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Positive and Negative Control of the Expression of Parathyroid Hormone (PTH)/PTH-Related Protein Receptor Via Proximal Promoter P3 in Human Osteoblast-Like Cells*

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ABSTRACT

Three promoters, P1, P2, and P3, regulate the expression of the receptor for the human PTH/PTH -related protein. The P3 promoter, proximal to the gene, seems to be turned on in many tissues and to be the most active of the three in the human adult kidney. P3 is also active in human osteoblastic SaOS-2 cells. Its structure to function relationship is, however, still poorly understood. To address this issue we assayed, in transiently transfected SaOS-2 cells, the expression of reporter gene constructs containing truncated P3 promoter fragments and substitution mutants. We thus localized *cis*-acting elements essential for P3 promoter activity and identified two key Sp1 binding sites. We also found in the 5′-untranslated exon U4, transcribed from promoter P3, an element that inhibits the expression of the receptor and is not promoter specific. This study provides new insights into PTH receptor expression in human osteoblast-like cells. (*J Clin Endocrinol Metab* **85:** 3376–3382, 2000)

PTH IS THE main regulator of calcium and phosphate homeostasis; the PTH-related protein (PTHrP) plays a major role in bone development (1, 2). Both hormones bind to the same receptor (PTH-R), which belongs to a family of membrane-associated receptors that contain seven transmembrane domains and are coupled to G proteins (3). The PTH-R complementary DNA has been isolated from opossum (3), rat (4), mouse (5), pig (6), and human (7, 8). There is a high degree of homology for the receptor across these species. The human, rat, and mouse genes share a common organization of 14 coding exons (9). The first coding exon is called exon S and can be preceded by various 5′-untranslated exons transcribed from different promoters (Fig. 1). In the human gene, the synthesis of PTH-R is under the control of three distinct promoters, P1, P2, and P3. The 5′-untranslated region (5′UTR) of the messenger ribonucleic acid (mRNA) generated by P1 comprises the exons U1 and U2 spliced to exon S. The 5′UTR of the mRNA generated by P2 is formed by splicing exon U3 to exon S. Finally, the proximal promoter P3 generates a 5′UTR including exon U4 coupled to exon S (10). The regulation of these three promoters is still poorly elucidated. P1 activity appears to be restricted to the kidney, whereas P2 and P3 are active in a variety of tissues, including kidney and bone cells (8, 10–12). P2 has been reported to be the only promoter active in fetal tissues at midgestation (13). The recently described promoter P3 drives the synthesis of abundant mRNA in kidney and is also active in human osteoblastic SaOS-2 cells (10, 11). The promoter P3 belongs to the family of TATA-less promoters that lie in GC-rich regions and generate mRNAs highly structured in their 5′UTR. To study the important elements contributing to the activity of P3 we used reporter gene constructs containing various fragments of the 5′-sequence flanking the gene. We identified a region essential for P3 activity and containing two key Sp1 binding sites. In addition, we found that the 5′-nontranslated exon U4 contains a strong negative regulator element for PTH-R gene expression.

Materials and Methods

DNA methodology

Cloning, ligations, transformations in *Escherichia coli* XL1 blue strain, and DNA minipreparations were carried out as reported by Sambrook *et al.* (14). Plasmid DNA was prepared using QIAGEN kits (Basel, Switzerland). When used in transient transfections, the plasmid DNA was phenol-chloroform extracted and ethanol precipitated before use. Plasmid DNA was sequenced using a T7 sequencing kit (Pharmacia Biotech, Dubendorf, Switzerland). Unless otherwise indicated, PCR was performed using *Taq* DNA polymerase from QIAGEN. The reactions were carried out as recommended by the supplier, using Q solution. The temperature cycling protocol was 94 C for 1 min, 55 C for 20 s, and 72 C for 1 min for 35 cycles. The reactions were concluded by a 10-min elongation at 72 C. Oligonucleotides were purchased from MWG-Biotech GmbH (Ebersberg, Germany). All reagents were of the highest purity available from laboratory suppliers.

Reporter gene constructs

We inserted various DNA fragments from the 5′-flanking region of exon S into the multiple cloning site of the promoterless firefly luciferase expression vector pGL3-enhancer (Promega Corp., Zurich, Switzerland). The convention for sequence coordinates is that +1 is the first base of the coding sequence in exon S (Fig. 1).

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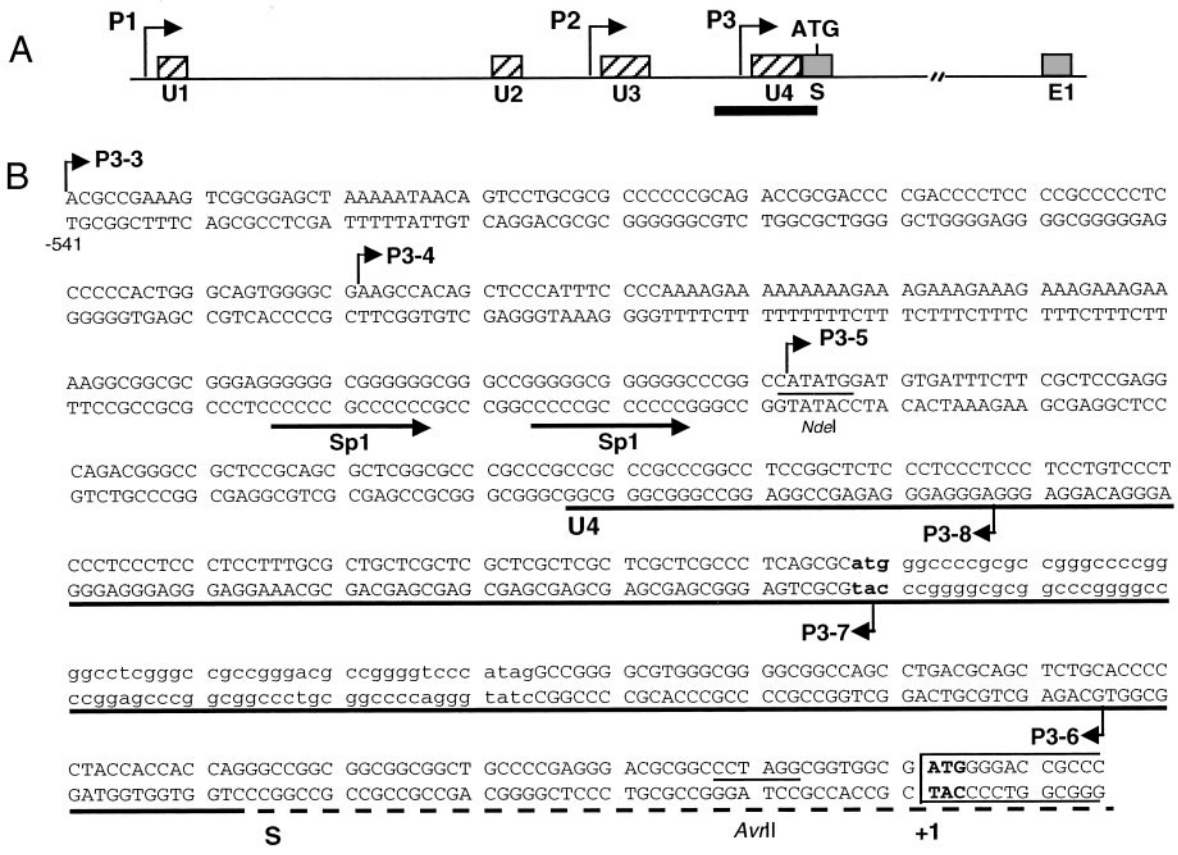

FIG. 1. A, Schematic representation of the promoter region of the PTH-R gene showing the localization of the three promoters P1, P2, and P3. *Hatched boxes*, Nontranslated exons U1-U4; *gray boxes*, coding exons S and E1. The nucleotide sequence of the region *underlined by a thick black line* is presented in B. B, Nucleotide sequence of the P3 region. The first base of the coding sequence of the PTH-R has been designated as coordinate +1. The 5′-end of the progressive 5′-deletions and the 3′-end of the progressive 3′-deletions in the P3 region are indicated by *bent arrows above* and *below* the sequence, respectively. The name of the plasmid corresponding to the resulting constructs (as depicted in Fig. 3) is indicated next to the *arrows*. The two essential Sp1 sites are marked by *arrows*. The *underlined* sequence is that of exon U4, and the *dotted underlined* sequence indicates exon S. The small open reading frame (ORF4) located in exon U4 is in lower case. The two restriction sites *Nde*I and *Avr*II used in plasmid constructions are indicated. The framed sequence shows the beginning of the coding sequence of the PTH-R.

5′-End deletions of P3. Plasmids P3–1 and P3–5 (Fig. 2A) are the previously described plasmids P3a and P3b (11). The *Sac*I-*Avr*II fragment was cloned in pGL3-enhancer (pGL3-en.) and cut by *Sac*I and *Nhe*I, leading to plasmid P3–1. We used the oligos D2, D3, and D4 as forward primers and the commercial primer GL-pr-2 as reverse primer to generate PCR fragments (Table 1). The DNA template was plasmid P3–1. The oligos D2, D3, and D4 contain an additional *Sac*I site in their 5′-end. We replaced the *Sac*I-*Nde*I fragment of P3–1 by the PCR fragments digested by *Sac*I and *Nde*I to obtain plasmids P3–2, P3–3, and P3–4 (Table 1 and Fig. 2A). Plasmid P3–5 resulted from deletion of the restriction fragment *Sac*I-*Nde*I from P3–1.

3′-End deletions of P3. The oligo D4 was used as forward primer, and the oligos D6, D7, and D8 were used as reverse primers to generate PCR fragments. The oligos D6, D7, and D8 contain an additional *Bgl*II site in their 5′-ends (Table 1). We replaced the *Nde*I and *Bgl*II fragment of P3–1 by the PCR fragments digested by *Nde*I and *Bgl*II to obtain plasmids P3–6, P3–7, and P3–8 (Table 1 and Fig. 2A). Deletion of the restriction fragment *Nde*I-*Bgl*II in plasmid P3–1 and P3–5 leads, respectively, to plasmids P3–9 and P3–10.

Site-directed mutagenesis. To mutate the distal Sp1 site (Figs. 1A and 3A), we amplified the DNA fragments located upstream and downstream from the Sp1 site using two sets of primers: first D4 (see Table1) and D64 (CTGCAGGGATCCCCCTCCCGCGCCGCCTTT) and then D65 (CTGCAGGGATCCGGGGCGGGCCGGGGGCGGGGGG) and Gl-pr2 (see Table 1). The DNA template was P3–4. Primers D64 and D65 contain a *Bam*HI site in their 5′-ends (*underlined*). The PCR fragment (D4; D64) was digested by *Sac*I and *Bam*HI, and the PCR fragment (D65; Gl-pr2) was digested by *Bam*HI and *Bgl*II. The fragments were ligated together and inserted into the pGL3-en. vector cut by *Sac*I and *Bgl*II. Thus, in the resulting plasmid, P3–4 m1, the wild-type sequence, GGGGGG-CGGGGG, was replaced by the mutated sequence, GGGGGGATCCGG, containing a *Bam*HI restriction site.

To mutate the proximal Sp1 site we performed a PCR amplification on P3–4 using primer D4 and primer D66 (CACATC*CATATG*-GCCGGGCCCCGGATCCCCGGCCCCGCCCCCCGCCCCCCT), which overlaps the *Nde*I site (*italics*; see also Fig. 1) and contains mismatches (*Bam*HI site; *underlined*) at the level of the site to be mutated. In the resulting plasmid, P3–4 m2, the wild-type sequence (GGGGGG-CGGGGG) was replaced by the mutated sequence (GGGGGGATC-CGG), containing a *Bam*HI restriction site.

To construct the double mutant we used oligos D4 and D67, (CACATC*CATATG*GCCGGGCCCCGGATCCCCGGCCCCGCCCCGGAT-CCCCCT). PCR was performed on plasmid P3–4 m1. We replaced the *Sac*I-*Nde*I fragment of P3–4 by the PCR fragment digested by *Sac*I and *Nde*I to obtain plasmid P3–4 m3, in which both Sp1 sites are mutated.

To mutate the ATG start codon of open reading frame 4 (ORF4; Figs. 1A and 4A) we amplified DNA fragments located upstream and downstream of the ATG codon using two sets of primers: first D4 (see Table1) and D69 (GCAGCCTAGGCGCTGAGGGCGAGCGA) and then D70 GCAGCCTAGGCCCCGCGCCGGGCCCCGGGG) and Gl-pr2 (see Table 1). The DNA template was P3–1. Primers D69 and D70 contain an *Avr*II site in their 5′-ends. The PCR fragment (D4; D69) was digested by *Nde*I and *Avr*II, and the PCR fragment (D70; Gl-pr2) was digested by

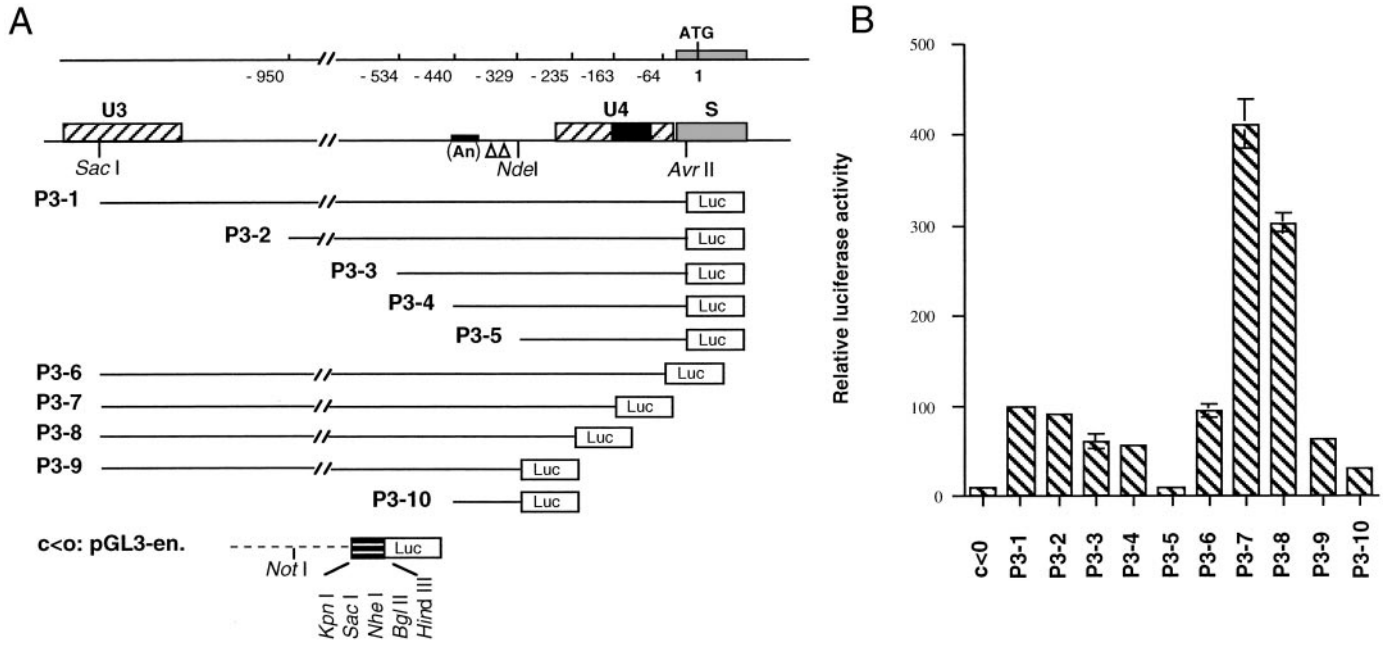

FIG. 2. A, 5′- and 3′-deletions in the P3 region. Coordinate +1 corresponds to the first base of the PTH-R coding sequence, in exon S. The A-T-rich region is indicated by (An). The Sp1 binding sites are indicated by *open triangles*. The *black box* within exon U4 represents ORF4. Luciferase reporter recombinants P3–1 to -9 tested in the reporter gene assays are shown along with the promoterless parental vector pGL3-enhancer (c<0). The main restriction sites of PGL3-enh. used for cloning are indicated. B, Promoter activities of the various P3 DNA fragments in SaOS-2 cells as measured by luciferase assays in transiently transfected cells. *Cis*-acting elements located between bp −440 and bp −329 are essential for P3 activity. The luciferase expression is increased when the gene fusion occurs upstream of ORF4 in exon U4. Luciferase activity was measured and standardized for the activity of the internal control, as indicated in *Materials and Methods*. The luciferase activity of plasmid P3–1 is taken as 100% value. Results represent the mean ± SD of triplicates.

TABLE 1. Sequence and position of primers used to amplify and clone various P3 fragments and name of resulting plasmids

Forward primers	Position (bp)	Reverse primers	Position (bp)	Plasmids
D2: TCGAGGAGCTCGAGGAGTGACCGAAACGG	−950, −932	G1-pr2: CTTTATGTTTTTGGCGTCTTCCAT	luc. gene	P3-2
D3: TGCAGGAGCTCACGCCGAAAGTCGCGGAGC	−541, −524	idem		P3-3
D4: TGCAGGAGCTCAAGCCACAGCTCCCATTTC	−440, −421	idem		P3-4
D4: TGCAGGAGCTCAAGCCACAGCTCCCATTTC	−440, −421	D6: GCAGAGATCTGCAGAGCTGCGTCAGGCT	−66, −84	P3-6
idem		D7: GCAGAGATCTATGCGCTGAGGGCGAGCGA	−163, −181	P3-7
idem		D8: GCAGAGATCTAGGGAGGGAGAGCCGGAGG	−235, −254	P3-8

*Sac*I and *Bgl*II restriction sites in forward and reverse primers respectively are *underlined*.

*Avr*II and *Bgl*II. We replaced the *Nde*I-*Bgl*II fragment of P3–1 by the PCR fragments digested and ligated together. In the resulting plasmid, P3–1 m4 (Fig. 4A), the ATG start codon of ORF4 was changed into the CTA triplet, creating an *Avr*II restriction site. In each case the cloned PCR fragments were sequenced to verify the absence of other mutations.

Other constructions. A *Not*I-*Hin*dII fragment containing the promoter region of the Glvr-1 gene [from construct −192, named Pr-01 in this work (15)] was inserted into plasmid P3–5, which was digested with *Not*I and *Nde*I, after blunting the *Hin*dIII site of the Glvr-1 promoter fragment and the *Nde*I site in plasmid P3–5 (plasmid Pr-02; Fig. 5A). (On request, the authors will supply additional details on plasmid constructions.) The pRL-TK vector (Promega Corp.) provides constitutive expression of Renilla luciferase and was used in transfection assays as an internal control.

Transient transfection assays

Human osteosarcoma cells SaOS-2 were grown in DMEM/F-12 medium (Life Technologies, Inc., Basel, Switzerland) supplemented with 100 IU/mL penicillin, 100 μg/mL streptomycin, and 10% FCS (Amimed, Muttenz, Switzerland). Seventy-two hours after plating, the cells were seeded in 6-well tissue culture clusters at a density of 200,000 cells/well. The cells were transfected 24 h later, using the standard method of calcium phosphate-DNA precipitation in *N,N*-bis-(2-hydroxyethyl)-2-aminoethanesulfonic acid buffer (16). Cells were transfected with 2 μg PTH-R promoter constructs and 0.25 μg pRL-TK vector as an internal control. After 24 h in 3% CO_2 at 35 C, the cells were washed twice in PBS without Mg^{2+} and without Ca^{2+} and put back to standard culture conditions for 48 h. We harvested the cells in 300 μL lysis buffer from the Dual-Luciferase Reporter Assay System (Promega Corp.), and we used 20 μL of the lysate to measure firefly and Renilla luciferase activities (the latter was used to normalize for transfection efficiency). We took the luciferase activity of the reporter plasmid P3–1 as the 100% value. Results are the mean ± SD of triplicate determinations and are representative of at least four independent experiments. Absolute values of the light units of firefly luciferase activity for the control groups are 5578 ± 620 in Exp 2–4 and 7975 ± 918 in Exp 5.

Gel retardation

The binding reaction mixture (15 μL final volume) contained approximately 2 ng ^{32}P end-labeled DNA, 0.1 footprinting unit (fpu) purified Sp1 protein (Promega Corp.), and 50 ng poly(dI-dC) in binding buffer [50 mmol/L Tris-glycine (pH 9.3), 1 mmol/L ethylenediamine tetraacetate (pH 8), 200 μg/mL BSA, 70 mmol/L KCl, 7 mmol/L $MgCl_2$, 3 mmol/L $CaCl_2$, and 1 mmol/L β-mercaptoethanol]. Incubation was carried out at 16 C for 20 min; 3 μL loading buffer (50% glycerol and 1 mg/mL BSA) were added before loading onto a 7.5% acrylamide non-

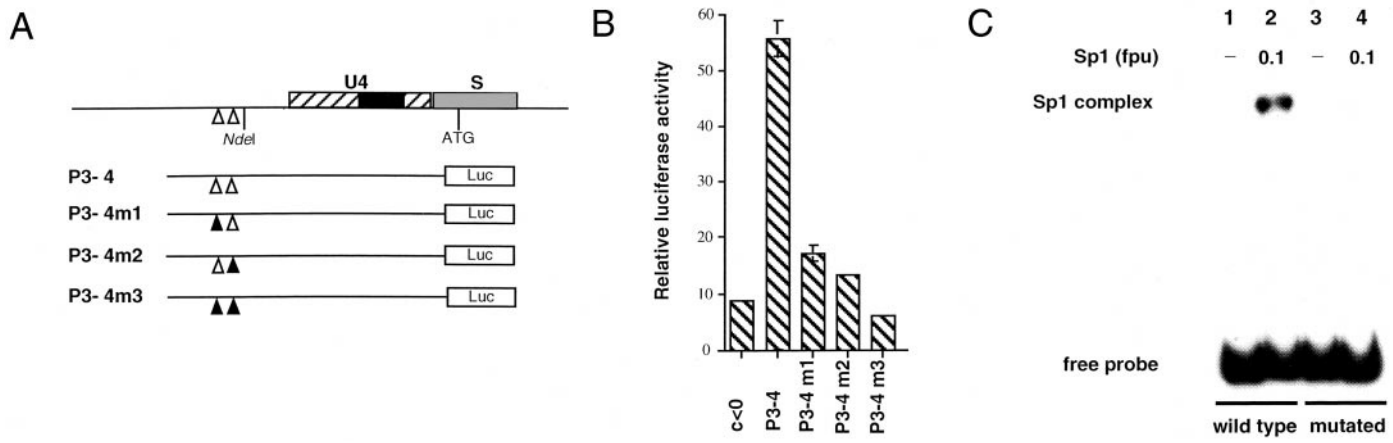

FIG. 3. A, Mutations in the P3 promoter. The wild-type Sp1 binding sites are indicated by *open triangles*, and the mutated sites by *closed triangles*; the sequence of each Sp1 binding sites, GGGGGGCGGGGG, was replaced by the sequence GGGGGGATCCGG. B, Mutated reporter activity as provided by luciferase assays of transiently transfected cells. Mutations in Sp1 elements abolish P3 activity. C, Bandshift assay. The purified Sp1 protein binds to the wild-type DNA segment (bp −440 to bp −329), but not to the corresponding segment in which both Sp1 sites have been mutated. Lanes 1 and 3, No protein; lanes 2 and 4, purified Sp1 (0.1 fpu).

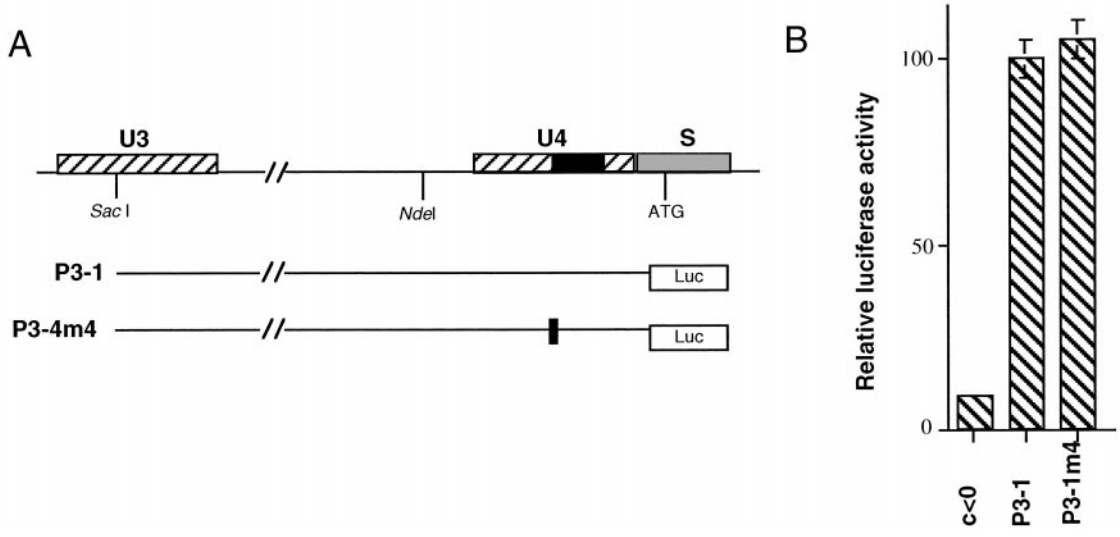

FIG. 4. A, Mutation of the start codon of ORF4. The *black box* in exon U4 indicates the ORF4. The ATG start codon was changed into CTA triplet in plasmid P3–1 m4. *Black rectangle* indicates the presence of the mutation. B, Mutated reporter activity as provided by luciferase assays of transiently transfected cells. The mutation does not affect luciferase expression.

denaturing gel [running buffer: 50 mmol/L Tris-glycine (pH 9.4) and 0.1 mmol/L ethylenediamine tetraacetate]. Electrophoresis was performed at 4 C for 3 h at 12 V/cm. The gel was dried and autoradiographed.

Computer analysis

A search for consensus transcription factor binding sites was performed using MatInspector V2.2 (17). Evaluation of the U4 RNA folding free energy was performed using Michael Zuker's FOLDRNA program in the Wisconsin Package (version 9.1, Genetics Computer Group, Madison, WI).

Results

Sequence of the P3 promoter region

In view of discrepancies between the two reported sequences of the P3 promoter region (10, 11), we repeated the sequencing of some 600 bp upstream of exon S, using our genomic clone. This analysis mostly confirmed the sequence we have previously published (11), except for three errors that are now corrected (at bp −367 a G was missing; at bp −197 and 191 two additional T have been removed; Fig. 1). Sequencing of our clone revealed the presence of a small open reading frame (ORF4, bp −164 to −108; Fig. 1) in the U4 exon, shorter than that described by Bettoun *et al*. (10).

Analysis of the P3 promoter region by progressive deletions

We previously reported on the promoter activity of the P3 region (11). To localize within that region *cis*-acting elements that are essential to the transcription of the gene, we fused various fragments of the 5′-flanking region of exon S to the luciferase gene in the pGL3 enhancer vector (Figs. 1 and 2). The relative luciferase activities of the different constructs are shown in Fig. 2.

Progressive 5′-deletions resulted in decreased luciferase activity (Fig. 2, plasmids P3–1 to P3–5). We found that the

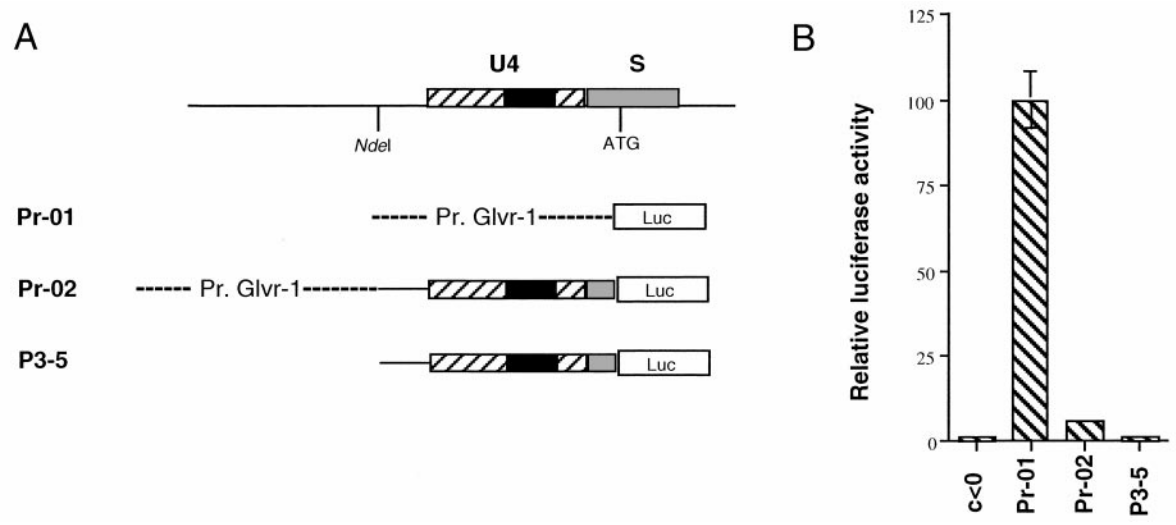

FIG. 5. A, Insertion of the receptor 5′-UTR region between the Glvr-1 promoter and the luciferase gene. The Glvr-1 promoter is indicated by *dotted line*. B, Reporter activity as provided by luciferase assays of transiently transfected cells. Insertion of the U4 region downstream of the Glvr-1 promoter decreases drastically the luciferase expression.

fragment between bp −440 and bp −329 was essential to retain a significant level of P3 expression (plasmids P3–4 and P3–5); when the inserted genomic fragment started at bp −329 (*Nde*I restriction site), no luciferase activity was detected. Remarkable features of this region, between bp −440 and −329, are a stretch of As and two canonical binding sites for the transcription factor Sp1. We noticed that additional elements located upstream of this essential region are necessary for full expression of P3 activity (Fig. 2, plasmids P3–1 to P3–4).

Starting from the reporter plasmid P3–1 we then performed 3′-deletions within exon U4. Luciferase activity was not changed when the fusion with the luciferase gene occurred at bp −66 (plasmid P3–6). When, however, the fusion occurred at bp −163 or −237 (plasmids P3–7 and P3–8), the reporter gene activity was markedly increased. These results suggest the existence of a negative regulatory element of PTH-R expression within exon U4. When the fusion with the reporter gene occurred at the *Nde*I restriction site in the genomic DNA, close to the 5′-end of exon U4 (plasmid P3–9), luciferase activity dropped markedly, indicating that the fragment located between the *Nde*I restriction site and the beginning of exon U4 contains elements important for P3 activity. Plasmid P3–10 resulted from combined 3′- and 5′-deletions and retained the P3 region between bp −440 and bp −329. It exhibited a low level of luciferase activity, which is consistent with all of the above-mentioned observations.

Mutations of the Sp1 sites located in the essential promoter region

As reported in the previous section, activity of the P3 promoter needs the presence of *cis*-acting elements localized between bp −440 and bp −329 (plasmids P3–4 and P3–5). By computer analysis we found two perfect consensus binding sites for the transcription factor Sp1 in this segment (Fig. 1). To evaluate the functional roles of these Sp1 sites, we replaced them, singly and jointly, by nonspecific sequences of equal size (plasmids P3–4 m1, P3–4 m2, and P3–4 m3; Fig. 3A). When either site was mutated, reporter gene expression decreased markedly; when both were mutated, P3 activity was completely abolished (Fig. 3, A and B). These results demonstrate that these Sp1 sites are needed for the activity of the P3 promoter in SaOS-2 cells.

Bandshift assays

We monitored the binding of purified Sp1 protein to a DNA fragment containing the two putative Sp1 binding sites, *i.e.* the *Sac*I-*Nde*I restriction fragment (bp −440 to −329) of plasmid P3–4. We purified and ^{32}P end-labeled the wild-type fragment (from plasmid P3–4) and the corresponding mutated fragment (from plasmid P3–4 m3) in which both Sp1 binding sites were mutated. In a bandshift experiment the wild-type fragment was shifted by 0.1 fpu purified Sp1 protein (Fig. 3C, lane 2). Under the same conditions there was no detectable attachment of the protein to the mutated fragment (lane 4).

Mutation of the ORF4 start codon

Results from the reporter gene constructs showed the presence of a negative regulatory element located in exon U4 (Fig. 2, P3–1 compared to P3–7 and P3–8). This inhibition could be due to either a decreased level of transcription or an impaired translation of the messenger. To determine whether transcription of ORF4 could impair translation of the messenger we mutated the start codon of ORF4, replacing ATG by CTA. We did not detect any significant change in luciferase expression (plasmid P3–1 m4; Fig. 4), suggesting that translation of ORF4 is not responsible for the low reporter gene expression.

The U4 region of PTH-R decreases expression of a heterologous gene

To analyze the effect of the U4 region on gene expression in a different context, we constructed a reporter gene in which the fragment of the 5′-flanking region of the PTH-R

gene containing exon U4 was inserted between a heterologous promoter and the luciferase-coding region (Fig. 5). As heterologous promoter, we used the promoter of the human Glvr-I gene, which codes for a phosphate transporter/retrovirus receptor and is well expressed in the SaOS-2 cell line (15). The U4 region does not have any promoter activity by itself (Fig. 5, P3–5). Insertion of the U4 fragment downstream of the Glvr-I gene promoter markedly inhibited the expression of luciferase (Fig. 5). This indicates that the negative effect of the U4 region is not specific to the PTH-R P3 promoter.

Discussion

Multiple promoters offer the possibility of selective tissue-specific gene expression at various developmental stages. In the case of the PTH-R gene, it has been reported that the activity of the P1 promoter is restricted to adult kidney, that the P2 promoter displays a widespread activity and is the only one operating in the fetal period at midgestation, and that the P3 promoter seems to be turned on in many tissues and to be the most active of the three in the human adult kidney (10, 11, 13). All three promoters lack a TATA element. The 5′-flanking region of the PTH-R gene is GC rich and contains multiple Sp1 consensus binding sequences in both P2 and P3 promoters regions and their corresponding 5′UTR. In the case of P3, in addition to being GC rich, the region contains numerous short direct repeats in and around the promoter sequence and in exon U4. The P3 promoter is located in the 5′-region immediately upstream of exon S, which contains the translation start codon. The 5′-end of the mRNA generated by P3 is composed of the untranslated exon U4 and exon S (10).

Transient transfection assays in the osteoblastic-like cell line SaOS-2, in which the PTH-R receptor is highly expressed (18), allowed us to identify genetic elements involved in the expression of the protein from the P3 promoter. We found that the DNA sequence between bp −440 and −329 possesses sites essential for P3 activity. This DNA fragment contains a stretch of As followed by two perfect consensus Sp1 binding sites, and we found that it does bind purified Sp1 protein *in vitro*. The functional roles of these two Sp1 binding elements were established by monitoring the reporter activity of constructs containing single or combined mutations of theses two sites. In each case we saw a significant loss of activity, demonstrating the key roles of these sites. The Sp1 protein belongs to a large family of zinc finger transcription factors interacting with GC box elements and capable of activating transcription in mammalian cells. Therefore, the identification of factors interacting *in vivo* with putative Sp1 binding sites is complicated by the multiplicity of such proteins (see Ref. 19 for a review). Our findings show that among the putative transcription factors that could be involved in P3 activity in the SaOS-2 cells, Sp1 is a good candidate.

Other features of this region, yet to be investigated, might be implicated in P3 activity. As such sequences are known to distort DNA structure, it is possible that the stretch of As participates in a specific topology necessary for P3 expression. Computer analysis also revealed the presence of a potential binding site for the activating protein-2 transcription factor between the Sp1 sites and exon U4, which might be implicated in P3 activity.

We detected the presence of a negative regulatory element located in exon U4. In our gene reporter system the luciferase gene expression is evaluated by measuring the amount of active luciferase synthesized. Therefore, the observed change in gene expression could reflect changes in mRNA levels or changes in the amount of protein produced from the messenger. As it has been reported that mRNA translation can be impaired by the presence of short ORF in the 5′UTR of transcripts (20, 21) we mutated the ATG start codon of ORF4. This mutation did not abolish the negative effect, eliminating this possibility. To better appreciate the intrinsic effect of the U4 inhibitor element we inserted the 5′-flanking region of PTH-R between a heterologous promoter and the luciferase reporter gene. There was a drastic drop in luciferase expression showing that the negative regulatory is not specific to the PTH-R P3 promoter.

As reported by Kozak (20), many mRNAs with GC-rich leader sequences include mRNAs for a variety of proteins essential for the cell viability such as GH receptors, protooncogenes, and others. She suggested that the production of these critical regulatory protein is throttled at the level of translation. For instance, the *N*-methyl-D-aspartate receptor is a typical example of down-regulation of translation by the presence of a highly structured 5′UTR (22). For PTH-R, the mRNA generated by the P3 promoter presents an encumbered leader sequence: computer analysis revealed for the folding of U4 RNA an elevated free energy in the range of ΔG of −80 kcal/mol. We, therefore, favor the hypothesis of a translation impaired by mRNA secondary structures located in exon U4. We cannot, nevertheless, exclude the presence in the leader sequence of a site responsible for a transcriptional down-regulation.

In conclusion, our data showed that in SaOS-2 cells the activity of promoter P3 depends on the integrity of two Sp1 binding sites. In addition, we demonstrated the presence of a negative regulatory element of PTH-R expression in the 5′-untranslated exon 4 generated by the P3 promoter.

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