The transcription factors SP1 and MAZ regulate expression of the parathyroid hormone/parathyroid hormone-related peptide receptor gene

L J S Williams and A B Abou-Samra

Endocrine Unit, Massachusetts General Hospital and Harvard Medical School, Boston, Massachusetts 02114, USA

(Requests for offprints should be addressed to A B Abou-Samra, Endocrine Unit, Bulfinch 327, Massachusetts General Hospital, Boston, Massachusetts 02114, USA;
Email: samra@helix.mgh.harvard.edu)

ABSTRACT

The parathyroid hormone (PTH)/PTH-related peptide (PTHrP) receptor regulates extracellular calcium concentrations and is therefore important for mineral homeostasis. ROS 17/2.8 cells, a rat osteoblast-like osteosarcoma cell line, express the PTH/PTHrP receptor and provide a good model for examining the transcriptional regulation of its gene. The rat PTH/PTHrP receptor gene has two promoters, U1 and U3, which were shown to be important for its expression. Using extracts from ROS 17/2.8 cells, we have demonstrated two regions (termed FP1 and FP2) of nuclear protein/DNA interaction within promoter sequences previously shown to be important for the activity of the U3 promoter. Nuclear extracts from rat 2

fibroblasts, which do not express the PTH/PTHrP receptor, produced one site of protein/DNA interaction which was found at a position on the promoter identical to the position of FP1 produced by a ROS 17/2.8 nuclear extract. Mutation of these two sites of protein/DNA interaction resulted in reduced U3 promoter activity. Furthermore, we have demonstrated that the transcription factors SP1 and MAZ regulate U3 promoter expression and have shown their functional significance using mutational analysis. These data demonstrate that SP1 and MAZ bind to the PTH/PTHrP receptor promoter and that they are involved in cell-specific expression of its gene product.

Journal of Molecular Endocrinology (2000) **25**, 309–319

INTRODUCTION

The parathyroid hormone (PTH)/PTH-related peptide (PTHrP) receptor regulates extracellular calcium levels. It is a G-protein-linked receptor which has a similar affinity for both PTH and PTHrP (Jüppner *et al.* 1991, Abou-Samra *et al.* 1992). Two other receptor subtypes have been identified. The PTH2 receptor has a higher specificity for PTH compared with PTHrP (Usdin *et al.* 1995), whereas the PTH3 receptor, recently cloned from zebrafish, has a higher specificity for PTHrP (Rubin & Jüppner 1999). The PTH/PTHrP receptor has been ablated in mice and it has been demonstrated that the receptor is required for normal bone development (Lanske *et al.* 1996). The PTH/PTHrP receptor is therefore important for bone development and calcium homeostasis. An understanding of how the PTH/PTHrP receptor

is regulated at the molecular level is therefore extremely important for elucidating how calcium homeostasis is maintained.

The PTH/PTHrP receptor has been cloned from multiple species including rats, humans, *Xenopus* and mice (Abou-Samra *et al.* 1992, Schipani *et al.* 1993, Bergwitz *et al.* 1994, McCuaig *et al.* 1994) and shows a high degree of homology among these organisms. The rat receptor gene is composed of 14 coding and 3 non-coding exons, and a similar gene structure is found in humans and in mice (Kong *et al.* 1994). The non-coding exons U1, U2 and U3 (exon U3 being the furthest 5') are at the 5'-end of the gene. Two promoters were identified in the rat PTH/PTHrP receptor gene with transcription start sites being demonstrated at the beginning of U1 and U3 (Joun *et al.* 1997). The U1 promoter is GC rich and is used in many tissues, but its highest activity is observed in bone. The U3 promoter has a

TATA-like sequence; however, it displays a more tissue-specific expression pattern and is primarily transcribed in the kidney and the ovary (Ureña *et al.* 1993). Differential promoter usage and alternate splicing are thought to be responsible for the tissue-specific expression of the gene (Ureña *et al.* 1993).

It has been demonstrated that the PTH/PTHrP receptor and other markers of osteoblastic function, including alkaline phosphatase and osteocalcin, are highly expressed in the rat osteoblastic osteosarcoma cell line, ROS 17/2.8 (Ducy & Karsenty 1995). Transfection of U1 or U3 promoter reporter constructs showed that both promoters were active in ROS 17/2.8 cells, which also express the endogenous PTH/PTHrP receptor gene. Interestingly, the U3 promoter constructs showed higher expression in ROS 17/2.8 cells than in non-osteoblastic cells (Giannoukos *et al.* 1999). The region between -206 and the transcription start site of the U3 promoter was identified, by transient transfection experiments of a U3 promoter 5'-deletion series, as being important for expression in ROS 17/2.8 cells. Furthermore, heterologous promoter constructs made up of promoter fragments between -432 and either -138 (-138 in this paper corresponds to -136 in Giannoukos *et al.* (1999)) or -76 fused to the SV40 promoter indicated that cell-specific activity was maintained by 3' deletion to -76 but not by deletion to -138. This indicated the presence of an important region for PTH/PTHrP receptor expression between -206 and -76 of U3 (Giannoukos *et al.* 1999).

In this paper, we demonstrate that ROS 17/2.8 nuclear proteins bind specifically to the -206/-76 region of the U3 promoter, that this binding is functionally important for promoter activity, and that the transcription factors SP1 and MAZ interact with this region of the U3 promoter.

MATERIALS AND METHODS

PTH/PTHrP-receptor promoter-luciferase constructs

The construct pXP2 and the PTH/PTHrP receptor promoter constructs pU3-LUC(-206) and pUX8/U1-LUC have been described previously (Giannoukos *et al.* 1999). The construct pU3-LUC(-206) was mutated at FP1 and/or FP2 using a QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, California, USA) and constructs were verified by sequencing. The oligonucleotides used to create the mutations were as follows (showing the coding strand only, with mutations shown in bold): FP1 m(7-11)-

GCTCACTGGAGGTTT**CTAGGG**CCTGGTGC
CTTGAAGGCAAACCCATGAG, FP1 m(12-16)-
GCTCACTGGAGGTTGAGCTT**TAAGGG**TGC
CTTGAAGGCAAACCCATGAGG, FP2 m(6+7)-
CATGAGGGTTGATTGGAGGGCCAGCTCA
GGACTTCAG and FP2 m(8+9)-CATGAGGGTT
GAGGTTAGGGCCAGCTCAGGACT TCAG.

Cell culture and transfection

ROS 17/2.8 cells were cultured in Ham's F12 medium supplemented with 5% fetal bovine serum (FBS), 1% penicillin and 1% streptomycin. Rat 2 fibroblast and COS-7 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 5% FBS, 1% penicillin and 1% streptomycin. The cells were incubated at 37 °C in a humidified atmosphere containing 95% air and 5% CO₂. Transfection conditions using lipofectin were as previously described for ROS 17/2.8 and rat 2 cells. COS-7 cells were transfected similarly to rat 2 cells. Assays were carried out using the Luciferase Dual-Reporter system (Promega, Madison, WI, USA), and, to correct for transfection efficiency pRL-TK (Promega), a renilla construct under the control of the thymidine kinase promoter was used. Briefly, cells were lysed in 250 µl Passive Lysis Buffer supplied by the manufacturer (Promega). Cell lysate (20 µl) was mixed with 100 µl Luciferase Assay Reagent (Promega) and luciferase activity was measured using a Monolight 2010 Luminometer (Analytical Luminescence Lab., San Diego, CA, USA). Stop & Glow reagent (100 µl; Promega) was then added to quench the luciferase activity of the test plasmid and activate the renilla reporter construct; the renilla luminescence was then measured.

Nuclear extracts

Nuclear extracts from ROS 17/2.8 and rat 2 cells were prepared essentially as described by Dignam (Dignam *et al.* 1983). Cells were washed with PBS, scraped from flasks in PBS, pooled and centrifuged for 10 min at 4 °C in a SL-50T rotor (Sorvall, Newtown, CT, USA) at 500 *g*. Cells were resuspended in PBS and centrifuged again as above. The cell pellet was resuspended in 5 volumes of cell lysis buffer (10 mM Hepes, pH 7.9, 1.5 mM MgCl₂, 10 mM KCl and 0.5 mM dithiothreitol (DTT)) for 10 min at 4 °C and centrifuged. The pellet was then resuspended in 2 volumes of cell lysis buffer and the cells were lysed with 10 strokes of the B-pestle of an all-glass dounce homogenizer (Wheaton, Millville, NJ, USA). Cells were centrifuged at 4 °C for 20 min in an SL-50T rotor at 14 500 *g*. The pelleted nuclei

FIGURE 1. Rat PTH/PTHrP receptor regulatory regions. The rat PTH/PTHrP receptor promoter structure is shown in the upper part of the figure. The three non-coding exons U1, U2 and U3 are indicated as well as the two transcription start sites (bent arrows). The sequence of the PTH/PTHrP receptor promoter between -206 and the U3 transcription start site is shown below. The transcription start is indicated by the bent arrow, the footprinted regions FP1 and FP2, formed by ROS 17/2.8 nuclear extract, are shown by the boxed regions and the nucleotides identified as most important for the nuclear protein/DNA interaction are shown in bold type.

were resuspended in 2 ml nuclear lysis buffer (20 mM Hepes, pH 7.9, 25% glycerol (v/v), 0.42 M NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.5 mM phenylmethylsulphonyl fluoride (PMSF), and 0.5 mM DTT) and lysed by 10 strokes of the B-pestle of an all-glass dounce homogenizer. The lysate was stirred for 30 min at 4 °C and centrifuged at 14 500 *g* for 30 min in an SL-50T rotor. The supernatant was dialyzed against 50 volumes dialysis buffer (20 mM Hepes, pH 7.9, 20% glycerol (v/v), 0.1 M KCl, 0.2 mM EDTA, 0.5 mM PMSF and 0.5 mM DTT). The extract was aliquoted and snap-frozen in dry ice and stored at -70 °C.

DNase I protection analysis

The sequence between -206 and the transcription start of exon U3 is shown in Fig. 1. The 5'-end of a HindIII/XbaI pU3-LUC(-206) DNA fragment was end-labeled using [α^{32} P]dATP and the Klenow fragment of DNA polymerase I. Labeled probe was pre-incubated with nuclear extract (50 μ g protein) and poly d(I-C)(I-C) (2 μ g) in the reaction buffer (10% glycerol, 10 mM Tris-HCl, pH 7.5, 2.5 mM MgCl₂, 0.1 mM EDTA, 7.5 mM KCl, 2.5 mM DTT and 1 mM CaCl₂) for 20 min on ice. DNase I protection assays were performed using 0.5 U (no extract) or 1 U (extract) RQ1 RNase-free DNase I (Promega) either in the presence or in the absence of a 100-fold molar excess of the unlabeled HindIII/XbaI fragment. Reactions were electrophoresed on

an 8% polyacrylamide sequencing gel in 1 $\times$ Tris borate-EDTA. Maxam-Gilbert sequencing reactions (Maxam & Gilbert 1980) were performed in parallel on the HindIII/XbaI DNA fragment to determine the nucleotide position on the DNA fragment.

Electromobility shift assays

Double-stranded oligonucleotides encoding the DNase-I-protected regions FP1 and FP2 and an SP1 binding site were radiolabeled using [α^{32} P]dATP and the Klenow fragment of DNA polymerase I. The sequences of the double-stranded oligonucleotides used were as follows: FP1, 5'-GAGGTTGAGCTGGCCTGGTGCCT TGAAGGCAAACCC-3' (-164 to -130 of the U3 PTH/PTHrP receptor gene promoter); FP2, 5'-GAGGGTTGAGGGGAGGGCCAGCTC-3' (-126 to -102 of the U3 PTH/PTHrP receptor gene promoter); CCAAT/enhancer binding protein (C/EBP), 5'-TCGATCAAACGTTGTGTAAGG-3' (Park *et al.* 1993); SP1, 5'-TCGAGAT TCGATCGGGGCGGGGCGAGC-3'; and MAZ, 5'-GATCTTCTTCCCTCCCCCTTCCCGCGCC GGGCGCGT-3' (Parks & Shenk 1996). ROS 17/2.8 or rat 2 nuclear extract (8 μ g) were pre-incubated with poly d(I-C)(I-C) (3 μ g) in a buffer containing 4 mM Tris-HCl (pH 7.5), 100 mM KCl, 5 mM MgCl₂, 1 mM EDTA, 10% glycerol and 0.02% Triton X-100 for 20 min at room temperature.

For competition and supershift experiments, either a 10-fold or a 100-fold molar excess of competitors or SP1 antisera (Santa Cruz Biotechnology, CA, USA) was added prior to the addition of the probe.

RESULTS

Cell-specific complexes are formed by ROS 17/2.8 nuclear extract on the PTH/PTHrP receptor U3 promoter

An important regulatory region of the U3 promoter between positions -206 and -76 has been previously shown to be essential for expression in ROS 17/2.8 cells, compared with that in rat 2 fibroblasts (Giannoukos *et al.* 1999). The U3 activity decreased dramatically when the U3 promoter was deleted to -138 (Giannoukos *et al.* 1999). DNase I protection analysis using a -206 to $+75$ U3 promoter fragment and a ROS 17/2.8 nuclear extract demonstrated two protected regions located at positions -164 to -137 (FP1) and -122 to -104 (FP2). The protected regions FP1 and FP2 were competed off by a 100-fold molar excess of specific DNA competitor (Fig. 2). In contrast, the rat 2 fibroblast nuclear proteins formed only one footprint which was identical to FP1 (Fig. 2). This suggests that the FP2 region was protected specifically with the nuclear extract from ROS 17/2.8 cells but not with the nuclear extract from rat 2 fibroblasts, and that the FP1 protein complexes are not cell-specific.

Characterization of FP1 and FP2 by electromobility shift assays

To identify the specificity of the nuclear protein interactions with FP1 and FP2, electromobility shift assay (EMSA) experiments were carried out using oligonucleotides that encompassed these sites. Oligonucleotide FP2 bound nuclear factors only from ROS 17/2.8 cells (Fig. 3A), not those from rat 2 fibroblasts (Fig. 3B), confirming the DNase I protection assay results. The complexes formed were competed with partially by a 10-fold molar excess and fully by a 100-fold molar excess of the FP2 oligonucleotide. Interestingly, examination of the nucleotide sequence of the footprinted region FP2 identified a similarity to known binding sites for the transcription factors SP1 and the *c-myc*-associated zinc-finger binding protein (MAZ). To clarify whether SP1 or MAZ is present in the protein/DNA complexes formed on FP2, competition experiments using known binding sites for these factors were performed; these resulted in ablation of the protein complexes (Fig. 3A).

FIGURE 2. The ROS 17/2.8 nuclear extract forms complexes with the U3 promoter. DNase I protection analysis of the $-206/+75$ fragment of the rat PTH/PTHrP receptor. Lanes: 1, Maxam-Gilbert sequencing reaction; 2, DNase I digestion of labeled probe in the absence of nuclear extract; 3 and 4, DNase I digestion of labeled probe incubated in the presence of ROS 17/2.8 nuclear extract; 5 and 6, DNase I digestion of labeled probe incubated in the presence of rat 2 nuclear extract; 4 and 6, competition analysis with a 100-fold molar excess of cold probe ($-206/+75$).

However, an oligonucleotide encompassing the C/EBP binding site did not compete even at a 100-fold molar excess (Fig. 3A). These data suggest that SP1 and/or MAZ may interact with FP2.

FIGURE 3. Interaction of ROS 17/2.8 and rat 2 nuclear proteins with FP1 and FP2. (A and B) DNA/protein complexes formed by ROS 17/2.8 (A) or rat 2 fibroblast (B) nuclear extract and the FP2 oligonucleotide probe. (C and D) DNA/protein complexes formed by ROS 17/2.8 (C) or rat 2 fibroblast (D) nuclear extract and the FP1 oligonucleotide probe. (A–D) Lanes: 1, no nuclear extract added; 2–12, 8 µg nuclear extract added; 3, 5, 7, 9 and 11, a 10-fold molar excess of competitor oligonucleotide added.; 4, 6, 8, 10 and 12, a 100-fold molar excess of competitor oligonucleotide added. C/EBP, oligonucleotide containing the binding site for C/EBP transcription factor; FP1, oligonucleotide encompassing the FP1 binding site; FP2, oligonucleotide encompassing the FP2 binding site; SP1 and MAZ, oligonucleotides encompassing binding sites for the transcription factors SP1 and MAZ.

In contrast, the oligonucleotide FP1 specifically bound nuclear proteins from both the ROS 17/2.8 cells (Fig. 3C) and rat 2 fibroblasts (Fig. 3D). Complexes formed by both nuclear extracts were

competed by the oligonucleotide FP1 at a 10-fold molar excess. The complexes formed by the rat 2 fibroblast nuclear extracts were not competed with by any other competitor. The higher-mobility

FP1

wt	ACTG	GAGGTTGAGCTGGCCTGGTGCCTTGAA	GGCAA
m1-6	ACTG	<u>TCTTGGG</u> GAGCTGGCCTGGTGCCTTGAA	GGCAA
m7-11	ACTG	GAGGTT <u>TCTAGG</u> GCCTGGTGCCTTGAA	GGCAA
m12-16	ACTG	GAGGTTGAGCTT <u>TAAGGG</u> TGCCTTGAA	GGCAA
m17-22	ACTG	GAGGTTGAGCTGGCCTT <u>TGTAATT</u> GAA	GGCAA
m23-27	ACTG	GAGGTTGAGCTGGCCTGGT <u>GCCGGTCC</u>	GGCAA

FP2

wt	TGAGG	GTTGAGGGGAGGGCCCAGC	TCAG
m1-5	TGAGG	<u>TGGTCGGGG</u> AGGGCCCAGC	TCAG
m6-9	TGAGG	GTTGATTTT <u>AGGGCCC</u> AGC	TCAG
m10-14	TGAGG	GTTGAGGGGCTTT <u>ACCAGC</u>	TCAG
m15-19	TGAGG	GTTGAGGGGAGGG <u>CAACTA</u>	TCAG
m7-13	TGAGG	GTTGAGTTTCTTT <u>CCCAGC</u>	TCAG
m6+7	TGAGG	GTTGATTGGAGGG <u>CCCAGC</u>	TCAG
m8+9	TGAGG	GTTGAGGTTAGGG <u>CCCAGC</u>	TCAG
m10+11	TGAGG	GTTGAGGGGCTGG <u>CCCAGC</u>	TCAG
m12+13	TGAGG	GTTGAGGGGAGTT <u>CCCAGC</u>	TCAG
m13+14	TGAGG	GTTGAGGGGAGGT <u>ACCAGC</u>	TCAG

FIGURE 4. Sequences of wild-type (wt) and mutant (m) FP1 or FP2 oligonucleotides used in gel mobility shift experiments. The footprinted region is boxed and the mutated nucleotides are underlined.

complexes formed by the ROS 17/2.8 nuclear extract and FP1 were competed by the oligonucleotides with binding sites for FP2 and MAZ, and less well by SP1. The SP1 competitor oligonucleotide partially competed with the upper set of complexes at a 100-fold molar excess (Fig. 3C).

Identification of the nucleotides that are important for U3 promoter activity

In order to investigate the functional specificity of FP1 and FP2 within the U3 promoter, oligonucleotides with 2–7 nucleotide mutations within FP1 and FP2 (Fig. 4) were used for competition in EMSA experiments to identify the nucleotides that are necessary for the interaction between the U3 promoter and the nuclear proteins. The EMSA showed that, for FP1, nucleotides 7–11 and 12–16 are the most important regions for protein/DNA interactions, as these mutant oligonucleotides did not compete for complex formation (Fig. 5A).

Oligonucleotides with mutations at positions 17–22 competed to some extent, whereas the mutations at positions 1–6 or 23–28 fully competed for complex formation (Fig. 5A). The nucleotides important for protein/DNA interactions within FP2 are located at positions 6–9 and 10–14, as oligonucleotides with mutations at these sites did not compete nuclear protein/DNA complex formation (Fig. 5B). For further mapping of important nucleotides within the 6–14 region of FP2, additional two-nucleotide mutations were created (Fig. 4). These two-nucleotide mutations were made at the following positions: 6+7, 8+9, 10+11, 12+13 and 13+14. The 6+7 and 8+9 mutations did not compete with complex formation (Fig. 5C), indicating that these sites within FP2 are important for binding to the nuclear proteins. The mutations at 10+11, 12+13 and 13+14 all competed with complex formation (Fig. 5C), indicating that these sites are less important for complex interaction. Overall, the nucleotides important for FP1 interaction with ROS

FIGURE 5. FP1 and FP2 nucleotides important for binding and expression. Electromobility shift competition experiments designed to identify nucleotides important for the interaction of either FP1 (A) or FP2 (B and C) and ROS 17/2.8 nuclear proteins. (A, B and C) Lane 1 had no nuclear extract added and the remaining lanes had 8 μ g nuclear extract added. (A) Lanes: 3, 5, 7, 9, 11, 13 and 15, a 10-fold molar excess of competitor oligonucleotide added; 4, 6, 8, 10, 12, 14 and 16, a 100-fold molar excess of competitor oligonucleotide added. (B) Lanes: 3, 5, 7, 9 and 11, a 10-fold molar excess of competitor oligonucleotide added; 4, 6, 8, 10 and 12, a 100-fold molar excess of competitor oligonucleotide added. (C) Lanes: 3, 5, 7, 9, 11, 13, 15, 17 and 19, a 10-fold molar excess of competitor oligonucleotide added; 4, 6, 8, 10, 12, 14, 16, 18 and 20, a 100-fold molar excess of competitor oligonucleotide added. (D) Transient transfections in ROS 17/2.8 and rat 2 cells using pU3-LUC(-206) and pU3-LUC(-206) with either FP1, FP2 or both mutated and pU3-LUC(-138) as indicated. Each column represents the mean of triplicate samples and the bars represent the standard deviation.

17/2.8 nuclear extract are at positions 7–11 and 12–17, whereas those most important for the FP2 interaction are at positions 6+7 and 8+9.

To determine the functionality of FP1 and FP2 for U3 promoter activity in ROS 17/2.8 cells and rat 2 fibroblasts, we mutated sites within the functional PTH/PTHrP receptor promoter construct, pU3-LUC(–206). This construct contains 206 nucleotides 5' of the transcription start site fused to a luciferase reporter gene. This is the shortest construct that maintains promoter activity in ROS 17/2.8 cells; further 5' deletion to –138 resulted in a significant loss of reporter activity (Giannoukos *et al.* 1999). FP1 was mutated between positions 7–11 or 12–16 and FP2 was mutated at positions 6+7 or 8+9 in the background of pU3-LUC(–206). Double mutations in FP1 and FP2 were also created to produce mutations at either FP1 m7–11 and FP2 m6+7 or at FP1 m12–17 and FP2 m8+9. All mutations reduced promoter activity in ROS 17/2.8 cells (Fig. 5D); however, the mutations at FP2 were most effective and consistently reduced promoter activity by approximately two-thirds (Fig. 5D). The FP1 mutations also reduced the activity, but to a lesser extent. The FP1 and FP2 double mutations did not have additive effects beyond those of the FP2 mutation alone. Rat 2 fibroblast luciferase activity was consistently below that of the ROS 17/2.8 cells (Fig. 5D), as has been demonstrated previously (Giannoukos *et al.* 1999).

Roles of SP1 and MAZ in U3 promoter activity

To investigate further as to whether SP1 bound to FP2, electromobility supershift experiments were carried out. The addition of either goat polyclonal (antibody A) or mouse monoclonal (antibody B) SP1 antisera to the ROS 17/2.8 nuclear extract resulted in a supershifted complex and complexes that were not supershifted (Fig. 6A). In addition, the ROS 17/2.8 nuclear extract bound to an SP1 probe and this also produced a supershifted complex when SP1 antisera were included in the reaction (Fig. 6A). These results indicate that SP1 and additional unidentified factors bind to the FP2 region on the U3 promoter. In addition, FP1 complexes were not supershifted by SP1 antisera, confirming that SP1 does not bind directly to FP1 (data not shown).

The involvement of SP1 and MAZ transcription factors in the expression of the U3 promoter was tested further using CGN expression vectors for SP1 and MAZ that express to high levels in COS-7 cells. Cells were transiently co-transfected with

pU3-LUC(–206) and the CGN expression vectors encoding SP1 or MAZ. These results showed that SP1 and MAZ induced expression of the pU3-LUC(–206) construct and that co-expression of MAZ resulted in a higher induction of promoter activity than that obtained with SP1 (Fig. 6B). When the mutated pU3-LUC(–206) constructs were used in co-transfection experiments with the SP1 and MAZ expression constructs there was a similar level of induction of luciferase activity by the FP1 m12–16 construct relative to the wild-type pU3-LUC(–206) construct (Fig. 6C). However, in the FP2 m6+7 mutation and the FP1/FP2 double mutation, the induction by SP1 and MAZ was much less than that for pU3-LUC(–206). There was also an induction of luciferase activity by SP1 and MAZ on pU3-LUC(–138), but this was less than that obtained with the mutants (Fig. 6C). Therefore, the mutation within FP1 had little effect on the ability of SP1 and MAZ to induce pU3-LUC(–206) luciferase expression, whereas the FP2 mutation did inhibit the ability of SP1 or MAZ to activate pU3-LUC(–206).

DISCUSSION

The region of the PTH/PTHrP receptor gene U3 promoter between –206 and –76 has been previously identified as being important for its activity in osteoblast-like cells (Giannoukos *et al.* 1999). This work has further characterized this region and has clearly shown that there are two distinct nuclear protein/DNA complexes formed on the U3 promoter, which we termed FP1 and FP2. It is interesting to note that these two sites flank nucleotide –138 (with FP1 located 5' and FP2 3' of –138 respectively) and that a 5' deletion of the promoter to –138, in transient transfection assays, resulted in a loss of luciferase reporter expression in ROS 17/2.8 cells (Giannoukos *et al.* 1999). These experiments show the functional importance of factors binding to this region. FP1 bound nuclear extract from ROS 17/2.8 cells and rat 2 fibroblasts, whereas FP2 specifically interacted with ROS 17/2.8 nuclear proteins. These results indicate that factor(s) binding to the FP2 region may be responsible for the increased activity of the U3 promoter in ROS 17/2.8 cells relative to rat 2 fibroblasts. Gel mobility shift analysis of the complexes formed on the FP2 oligonucleotide identified SP1 binding to the FP2 oligonucleotide, and indicated that the transcription factor MAZ may play a role in promoter activity.

Mutational analysis of pU3-LUC(–206) identified bases within FP1 and FP2 which reduced the

FIGURE 6. The role of SP1 and MAZ in U3 promoter activity. (A) Electromobility supershift experiment using SP1 antisera. The radiolabeled oligonucleotide probes used were SP1 (lanes 1–4) and FP2 (lanes 5–8). ROS 17/2.8 nuclear extract was added in lanes 2–4 and 6–8. Two SP1 antibodies were used: antibody A (Ab A) was goat polyclonal SP1 (used in lanes 3 and 7); antibody B (Ab B) was mouse monoclonal SP1 antiserum (used in lanes 4 and 8). (B) Transient co-transfections of COS-7 cells using pU3-LUC(–206) and an expression vector with no inserted gene (vector), the vector with an epitope-tagged SP1 or MAZ gene. The luciferase activity is expressed relative to the activity observed for co-transfection of pU3-LUC(–206) and vector alone. Three representative experiments are shown. (C) Co-transfection of pU3-LUC(–206) or pU3-LUC(–206) constructs mutated within FP1, FP2, or both, with the empty SP1, MAZ or empty expression vector as indicated. The experiment was carried out in triplicate. Promoter activity is indicated as the mean luciferase expression. The bars indicate the standard deviation.

transcriptional activity of the promoter and indicated that FP2 is more important than FP1 for PTH/PTHrP receptor gene expression. Analysis of the FP1 sequence for known transcription factor binding sites identified the transcription factors AP4, NF1 and USF as candidates for binding (Heinemeyer *et al.* 1999). Further work is required to determine the significance, if any, of these factors in the expression of the PTH/PTHrP receptor. The FP2 mutations resulted in a greater decrease in promoter activity than that caused by the two FP1 mutations. Furthermore, combined mutations of FP1 and FP2 did not alter promoter activity compared with FP2 alone, thereby indicating the importance of FP2 for U3 promoter activity. SP1 was identified and MAZ was implicated in binding to the FP2 site; transfection experiments confirmed the actions of SP1 and MAZ on U3 promoter activity. Other factors may bind, as the SP1 antibody did not supershift all of the complexes formed on FP2.

The transcription factor MAZ (also called ZF87/Pur-1 and SAF-1) has been cloned from humans (Bossone *et al.* 1992, Pycr *et al.* 1992), mice (Kennedy & Rutter 1992) and rabbits (Ray & Ray 1998) and is present in most, if not all, tissues (Bossone *et al.* 1992, Kennedy & Rutter 1992, Pycr *et al.* 1992, Parks & Shenk 1996). Despite being a ubiquitously expressed factor, MAZ has also been associated with tissue-specific expression of genes, e.g. the serotonin 1a receptor gene (Parks & Shenk 1996) and the insulin gene (Kennedy & Rutter 1992), although the way in which MAZ influences this tissue-specific expression is not understood. SP1 is another ubiquitously expressed transcription factor (Saffer *et al.* 1991). It is thought to have various modes of action, including the regulation of basal transcription, and it has also been implicated in tissue-specific gene activity. The fact that SP1 is ubiquitous implies that it must interact with cell-specific factors to regulate genes in a cell-specific manner. SP1 controls basal transcription via the interaction of its glutamine-rich activation domain with the TATAA-binding protein-associated factors (Gill *et al.* 1994). It would be interesting to determine whether transcription of the PTH/PTHrP receptor is mediated by an SP1/TAF interaction. The fact that rat 2 fibroblasts do not exhibit any binding to FP2 either in DNase I protection or in gel mobility shift analysis raises the possibility that perhaps other, more bone-specific, factors interact/mediate the interaction of SP1 with FP2, thus resulting in the increased activity of the U3 promoter in the ROS 17/2.8 cells. Indeed, SP1 has been shown to be involved in regulating specific genes in a wide variety of situations, e.g. SP1 may

mediate the response of epidermal growth-factor stimulation of the gastrin gene (Merchant *et al.* 1995) or environmental stimuli, such as phorbol esters, on the platelet thromboxane receptor gene (D'Angelo *et al.* 1996). MAZ and SP1 both regulate the phenylethanolamine N-methyltransferase (PNMT) gene and it was suggested that SP1 may potentially contribute to the tissue-specific expression of the PNMT gene in Neuro2A cells and that competition with MAZ may confer an additional tissue-specific control (Her *et al.* 1999). It is also suggested that SP1 may play a role in bone-cell gene expression, e.g. in the expression of the type X collagen gene in hypertrophic chondrocytes (Long *et al.* 1998). SP1, therefore, may only bind to FP2 in the presence of specific osteoblastic proteins in ROS 17/2.8 cells. SP1 may be required to relay the transcriptional signal to the RNA polymerase II complex, thus increasing the cell-specific transcription of the gene.

MAZ and SP1 are often found regulating the same genes and even bind to the same sites, e.g. SP1 can bind to three of the four MAZ binding sites of the serotonin 1a receptor (Parks & Shenk 1996). Co-transfection experiments using pU3-LUC(−206) and either the expression plasmids for MAZ, SP1, or both, in COS cells showed that MAZ activated the gene more strongly than SP1 and both SP1 and MAZ together did not increase the activity further. This lack of an additive effect indicates that these factors act via the same pathway/route to induce transcription of the U3 promoter. In conclusion, we have identified SP1 and MAZ transcription factors as binding to the U3 promoter and have shown that they do enhance expression of the pU3-LUC(−206) luciferase construct.

ACKNOWLEDGEMENTS

We thank Thomas Shenk (Princeton University) for the gift of pCGN, pSP1-CGN and pMAZ-CGN. We would also like to thank Drs Henry Kronenberg and Marie Demay for helpful comments and critical reading of the manuscript. This work is supported by NIH Grant DK45485.

REFERENCES

- Abou-Samra AB, Jüppner H, Force T, Freeman MW, Kong XF, Schipani E, Ureña P, Richards J, Bonventre JV, Potts JJ, Kronenberg HM & Segre GV 1992 Expression cloning of a common receptor for parathyroid hormone-related peptide from rat osteoblast-like cells: a single receptor stimulates intracellular accumulation of both cAMP and inositol triphosphates and increases intracellular free calcium. *PNAS* **89** 2732–2736.

- Bergwitz C, Klein P, Kohno H, Forman SA, Lee K, Rubin D & Juppner H 1998 Identification, functional characterization, and developmental expression of the two nonallelic parathyroid hormone (PTH)/PTH related peptide receptor isoforms in *Xenopus laevis* (Daudin). *Endocrinology* **139** 723–732.
- Bossone SA, Asselin C, Patel AJ & Marcu KB 1992 MAZ, a zinc finger protein, binds to c-MYC and C2 gene sequences regulating transcriptional initiation and termination. *PNAS* **89** 7452–7456.
- D'Angelo DD, Oliver BG, Davis MG, McCluskey TS & Dorn GW II 1996 Novel role for Sp1 in phorbol ester enhancement of human platelet thromboxane receptor gene expression. *Journal of Biological Chemistry* **271** 19696–19704.
- Dignam JD, Lebovitz RM & Roeder RG 1983 Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian cells. *Nucleic Acids Research* **11** 1475–1489.
- Ducy P & Karsenty G 1995 Two distinct osteoblast-specific cis-acting elements control expression of a mouse osteocalcin gene. *Molecular and Cellular Biology* **15** 1858–1869.
- Giannoukos G, Williams LJS, Chilco PJ & Abou-Samra AB 1999 Characterization of an element within the rat parathyroid hormone/parathyroid hormone-related peptide receptor gene promoter that enhances expression in osteoblastic osteosarcoma 17/2.8 cells. *Biochemical and Biophysical Research Communications* **258** 336–340.
- Gill G, Pascal E, Tseng ZH & Tjian R 1994 A glutamine-rich hydrophobic patch in transcription factor Sp1 contacts the dTAFII110 component of the *Drosophila* TFIID complex and mediates transcriptional activation. *PNAS* **91** 192–196.
- Heinemeyer T, Chen X, Karas H, Kel AE, Kel OV, Liebich I, Meinhardt T, Reuter I, Schacherer F & Wingender E 1999 Expanding the TRANSFAC database towards an expert system of regulatory molecular mechanisms. *Nucleic Acids Research* **27** 318–322.
- Her S, Bell RA, Bloom AK, Siddall BJ & Wong DL 1999 Phenylethanolamine N-methyltransferase gene expression. Sp1 and MAZ potential for tissue-specific expression. *Journal of Biological Chemistry* **274** 8698–8707.
- Joun H, Lanske B, Karperien M, Qian F, Defize LH & Abou-Samra AB 1997 Tissue-specific transcription start sites and alternative splicing of the parathyroid hormone (PTH)/PTH-related peptide (PTHrP) receptor gene: a new PTH/PTHrP receptor splice variant that lacks the signal peptide. *Endocrinology* **138** 1742–1749.
- Juppner H, Abou-Samra AB, Freeman M, Kong XF, Schipani E, Richards J, Kolakowski LJ, Hock J, Potts JJ, Kronenberg HM & Segre GV 1991 A G protein-linked receptor for parathyroid hormone and parathyroid hormone-related peptide. *Science* **254** 1024–1026.
- Kennedy GC & Rutter WJ 1992 Pur-1, a zinc-finger protein that binds to purine-rich sequences, transactivates an insulin promoter in heterologous cells. *PNAS* **89** 11498–11502.
- Kong XF, Schipani E, Lanske B, Joun H, Karperien M, Defize LH, Juppner H, Potts JJ, Segre GV, Kronenberg HM & Abou-Samra AB 1994 The rat, mouse, and human genes encoding the receptor for parathyroid hormone and parathyroid hormone-related peptide are highly homologous. *Biochemical and Biophysical Research Communications* **200** 1290–1299.
- Lanske B, Karaplis AC, Lee K, Luz A, Vortkamp A, Pirro A, Karperien M, Defize LH, Ho C, Mulligan RC, Abou-Samra AB, Juppner H, Segre GV & Kronenberg HM 1996 PTH/PTHrP receptor in early development and Indian hedgehog-regulated bone growth. *Science* **273** 663–666.
- Long F, Sonenshein GE & Linsenmayer TF 1998 Multiple transcriptional elements in the avian type X collagen gene: identification of SP1 family proteins as regulators for high level expression in hypertrophic chondrocytes. *Journal of Biological Chemistry* **273** 6542–6549.
- McCuaig KA, Clark JC & White JH 1994 Molecular cloning of the gene encoding the mouse parathyroid hormone/parathyroid hormone-related peptide receptor. *PNAS* **91** 5051–5055.
- Maxam A & Gilbert W 1980 Sequencing end labeled DNA with base specific chemical cleavages. *Methods in Enzymology* **65** 499–560.
- Merchant JL, Shiotani A, Mortensen ER, Shumaker DK & Abraczinskas DR 1995 Epidermal growth factor stimulation of the human gastrin promoter requires Sp1. *Journal of Biological Chemistry* **270** 6314–6319.
- Park EA, Gurney AJ, Nizielski SE, Hakimi P, Cao Z, Moorman A & Hanson RW 1993 Relative roles of CCAAT/enhancer-binding protein β and cAMP regulatory element binding protein in controlling transcription of the gene for phosphoenolpyruvate carboxykinase (GTP). *Journal of Biological Chemistry* **268** 613–619.
- Parks CL & Shenk T 1996 The serotonin 1a receptor gene contains a TATA-less promoter that responds to MAZ and Sp1. *Journal of Biological Chemistry* **271** 4417–4430.
- Pyrce JJ, Moberg KH & Hall DJ 1992 Isolation of a novel cDNA encoding a zinc-finger protein that binds to two sites within the c-myc promoter. *Biochemistry* **31** 4102–4110.
- Ray A & Ray BK 1998 Isolation and functional characterization of cDNA of serum amyloid A-activating factor that binds to the serum amyloid A promoter. *Molecular and Cellular Biology* **18** 7327–7335.
- Rubin DA & Juppner H 1999 Zebrafish express the common parathyroid hormone/parathyroid hormone-related peptide receptor (PTH1R) and a novel receptor (PTH3R) that is preferentially activated by mammalian and fugu fish parathyroid hormone-related peptide. *Journal of Biological Chemistry* **274** 28185–28190.
- Saffer JD, Jackson SP & Annarella MB 1991 Developmental expression of Sp1 in the mouse. *Molecular and Cellular Biology* **11** 2189–2199.
- Schipani E, Karga H, Karaplis A, Potts JJ, Kronenberg HM, Segre GV, Abou-Samra AB & Juppner H 1993 Identical complementary deoxyribonucleic acids encode a human renal and bone parathyroid hormone (PTH)/PTH-related peptide receptor. *Endocrinology* **132** 2157–2165.
- Ureña P, Kong XF, Abou-Samra SA, Juppner H, Kronenberg HM, Potts JJ & Segre GV 1993 Parathyroid hormone (PTH)/PTH-related peptide receptor messenger ribonucleic acids are widely distributed in rat tissues. *Endocrinology* **133** 617–623.
- Usdin TB, Gruber C & Bonner TI 1995 Identification and functional expression of a receptor selectively recognizing parathyroid hormone, the PTH2 receptor. *Journal of Biological Chemistry* **270** 15455–15458.

RECEIVED 10 July 2000