimal stimulation of luciferase activity was obtained 48 h following transfection of cells (in 10 cm^2 wells) with 0·1 and 0·3 µg 1285P2-pLucB/wtmax construct DNA. Co-transfection with a β-galactosidase expression vector as an internal control for transfection efficiency were not performed because: (i) reproducibility of transfection efficiency measuring luciferase activity was demonstrated in preliminary experiments and in previous works from our laboratory describing measurement of activated signal transduction following transient transfections of cells with PTHR1 expression constructs (Jobert et al. 1998, Zhang et al. 1998); and (ii) DNA amounts above 500 ng/10 cm^2 wells in our transfection conditions lead to a decrease in luciferase activity, as previously observed (Jobert et al. 1998, Zhang et al. 1998).

In some experiments (reported in Fig. 4), 1285P2-pLucB/wtmax and /mumaz constructs (50–300 ng) were co-transfected with MAZ expression plasmid (pcDNA3-ZF87) (50 ng) or an equal amount (in moles) of pcDNA3 plasmid (i.e. 37 ng) (Invitrogen). pcDNA3-ZF87 MAZ expression plasmid was a kind gift of Dr David Hall (NIH, www.endocrinology.org)
Bethesda, MD, USA). Transfection and luciferase assays were performed as described above.

**MAZ and Sp1 phosphorothioate-modified double-stranded oligonucleotides**

Twenty-one phosphorothioate-modified DNA fragments containing the −45 to −39 putative MAZ site (ds-MAZ-oligo) (sense sequence: 5′-GGCG CGGGGGAGGAGGAGG), an Sp1 consensus motif (ds-Sp1-oligo) (sense sequence: 5′-ATTC GATCGGGGCGGGGCGAG), and a control sequence (sense sequence: 5′-GGGGGAGCGGA GCGGAGGAG) were synthesized by MWG Biotech (Courtabeuf, France).

For oligonucleotide transfections, SaOS-2 cells were seeded in 100 mm diameter plates at a density of 38 × 10³ cells/cm² in 10 ml culture medium. Twenty-four hours later, cells were transfected with 250 nM (final) double-stranded oligonucleotides using Oligofectamine (Invitrogen) according to the manufacturer’s protocol. After 72 and 96 h, transfusion medium was removed and plates were immediately frozen at −80 °C for subsequent RNA extraction and RNase protection analysis.

**RNA extraction**

Total RNA was extracted from cells grown in 100 mm diameter plates according to the method of Chomczynski & Sacchi (1987) and stored at −80 °C.

**RNase protection analysis**

RNase protection analysis was used to quantify P2-specific and PTHR1-coding mRNA. In order to synthesize a probe enabling the detection of both P2-specific PTHR1 transcripts and the coding sequence, cDNA was prepared from SaOS-2 cell total RNA, and a 330 bp fragment of the PTHR1 cRNA comprising a portion of U3 untranslated exon, i.e. P2 promoter-specific, was amplified using a forward oligonucleotide complementary to a sequence localized on U3 untranslated exon, and a reverse primer complementary to a sequence localized on E1. The sequences of the forward and reverse primers were 5′-ATCCCGAGAGCTCCA TGAGTC and 5′-TCCTTGAGCGCGCCCTTC GCACTG respectively. The PCR product was cloned into the pGEMTeasyVector (Promega).

The plasmid containing the human PTHR1 fragment was linearized and the riboprobe synthesized using T7 RNA polymerase.

RNase protection analyses were performed as described (Turner et al. 1995, Jobert et al. 1996, 1997) using 10 µg total RNA extracted from SaOS cells transfected with preparations of different double-stranded oligonucleotides as indicated. The unprotected PTHR1 probe migrated at 395 bp, and the P2-specific and [P1+P3]-specific protected fragments migrated at 330 and 209 bp respectively. The radioactivity present in the bands corresponding to the protected fragments of P2- and [P1+P3]-specific PTHR1 transcripts was quantified by electronic autoradiography (Instant Imager; Packard Instruments, Meriden, CT, USA). P2-specific PTHR1 transcript expression was expressed as the ratio of radioactivity present in the P2-specific protected band over that corresponding to total PTHR1 mRNA (i.e. P2+[P1+P3]-specific bands). In preliminary experiments, it was verified that total PTHR1 mRNA expression was not significantly different in cells transfected with the various preparations of double-stranded oligonucleotides.

**Nuclear proteins**

Nuclear extracts from SaOS-2 cells were prepared as described by Asubel (1994).

**Electrophoretic mobility shift assay (EMSA)**

Double-stranded oligonucleotides were labeled with [γ-32P]ATP using T4 polynucleotide kinase (Promega) (see above and Fig. 5 for oligonucleotide sequences). The binding reaction mixture (15 µl final volume) contained approximately 0·2 ng 32P-end-labeled double-stranded oligonucleotide, variable amounts of nuclear proteins (0·2–2 µg, as indicated) and 250 ng polydI-dC in binding buffer (50 mM Tris–glycine pH 9·3, 5 mM EDTA (pH 8), 200 µg/ml BSA, 70 mM KCl, 7 mM MgCl₂, 3 mM CaCl₂, 1 mM β-mercaptoethanol). For competition experiments a 20- or 100-fold molar excess of unlabeled double-stranded oligonucleotide was included in the DNA binding reaction. Incubation was carried out at 16 °C for 20 min; 3 µl loading buffer (50% glycerol, 1 mg/ml BSA) were added prior to loading on a 7-5% acrylamide non-denaturing gel (running buffer: 50 mM
Tris–glycine pH 9·4; 0·1 mM EDTA). Electrophoresis was performed at 4 °C for 3 h at 12 V/cm. The gel was dried and autoradiographed.

**Expression of results and statistical analysis**

Unless otherwise stated, values are expressed as means ± s.e.m. of two to seven experiments performed in triplicate. Statistical analyses were performed using one-way ANOVA. Comparisons between individual groups were made using the Tukey’s multiple comparison test. *P* values <0·05 were considered as significant.

**Results**

G/A mutation at position −40 in the proximal consensus MAZ site strongly impairs PTHR1 P2 promoter activity

‘Full-length’ P2 constructs in pLucB plasmid

The requirement for an intact putative −45 to −39 MAZ site (GGGAGGG) for P2 promoter activity was first demonstrated in the human osteoblast-like cell line (SaOS-2) and the rabbit renal cell line (RC.SV3A2) by transfection of the full-length P2 promoter sequence inserted in pGL3 pLucB (1285P2-pLucB) (Fig. 1). In SaOS-2 cells transfected with the wild-type sequence (1285P2-pLucB/wtmaz), reporter activity was significantly stimulated compared with that measured in cells transfected with the pLucB (5·3 ± 0·28-fold stimulation in 1285P2-pLucB/wtmaz-transfected SaOS-2 cells compared with pLucB-transfected SaOS-2 cells, *P* < 0·001) (Fig. 2A). In contrast, reporter activity in SaOS-2 cells transfected with the MAZ site mutated at position −40 (GGGAAG) of the P2 promoter sequence (1285P2-pLucB/mumaz) was significantly lower than that measured in cells transfected with the pLucB (1·5 ± 0·31-fold stimulation in 1285P2-pLucB/mumaz-transfected SaOS-2 cells, *P* < 0·001 compared with 1285P2-pLucB/wtmaz-transfected SaOS-2 cells, i.e. approximately 94% reduction).

Truncated P2 constructs in pLucE

Reporter activity was also significantly stimulated in cells transfected with a truncated wild-type P2 promoter sequence inserted in pLucE (760P2-pLucE/wtmaz) compared with that measured in cells transfected with the pLucE (72 ± 2·7-fold stimulation in 760P2-pLucE/wtmaz-transfected SaOS-2 cells compared with pLucE-transfected SaOS-2 cells, *P* < 0·001) (Fig. 3). The presence of the −40 G/A mutation in this shorter 760P2-pLucE/mumaz construct also significantly reduced reporter activity compared with that measured in cells transfected with 760P2-pLucE/wtmaz construct (4·3 ± 0·31-fold stimulation in 760P2-pLucE/mumaz-transfected SaOS-2 cells, *P* < 0·001 compared with 72 ± 2·7 in 760P2-pLucE/wtmaz-transfected SaOS-2 cells, i.e. approximately 94% reduction). The sequence lying between −109 and −37 bp, but not that between −760 and −109 bp, is essential for PTHR1 P2 promoter activity

In order to further assess the role of the putative MAZ sites for P2 promoter activity, we compared luciferase activities in SaOS-2 cells transiently transfected with 760P2-pLucE/wtmaz construct, and two further truncated P2 promoter sequences, 109P2-pLucE and 37P2-pLucE respectively comprising or not the MAZ sites (described in Fig. 1B). Reporter activity in SaOS-2 cells transfected with the 109P2-pLucE construct was similar to that in cells transfected with 760P2-pLucE/wtmaz construct (65 ± 3·7- and 72 ± 2·7-fold induction in reporter activity in cells transfected with 109P2-pLucE and 760P2-pLucE/wtmaz construct compared with pLucE reporter activity, *P* > 0·05) (Fig. 3). This result indicated that the region lying between −760 and −109 is not essential for basal
P2 activity in SaOS-2 cells. In contrast, further deletion of the P2 promoter sequence to position −37 led to 80% reduction in reporter activity, indicating that the region lying between −109 and −37 is essential for promoter activity (Fig. 3). As stated previously, remarkable features of this region are the two putative canonical binding sites for the transcription factor MAZ at positions −58/−52 and −45/−39 and a putative Sp1-binding site (Fig. 1C).

MAZ protein expression increases MAZ site mutated P2 promoter activity

The involvement of MAZ transcription factor in the induction of P2 promoter activity was tested by transiently co-transfecting cells with 1285P2-pLucB/wt\textsubscript{maz} or /mu\textsubscript{maz} constructs and pcDNA3 expression vector encoding MAZ. As documented previously in Fig. 2, reporter activity in SaOS-2 cells transfected with 1285P2-pLucB/wt\textsubscript{maz} and 1285P2-pLucB/mu\textsubscript{maz} transfected cells over RLU\textsubscript{s} measured in pLucB transfected cells. RLU\textsubscript{s} measured in cells transfected with pLucB plasmid alone: 5120±395·8 and 8064±585·4 RLU\textsubscript{s} well respectively in SaOS-2 and RC.SV3A2 cells. Results are the means±S.E.M of seven (SaOS-2) (n=21) and two (RC.SV3A2) (n=6) experiments performed in triplicate. ***P<0.001; ns: not significant. Constructs are described in the Methods section and Fig. 1. Cells were transiently transfected with the different constructs (300 ng/well) and luciferase activity measured 48 h following transfection as described in the Methods section.
construct was significantly lower than that measured in cells transfected with the 760P2-pLucE/\text{wt}_{\text{maz}} and the 109P2-pLucE were similar, while it was significantly lower in cells transfected with the shorter construct 37P2-pLucE ($P<0.05$). As observed for the full-length P2 promoter activity, the −40 G/A MAZ mutation impairs reporter activity of the shorter construct 760P2-pLucE. Results are expressed as fold induction and are the ratio of RLU measured in cells transfected with the P2 constructs over RLU measured in pLucE transfected cells; results are the means±S.D. of one experiment performed in triplicate. *$P<0.05$, ***$P<0.001$. The symbols inside the bars indicate comparison with results obtained in cells transfected with pLucE. RLU measured in cells transfected with pLucE plasmid alone: 18 892±3055 RLU/well. Constructs are described in the Methods section and Fig. 1. Cells were transiently transfected with the different constructs (300 ng/well) and luciferase activity measured 48 h following transfection as described in the Methods section.

**Role of MAZ in P2 promoter activity as determined by EMSA**

The involvement of the putative MAZ sites in P2 promoter activity was further investigated by EMSA of oligonucleotides derived from −52 to −33 bp region (thus covering −45 to −39 MAZ site) (Fig. 5A) with SaOS-2 cell nuclear extracts by monitoring the binding of increasing amount
(0.2–2 µg) of SaOS-2 nuclear protein extracts to DNA fragments containing the wild-type or mutated MAZ motif. Two complexes were formed. The protein present in complex I (slow moving) had the same affinity in experiments using the wild-type or mutated MAZ motif (Fig. 5B). In contrast, the protein present in complex II (fast moving) showed a higher affinity for the wild-type MAZ motif than for the mutated MAZ motif.

Using the wild-type MAZ labeled probe, the signal in complex I was totally competed away by both 100-fold molar excess of unlabeled wild-type and mutated MAZ probes (Fig. 5C, lanes 3 and 4), while the signal in complex II was totally competed away by 100-fold molar excess of unlabeled wild-type probe but only partially by 100-fold molar excess of mutated MAZ probes (Fig. 5C, lanes 3 and 4). Using the mutated MAZ labeled probe, complex I and II were of similar intensity (Fig. 5C, lane 6), and signals in both bands were competed away by 100-fold molar excess of unlabeled wild-type and mutated MAZ probes (Fig 5C, lanes 7 and 8). Taken together, these results further demonstrate that the mutated MAZ probe binds with a lower affinity to the protein II complex than the wild-type MAZ probe, and thus is more easily displaced.

As it is known that Sp1 can bind to a MAZ site, we tested the hypothesis that Sp1 is the protein in complex I. A decrease in complex I signal was observed in competition experiments using an Sp1 consensus unlabeled probe and either the wild-type or the mutated MAZ motif. Interestingly, the Sp1 consensus unlabeled probe favored protein attachment in complex II, suggesting that complex II is the MAZ protein (Fig. 5B, lanes 6 and 12). Using Sp1 consensus labeled probe, only complex I was detected (Fig. 5C, lane 10), and its intensity was competed away by 100-fold molar excess of unlabeled Sp1 consensus probe as well as wild-type and mutated MAZ probes (Fig. 5C, lanes 11–13). Similar results were obtained using Hela cell nuclear extracts using 100-fold molar excess of unlabeled wild-type probe (data not shown).
EMSA results suggest that the factors involved in the complexes I and II are Sp1 and MAZ respectively.

Effects of double-stranded nucleotide encompassing the −45 to −39 putative MAZ site on endogenous P2-specific PTHR1 mRNA transcript

In order to analyze the possibility that endogenously expressed MAZ regulates endogenous P2-specific PTHR1 mRNA transcript expression, the effects of double-stranded oligonucleotides encompassing the −39 to −45 putative MAZ site (ds-MAZ-oligo) were compared with those of double-stranded control oligonucleotides and ds-Sp1-oligo. Expression of MAZ mRNA in SaOS-2 cells was demonstrated beforehand by RT-PCR technique (data not shown).

Transfection of cells with ds-oligonucleotides (MAZ, Sp1 and MIX) induced a significant decrease in P2-specific PTHR1 mRNA transcript expression compared with that in cells transfected with Oligofectamine alone (Figs 6A and B). However, this decrease was more important in cells transfected with ds-MAZ-oligo than in cells transfected with ds-Sp1-oligo, and there was a...
Figure 6 Effect of 21 bp phosphorothioate-modified double-stranded DNA fragments on P2-specific PTHR1 transcript mRNA expression in SaOS-2 cells 96 h following transfection. (A) Examples of autoradiographs of RNase protection analysis showing PTHR1 mRNA expression. PTHR1 P2-specific and [P1+P3]-specific transcripts migrate at 330 and 209 bp as indicated by the arrows, the unprotected PTHR1 probe migrates at 395 bp (indicated by * on the panels). (B) Quantification of P2-specific PTHR1 transcript expression (P2-specific PTHR1 mRNA). P2-specific PTHR1 mRNA transcript expression is the ratio of the radioactivity measured in the P2-specific PTHR1 transcript signal over total PTHR1 mRNA expression, as described in the Methods section. P2-specific PTHR1 mRNA transcript expression was significantly lowered with double-stranded DNA fragments containing the −45 to −39 putative MAZ site (MAZ), compared with that in cells transfected with control oligonucleotides (MIX) (*P<0.05). It was not significantly modified in cells transfected with ds-Sp1-oligo (Sp1), as compared with that in cells transfected with ds-control-oligo (MIX) (P>0.05). P2-specific PTHR1 mRNA transcript expression in cells transfected with ds-control-oligo (Mix) was significantly decreased compared with that measured in non-transfected cells (nt) and cells transfected with Oligofectamine alone (Oi), indicating a non-specific effect of the control oligonucleotides. Cells were transfected with ds-MAZ-oligo (MAZ), ds-Sp1-oligo (Sp1), ds-control-oligo (MIX) and Oligofectamine alone (Oi), and P2-specific PTHR1 transcript measured by RNase protection assays as described in the Methods section. P2-specific transcript mRNA expression was also measured in non-transfected SaOS-2 cells (nt). Results are means±S.E.M. of 8–12 independently transfected culture wells in three or four experiments. Nt: non transfected; Oi: Oligofectamine alone; Mix/MIX: control oligonucleotides. *P<0.05, ***P<0.001, ns: not significant. AU: arbitrary units.
significant difference between P2-specific PTHR1 mRNA transcript expression in cells transfected with MAZ, but not Sp1, oligonucleotides, compared with that in cells transfected with MIX oligonucleotides (P < 0·05) (Fig. 6). Transfection of cells with Oligofectamine alone was associated with a slight (not significant) increase in P2-specific PTHR1 mRNA transcript expression compared with that in non-transfected cells. Similar results were obtained 96 h (Fig. 6) and 72 h (not shown) following cell transfection.

Discussion

PTHR1 P2 promoter sequence presents several properties which are compatible with its broad activity, such as the absence of TATA box consensus sequence, and a high G+C content, a situation which resembles that found for most housekeeping genes. In addition, several putative Sp1 and MAZ transcription factor binding sites are present in the P2 sequence. In this study, we (i) confirmed that the region of the human PTHR1 gene P2 promoter sequence lying between −109 and −37 bp 5′ of the U3 transcription start site is required for P2 minimal activity in osteoblast-like cells and in renal cells (Minagawa et al. 2000); (ii) demonstrated by mutational analysis that a putative MAZ site localized in this minimal promoter region at position −45/−39 bp is essential for human P2 activity; (iii) demonstrated that loss of activity resulting from the putative MAZ site mutation was partially reversed following MAZ overexpression; (iv) presented EMSA results supporting the possibility that one DNA–protein complex detected following binding of SaOS-2 and HeLa nuclear extracts to MAZ site-spanning oligonucleotides contained MAZ transcription factor; and (v) presented results supporting the possibility that endogenous MAZ regulates human P2-specific PTHR1 transcript expression in SaOS-2 cells.

In agreement with previous work (Minagawa et al. 2000), we demonstrated that the human P2 promoter region between bp −109 to −37 was essential for promoter activity. Remarkable features of this region are two canonical binding sites for the transcription factor MAZ at positions −58/−52 and −45/−39 and one for Sp1. The entire P2 sequence between U2 and U3 comprises six putative Sp1-binding sites, five of which are localized 5′ of the minimal promoter region, and six putative MAZ-binding sites, four of which are localized 5′ of the minimal P2 promoter region (Manen et al. 1998, Minagawa et al. 2000). The observation that promoter activity of P2 promoter sequences extending to −760 and −109 bp are similar supported the conclusion that the Sp1 and MAZ sites located in the region beyond the minimal promoter region are not essential for P2 promoter activity.

In sharp contrast, mutation of a single MAZ site localized at position −45 to −39 from the transcription start site of the untranslated exon U3 led to a near complete loss of P2 promoter activity in human osteoblast-like cells SaOS-2 and a 50% reduction in rabbit renal cells. This loss of promoter activity was observed for a truncated P2 promoter construct extending to bp −760 and cloned into pLucE and further confirmed using a full-length P2 promoter sequence cloned into pLucB in human osteoblast-like and rabbit renal cells. This supported a role for this MAZ site in the regulation of PTHR1 expression under physiological conditions, and suggested that MAZ is important for PTHR1 constitutive expression. Although a single mutation of the MAZ site was studied, its striking effect on P2 promoter activity clearly demonstrates the importance of the site for P2 promoter activity. It should be pointed out that the main information provided by our work is that disruption of a single putative MAZ binding site, the proximal one, is sufficient to disrupt human PTHR1 P2 promoter activity. These results underline the functional importance of this site, but do not provide information on interactions between additional sites in the PTH1R promoter.

Partial recovery of the decreased P2 promoter reporter activity associated with the −40 MAZ site mutation was obtained when MAZ protein was overexpressed in the cells. This result is in agreement with the hypothesis that the lower P2 activity probably results from a decreased affinity of the MAZ mutated sequence for the MAZ protein compared with the affinity of the wild-type MAZ sequence, a decreased affinity partially overcome following overexpression of MAZ protein. No increase in P2 promoter activity was obtained following overexpression of MAZ with wild-type P2 sequence in SaOS-2 cells and in renal cells, suggesting that endogenous MAZ expression is
already sufficient to maximally stimulate wild-type MAZ promoter construct activity.

Gel mobility shift assays identified two putative MAZ protein–DNA binding complexes arising from SaOS-2 and HeLa cell nuclear extracts. Careful analysis of competitive experiments using wild-type and mutated ds-MAZ-oligo, and ds-Sp1-oligo strongly suggested that the fast moving complex (complex II) indeed contained MAZ transcription factor. Interestingly, our results suggested that ds-Sp1-oligo allowed the detection of putative Sp1 protein–DNA complex, while ds-Sp1-oligo did not allow the detection of putative MAZ protein–DNA complex. This was unexpected because MAZ and Sp1 motifs are similar and interaction of MAZ with at least some Sp1-binding sites, and vice versa, has been previously demonstrated (Song et al. 2001). One explanation at least may explain the lack of interaction between ds-Sp1-oligo with putative MAZ protein-containing complex. The ds-Sp1-oligo sequence was chosen based on previously published work, which indeed demonstrated its interaction with Sp1 protein. However, in these studies, interaction with MAZ protein or MAZ-containing nuclear extract was not studied. It is therefore possible that the sequence of this oligonucleotide does not allow interaction with MAZ. Figure 7 presents a possible scheme to interpret our data. Wild-type MAZ oligo is able to bind to MAZ transcription factor, and to a lesser extent to Sp1 transcription factor. In contrast, the Sp1 site is not able to bind to MAZ transcription factor (but obviously able to bind to Sp1). Mutation of the MAZ-binding site results in a decreased affinity of the oligonucleotide for MAZ, without affecting affinity for Sp1 (which was already lower). It should be acknowledged that conclusive evidence for MAZ binding to PTHR1 P2 promoter was not provided in the present work, given the fact that MAZ antibodies were not available to us.

It is important to note that a single point mutation in the MAZ site totally abolished P2 activity in SaOS-2 cells. This result underlines the importance of this site for constitutive activity of this promoter in human osteoblast-like cells. Furthermore, the observation that in SaOS-2 cells competition of endogenously expressed MAZ binding to 21 bp specific DNA sequence encompassing the MAZ sites present in the proximal part of PTHR1 P2 promoter by specific double-stranded oligonucleotides was associated with a specific decrease in P2-specific PTHR1 transcript also supports a role for MAZ for P2-specific PTHR1 transcript basal expression. The promoter region of the rat PTHR1 was recently cloned (Kawane et al. 2001). The rat counterpart of the human U3 exon and its 5’ flanking sequence were demonstrated to be required for basal rat PTHR1 expression in osteoblast-like cells. This promoter sequence contains consensus Sp1 and MAZ motifs, shown to be required for PTH suppression of receptor gene expression (Kawane et al. 2001). Putative regulation of the rat PTHR1 P1 (designated U3 promoter) (but not rat PTHR1 P2) promoter activity by MAZ has been reported (Williams & Abou-Samra 2000). Taken together, our present and the previous studies point to an important role for MAZ acting
on different promoters in regulating PTHR1 expression.

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References


Amizuka N, Kwan MY, Goltzman D, Ozawa H & White JH 1999 Vitamin D3 differentially regulates parathyroid hormone/parathyroid hormone-related peptide receptor expression in bone and cartilage. Journal of Clinical Investigation 103 373–381.


McCuia KA, Clarke JC & White JH 1994 Molecular cloning of the gene encoding the mouse parathyroid hormone/parathyroid hormone-related peptide receptor. PNAS 91 5051–5055.


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