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Ras Responsive Element Binding Protein-1 (RREB-1) Down-Regulates hZIP1 Expression in Prostate Cancer Cells

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Abstract

BACKGROUND—Normal prostate accumulates extremely high levels of zinc compared to other soft tissues. In contrast, the level of zinc in the prostate decreases significantly in prostate cancer. We have shown that down-regulation of the expression of the zinc transporter hZIP1 in prostate cancer is an important event that is responsible for the decrease of zinc levels. However, the mechanism of hZIP1 down-regulation is not known. We have hypothesized that hZIP1 is down-regulated through transcriptional regulation.

METHODS—The hZIP1 promoter was studied using luciferase reporter assays, site-directed mutagenesis, gel shift, and ChIP assay.

RESULTS—We have characterized a promoter region, downstream of the transcription start site, responsible for repression of hZIP1 transcription. We demonstrate that this region contains a binding site for the Ras-Responsive Element Binding protein 1 (RREB-1) and that the binding of RREB-1 to the hZIP1 promoter is involved in the decrease of hZIP1 transcription in PC-3 cells.

CONCLUSION—The Ras pathway and activation of RREB-1 are involved in hZIP1 down-regulation and may play a role in the decrease of the transporter expression in prostate cancer.

Keywords

hZIP1; promoter; RREB-1; PC-3 cells

INTRODUCTION

The normal human prostate gland (peripheral zone) is characterized by its unique capability and function to accumulate high levels of zinc. This capability is lost in prostate cancer, which develops mainly in the peripheral zone [1–3]. This loss of zinc accumulation is an essential event that is responsible for the metabolic transformation that is necessary for prostate carcinogenesis. Zinc uptake by plasma membrane zinc transporters contributes to

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zinc accumulation by the prostate epithelial cells. We previously showed that hZIP1, a member of the ZIP (Zrt/Irt like protein) family of zinc transporters, functions in zinc uptake and accumulation by prostate cells [4]. In addition, we showed that hZIP1 is down-regulated and zinc content is decreased in adenocarcinomatous glands and in prostatic intraepithelial neoplasia lesions (PIN) compared to normal prostate tissue [5]. Thus the down-regulation of hZIP1 is an important event responsible for the decrease in prostate zinc level in cancer.

One possible mechanism for the decrease in hZIP1 level is regulation of hZIP1 gene expression at the transcriptional level. The public transcript for hZIP1 (NM_014437.3) from the Entrez Gene database is 2445 bp in length with five exons, three of which contain the coding sequence. However, many other transcripts have also been reported for hZIP1. These can be divided into three categories depending on the number of exons. Indeed, the reported mRNAs contain either three, four, or five exons with the majority being composed of four exons (http://dbtss.hgc.jp/). Due to the high number of transcripts reported, several promoter regions may be functional.

Makhov et al. [6] recently characterized the transcription start site (TSS) and the minimal promoter of hZIP1 in prostate cancer cells. The location of the TSS is consistent with a transcript containing four exons. In this report, we describe the characterization of a 3′ region of the hZIP1 promoter involved in the down-regulation of the transporter expression in PC-3 cells. We show evidence that the Ras-Responsive Element Binding protein 1 (RREB-1) binds to the 3′ region of the promoter and is involved in hZIP1 down-regulation in PC-3 cells.

MATERIALSANDMETHODS

5′RACE

In order to identify the TSS of the hZIP1 gene, the 5′-rapid amplification of cDNA ends (5′RACE) technique was used with the BD SMART RACE cDNA amplification kit (BD Biosciences Clontech). The following reverse primer, GCACACAGATGGGCACCAGGCTGCAGAG, located about 150 bp downstream of the translation initiation ATG was synthesized. Total RNA was extracted from PC-3 cells using Trizol Reagent from Invitrogen. The 5′RACE reactions and the control reactions were performed following the manufacturer's instructions. The products obtained were cloned into a pCR4-TOPO (Invitrogen) vector and analyzed by sequencing.

Cloning of the Promoter Sequences Into Reporter Vectors

Genomic DNA from PC-3 cells was extracted using Trizol from Invitrogen. Two pairs of primers were designed to amplify the 5′ region upstream of the two TSSs found for the hZIP1 gene: F1=AGTCGGTACCGAACTTGGACCAGAATCCAGG, R1=AGTCCTCG AGGCGGTGCCTGAAAGTCCGGG, F2=AGTCGGTACCACAGCAAACCGACCGG CGAG, and R2= AGTCCTCGAGTGAGCACCAGCAGCAGCACC. A *Kpn*I site and an *Xho*I site were added to the forward and reverse primers respectively for cloning purpose. The PCR reaction contained 750 ng of DNA, 0.2 μM of each primer, 0.3mM of dNTP, 1mMMgSO_4, Platinum *Pfx* polymerase buffer and 0.4 units of Platinum *Pfx* polymerase

(Invitrogen). After digestion with *Kpn*I and *Xho*I, the PCR products were sub-cloned into the pGL3-Basic vector from Promega. The resulting plasmids were named pGL3-Prom(−2309/−622), pGL3-Prom(−1596/+121), and pGL3-Prom(−1596/−622). Several other constructs were generated by digestions using these constructs as templates.

In order to refine the location of an inhibitory element, unidirectional digestions with the Exonuclease III/S1 Nuclease (*Exo*III/S1N) were performed. To obtain a construct compatible with the unidirectional digestion, the promoter was inserted into pGL3-Basic at the blunted *Kpn*I site. The resulting construct was then digested with *San*DI (sensitive to *Exo*III) and *Bmt*I (resistant to *Exo*III) and subjected to an *Exo*III/S1N digestion following the manufacturer's protocol (Fermentas).

Cell Culture and Transfections

PC-3 cells were maintained in RPMI 1640 medium with L-glutamine and HEPES (Gibco) supplemented with 10% fetal bovine serum and 1% penicillin– streptomycin mixture. PC-3 cells were plated in 12-well plates (100,000 cells/well) 24 h before the transfection. The cells were transfected with the different Firefly luciferase constructs and the phRG-TK vector using the Fugene HD transfection reagent from Roche Applied Science.

Quantification of the Luciferase Activities

Forty-eight hours after transfection, the cells were lysed in the wells using the Passive Lysis Buffer (Promega). Twenty microliters of the lysate were used to quantify the Firefly luciferase activity and the *Renilla* luciferase activity with the Dual Luciferase Reporter Assay System (Promega) following the manufacturer's protocol. The light produced by the luciferases was read in a TD-20/20 single-tube luminometer (Turner Biosystems) with 2 sec delay and 10 sec integrals. The Firefly luciferase activity values were corrected for transfection efficiency using the values obtained for the *Renilla* luciferase activity. The results are expressed as luciferase activity over basal activity obtained with the promoterless construct. N = 3 within each group for an experiment. Each experiment was repeated two or three times with similar results.

Electrophoretic Mobility Shift Assay (EMSA)

Nuclear extracts from PC-3 cells were obtained using NE-PER® Nuclear and Cytoplasmic Extraction Reagent (Pierce Biotechnology). The sequence of the biotin-labeled oligonucleotide (along with the reverse complement) and the corresponding non-labeled oligonucleotide is GCGATCCAGCACCCAAACTTACCCTGTCCA. The sequence of the oligonucleotide specific for the RREB-1 binding site is GGTCCCCCACCATCCCCCGCCATTTCCA [7]. Double strand probes were obtained by annealing equimolar amounts of the complementary oligonucleotides in a thermocycler at 95°C for 5 min followed by a decrease of 1°C every 1 min down to 25°C. The EMSA were performed using the LightShift® Chemilum in escent EMSA Kit (Pierce Biotechnology) following the manufacturer's recommendations. Briefly, the biotin-labeled probes were incubated with the nuclear extracts and poly(dI–dC) in the presence or absence of an excess of unlabeled probes. The complexes were then run through a 6% non-denaturing acrylamide

gel before transfer to a Biodyne® B Nylon Membrane (Pierce Biotechnology), UV cross-linked and detected with streptavidin HRP conjugate.

ChIP Assay

ChIP assays were performed using the EZ-Magna ChIP™ G kit from Millipore following the manufacturer's instructions. Briefly, following crosslinking with 1% formaldehyde, PC-3 cells were scraped off the 150-mm dish in PBS. After centrifugation, the pellets were resuspended in cell lysis buffer and homogenized for 10 strokes with a dounce homogenizer. The nucleus pellets obtained after centrifugation were resuspended and lysed in nuclear lysis buffer. The chromatin was sheared by sonication and immunoprecipitated overnight at 4°C in the presence of protein G magnetic beads and IgG or 2 μg of RREB-1 antibody (described in Ref. [8] and kindly provided by Dr. Leiter). After several washes, the DNA/protein complexes were eluted from the beads and the proteins digested by Proteinase K. The DNA was then purified using spin columns to obtain a final volume of 50 μl. DNA (2.5 μl or 2.5 μl water for the negative control) was used for each 25 μl PCR reaction performed with the Platinum® PCR SuperMix (Invitrogen). The primers specific for the hZIP1 promoter were 5′-AGACTCTTAGGGCCCCTCTCTT-3′ (forward) and 5′-GAGTGGGTGAAGCCAAAAAG-3′ (reverse). After the initial denaturating cycle at 94°C for 5 min, 32 cycles at 94°C for 30 sec, 55°C for 30 sec and 72°C for 1 min were performed. The PCR products were analyzed on a 2% agarose gel.

Site-Directed Mutagenesis of the RREB-1 Site

The constructs containing mutated RREB-1 binding sites were obtained using the QuikChange II Site-Directed Mutagenesis Kit (Stratagene) following the manufacturer's instructions. The primers were designed using the QuikChange® Primer Design Program (Stratagene). The sequence of the forward primer is GGAGCGATCCAGCACCTGA ACTTGTCCTGTCCAGCCCGGGC and the reverse primer is the reverse complement. The mutagenesis was performed with the construct Prom(−1507/−461) as a template. The successful mutants were confirmed by sequence.

siRNA Experiments

RREB-1 siRNA, Non-Targeting siRNA and siGLO Green Transfection Indicator were obtained from Dharmacon. Twenty-four hours after being plated in 96-well plates, PC-3 cells were co-transfected with 50nM siRNA or Non-Targeting siRNA and 50nM siGLO as an indicator for transfection efficiency using DharmaFECT 2 Transfection Reagent (Dharmacon) following the manufacturer's instructions for PC-3 cells. Twenty-four hours after the siRNA transfection, the cells were transfected with the luciferase vectors using Fugene HD transfection reagent. The luciferase assays were performed 24 h after the second transfection.

Alternatively, PC-3 cells in 96-well plates were co-transfected with 50nM siRNA or Non-Targeting siRNA and 50nM siGLO. Forty-eight hours after transfection, RNA was extracted from the cells with the QIAshredder and RNeasy Mini Kit (Qiagen). Five hundred nanograms of RNA were used as a template to synthesized cDNA with the First Strand

cDNA Synthesis Kit (Fermentas). Polymerase Chain Reaction to amplify RREB-1 and GAPDH was performed using Platinum® PCR SuperMix and the following primers:

RREB1-Forward: CACCACAGACACCAACAAGTTCAGTCC.

RREB1-Reverse: CTTCTCAGGGAACAGGTGGTAACTCC.

GAPDH-Forward: CCACCCATGGCAAATTCCATGGCA.

GAPDH-Reverse: TCTAGACGGCAGGTCAGGTCCACC.

RAS Experiments

The plasmids pCMV-RasV12 (constitutively active form of Ras) and pCMV-RasN17 (dominant-negative form of Ras) were obtained from Clontech. Twenty-four hours after plating in 96-well plates, PC-3 cells were co-transfected with the RAS plasmids, the different Firefly luciferase constructs and the *Renilla* luciferase vector using the Fugene HD transfection reagent (Roche Applied Science). The luciferase assays were performed 48 h post-transfection.

RESULTS

5′RACE

Our 5′RACE using PC-3 cell RNA revealed the presence of two TSSs. One TSS (TSS_1) is found 804 bp upstream of the translation initiation ATG codon and corresponds to a transcript with four exons (Fig. 1A). TSS1 is consistent with the TSS described by Makhov et al. [6] The second TSS (TSS_2) is located 84 bp upstream of the translation start codon of hZIP1 and corresponds to a transcript with three exons (Fig. 1B). As we have two potential TSSs, the numbering in this report considers the ATG as +1.

Characterization of the Promoter

From the 5′RACE results, we arbitrarily delimited a promoter region around each TSS to subclone into a Firefly luciferase reporter system. The primers to amplify the two putative promoters were chosen so that the forward and reverse primers were located about 1.5 kb upstream and about 200 bases downstream of each TSS. The sequences obtained after PCR were named Prom(−2309/−622) and Prom(−1596/+121). Additional constructs were obtained by PCR or restriction enzyme digestions using Prom(−2309/−622) and Prom(−1596/+121) as templates. The pGL3 vectors containing the different regions of the potential hZIP1 promoter were transfected into PC-3 cells along with the phRG-TK plasmid constitutively expressing the *Renilla* luciferase to correct the Firefly luciferase activity for transfection efficiency.

Our luciferase assays shown in Figure 2 locate the minimal promoter around TSS_1 (construct Prom(−1040/−721)) which confirms the results obtained by Makhov et al. [6] On the other hand, the region around the TSS_2 did not lead to luciferase expression (construct PromΔ(−1507/−276)). The comparison of the luciferase activities with Prom(−1596/+121) and Prom(−1596/−622) reveals the presence of an inhibitory region located downstream of the TSS_1 between base pairs −622 and +121 (Fig. 3). Since the mechanism of hZIP1 down-

regulation in prostate cancer is not known, we decided to focus on this inhibitory region as transcriptional regulation is a potential mechanism for the decrease of zinc transporter expression.

Inhibitory Region in hZIP1 Promoter

To locate more precisely the inhibitory element, we performed restriction enzyme digestions and Exonuclease III/S1 Nuclease digestions with Prom(−1596/+121) as a template. The luciferase assays with the new constructs shown in Figure 3 reveal that several regions downstream of the TSS_1 are involved in the down-regulation of the luciferase activity. Interestingly, the presence of a 30 bp sequence is sufficient to decrease the activity down to the level obtain with the construct Prom(−1596/+121). Indeed, similar high luciferase activities are obtained with the constructs Prom(−1596/−622) and Prom(−1507/−491) whereas the construct Prom(−1507/−461) lead to an activity near background (Fig. 3). These results locate a strong inhibitory element between base pairs −491 and −461. Therefore, we decided to further study this 30 bp sequence.

RREB-1 Binds to the Region −461/−491

Analysis of the promoter sequence located between the nucleotides −461 and −491 with MatInspector (www.genomatix.de) and TFSEARCH (http://www.cbrc.jp/research/db/TFSEARCH.html) reveals that the motif ACCCAAACTTACCC present in this region is a potential binding site for RREB-1. RREB-1 is a transcription factor acting downstream of the RAS–RAF–MEK–ERK pathway [7,9]. Interestingly, this pathway is activated in prostate cancer due to an increase in production of growth factors (e.g., TGFα, EGF, and KGF) [10]. These characteristics make RREB-1 a good candidate as a factor contributing to hZIP1 down-regulation in prostate cancer.

In order to verify that RREB-1 binds to this region, a 35 bp biotin labeled oligonucleotide was synthesized and used for Electrophoretic Mobility Shift Assay (EMSA) with nuclear extracts from PC-3 cells. The oligonucleotide corresponds to the sequence (−491/−457) of the hZIP1 promoter. The addition of nuclear extract to the reaction leads to the apparition of a band shift that is competed when an excess of unlabeled oligonucleotides is added (Fig. 4).

The potential RREB-1 binding site in the hZIP1 promoter is ACCCAAACTTACCC. Based on Thiaga-lingam et al. [7], CCCCAAACCACCCC is the consensus binding site sequence for RREB-1. Therefore, we mutated the hZIP1 promoter site to ACCTGAACTT*GT*CC in order to disrupt the core sequence. An excess of unlabeled oligonucleotides containing the mutated site has no effect on the shift (Fig. 4). This result suggests that the mutation is effective in preventing the binding of the factor. To confirm RREB-1 as the binding factor, we performed a gel shift with an excess of unlabeled oligonucleotide containing a specific site for RREB-1 [7]. Figure 4 shows that the oligonucleotide specific for RREB-1 competes with the band shift, further suggesting RREB-1 as the factor binding to the region −491/−461.

We mutated the RREB-1 site in the construct Prom(−1507/−461) as well to obtain the construct Prom(−1507/−461)Mut. These two constructs were then transfected into PC-3

cells for luciferase assays. Consistent with a role of RREB-1 in the reporter down-regulation, mutation of the binding site lead to a 10-fold increase in luciferase activity when compare to the construct with the wild-type RREB-1 site (Fig. 5).

We showed that RREB-1 binds to the hZIP1 promoter in vitro. Therefore, in order to confirm the binding of RREB-1 in vivo, we performed a ChIP assay. Chromatin/protein complexes from PC-3 cells were immunoprecipitated with a RREB-1 antibody [8]. After digestion of the proteins and purification of the DNA, a PCR amplifying the region −461/−491 of hZIP1 promoter was performed. Figure 6 shows that the eluate from the chromatin/protein complex immunoprecipitated with a RREB-1 antibody was enriched for hZIP1 promoter compared to the eluate obtained from the immunoprecipitation with the IgG. These results confirm the binding of RREB-1 to the hZIP1 promoter.

RREB-1 Knock-Down Effect on Luciferase Activity

Our results suggest that RREB-1 binds to the 3′ region of the hZIP1 promoter and is involved in hZIP1 down-regulation. To confirm the role of RREB-1, we decided to knock-down the transcription factor using siRNA. PC-3 cells were transfected with the siRNA or Non-Targeting siRNA and with the Firefly luciferase construct containing the potential RREB-1 site (Prom(−1507/−461)) and the construct with the mutated site. RREB-1 knock-down was verified by RT-PCR and the results are shown on Figure 7A. Treatment of the cells with siRNA for 48 h resulted in a 44% decrease for RREB-1 expression. Figure 7B shows a 1.5-fold increase in luciferase activity with Prom(−1507/−461) combined with RREB-1 siRNA compared to the control Non-Targeting siRNA while no significant change is observed with the mutated site. In addition, we also observed a significant increase in luciferase activity with the full length fragment (Prom(−1596/+121)) in cells treated with RREB-1 siRNA compared to control Non-Target siRNA (not shown). Since, the RREB-1 siRNA did not lead to a high level of knockdown of RREB-1 expression, we used another strategy to inhibit the transcription factor.

The RAS Pathway Is Involved in hZIP1 Down-Regulation

RREB-1 has been shown to be activated through the RAS–RAF–MEK–ERK pathway [9]. Therefore, in order to inhibit RREB-1 activity, we targeted the RAS pathway by expressing RAS-N17, a dominant-negative form of RAS [11]. PC-3 cells were co-transfected with the constructs containing the RREB-1 site or its mutated form and a plasmid expressing RAS-N17 or RAS-V12 (constitutively active form of RAS). Figure 8 shows that the inhibition of the RAS pathway and subsequently the inhibition of RREB-1 leads to a 2.5-fold increase in luciferase activity with the construct Prom(−1507/−461) containing only the RREB-1 site, further suggesting RREB-1 as a factor involved in hZIP1 down-regulation (Fig. 8). A slight increase in luciferase activity is seen with the construct containing the mutated RREB-1 site when RAS is inhibited. This can be due to the fact that the mutation does not completely prevent the binding of RREB-1. It would also explain the fact that the luciferase activity obtained with Prom(−1507/−461)Mut is 30% lower than the activity obtained with the Prom(−1507/−491) (Fig. 5).

DISCUSSION

hZIP1 is a zinc transporter that is ubiquitously expressed in a majority of tissues [12]. Several transcripts have been reported for hZIP1 and the TSSs for all of these can be found in the DataBase of Transcription Start Site site (http://dbtss.hgc.jp/). These DataBase sequences can be organized into three categories: transcripts with five exons, transcripts with four exons (most reported), and transcripts with only three exons. However, the varying number of exons in the reported transcripts does not alter the coding sequence. The variety of transcripts suggests that several potential promoter regions may be active. In a recent report, Makhov et al. [6] described the characterization of the core promoter of hZIP1 in PC-3 cells. The TSS they identified is located 836 bp upstream of the ATG codon and the mRNA corresponds to a transcript with four exons. Although our 5′RACE experiment identified two TSSs (−804 bp from a four exon transcript and −84 bp from a three exon transcript), luciferase assays located a minimal promoter only around the most 5′TSS. Therefore, our minimal promoter is consistent with the core promoter described by Makhov et al. [6]. Interestingly, our luciferase assays allowed us to identify the 3′ region of hZIP1 promoter as a region involved in the inhibition of hZIP1 expression.

Zinc depletion associated with hZIP1 down-regulation has been identified in prostate cancer [5]. However, the mechanism of decreased hZIP1 expression has not been characterized. Initially we considered promoter hypermethylation as a potential mechanism of hZIP1 silencing; however, analysis of the methylation status of the promoter in laser capture micro-dissected material from prostate cancer tissue sections and DNA from PC-3 and DU145 cells revealed that hypermethylation is not the mechanism for down-regulation (unpublished studies). In this report we focused on transcriptional regulation as the mechanism of hZIP1 silencing. Transcriptional down-regulation of tumor suppressor genes has been described in several types of cancer including lung cancer [13], colon cancer [14], and melanoma [15]. The up-regulation of specific transcription factors in cancer compared to normal tissues can lead to the silencing of tumor suppressor genes. Our luciferase assay experiments revealed the presence of several independent inhibitory elements in the 3′ region of the hZIP1 promoter including a strong inhibitory element located within a 30 bp sequence (−491/−461). Further analysis of the −491/−461 sequence combining in silico analysis, gel shift, ChIP, site-directed mutagenesis and luciferase assays suggests that RREB-1 binds to this sequence and is involved in hZIP1 transcriptional down-regulation. RREB-1 is a zinc finger transcription factor that has been described both as an activator [8] and a repressor [9] of transcription depending on the promoter that it binds. RREB-1 acts downstream of ERK in the RAS–RAF–MEK–ERK pathway [7,9,16]. This pathway has been shown to be up-regulated in prostate cancer progression [10,17,18]. Our results combined with the previous reports on RREB-1 would elucidate one of the mechanisms involved in hZIP1 down-regulation. Indeed, the up-regulation of the RAS pathway in prostate cancer compared to normal prostate would lead to the up-regulation of RREB-1. The transcription factor would bind to the hZIP1 promoter and inhibit the transcription of the transporter subsequently decreasing zinc uptake into the cells.

Zinc has been reported to induce a number of anti-tumor effects in prostate cancer cell lines and in prostate tumor xenografts; for example, increased apoptosis, and metabolic

bioenergetic alterations [19–23]. In addition, decreased zinc accumulation is a consistent and common observation in prostate cancer tissue. Thus the lost ability of prostate epithelia cells to accumulate zinc is a necessary metabolic transformation required for the development and progression of prostate malignancy. As such, we characterize zinc as a tumor suppressor agent in prostate cancer. Because of its relationship in the accumulation of zinc in normal prostate glands and its down-regulation in adenocarcinomatous glands, the mechanism and factors involved in the down-regulation of hZIP1 are essential to understanding the events associated with the development of prostate cancer.

hZIP1 expression is observed in several prostate cancer cell lines (e.g., PC-3, LNCaP, DU-145) under standard culture conditions. Therefore, the loss of hZIP1 expression in situ in peripheral zone malignant cells is not due to gene deletion or fatal mutations that eliminate gene expression, but is likely due to other silencing mechanisms. Elucidation of these silencing mechanisms may identify addition molecular targets for development of effective therapies against prostate cancer. Here we show that the Ras pathway and activation of RREB-1 are involved in hZIP1 down-regulation. However, we have also shown that the hZIP1 promoter region located downstream of the TSS1 contains several additional elements that seem independently able to reduce the transcription level. Therefore, the factors binding to these additional regions will need to be identified in order to fully understand the mechanism of hZIP1 down-regulation in prostate cancer cells.

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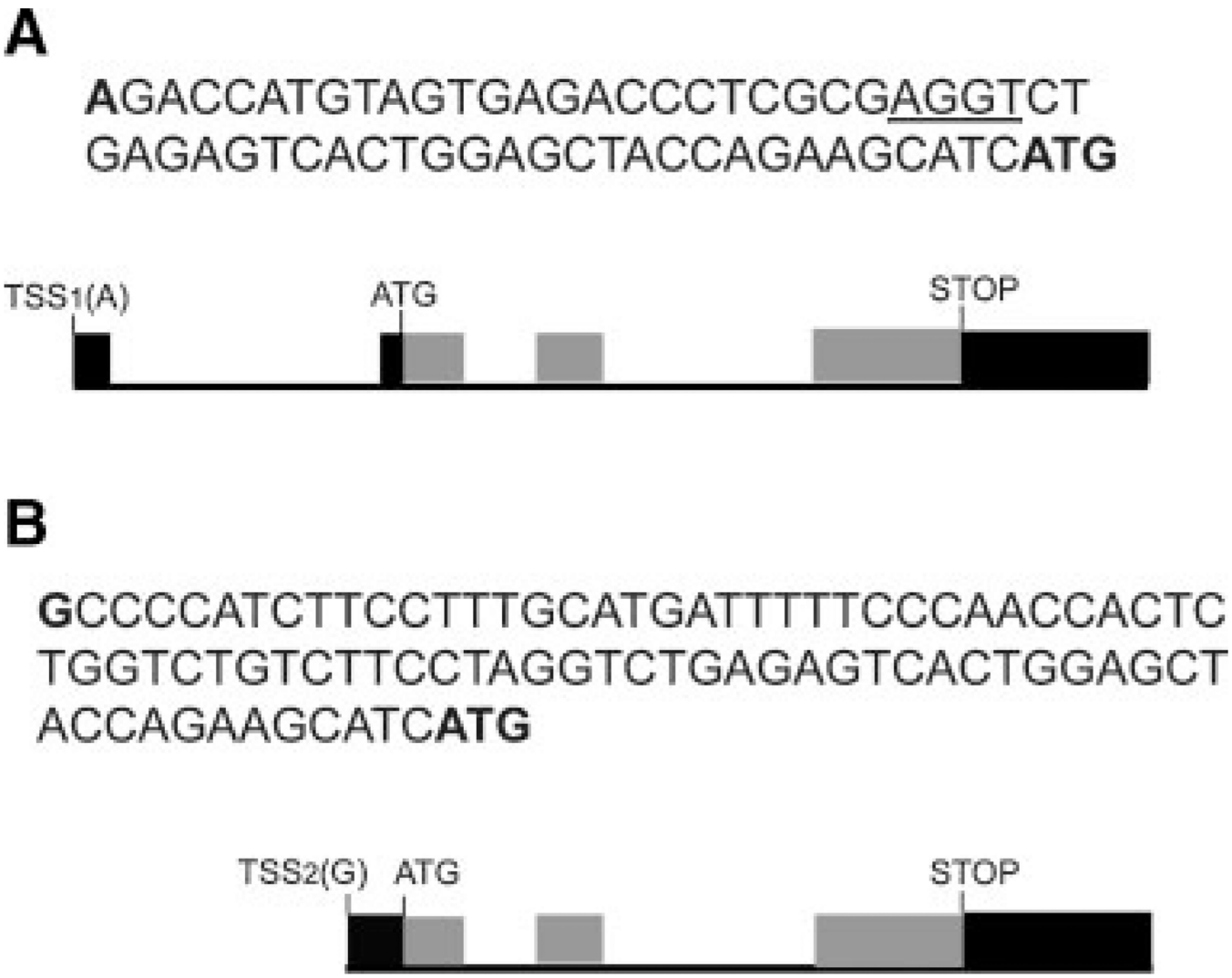

Fig. 1.

Results of the 5′RACE experiments. Total RNA fromPC-3 cells was used as a template to performed 5′RACE. The two sequences that were obtained are shown with a representation of the corresponding transcripts. The ATG codon is considered as +I. **A**: Sequence leading to a 4-exon transcript. The TSS_I and the start codon (ATG) are in bold. The four nucleotides underlined show the intron–exon limit. **B**: Sequence leading to a 3-exon transcript. The TSS_2 and the start codon (ATG) are in bold. TSS = Transcription Start Site. The exons are shown in black and the gray boxes show the coding region.

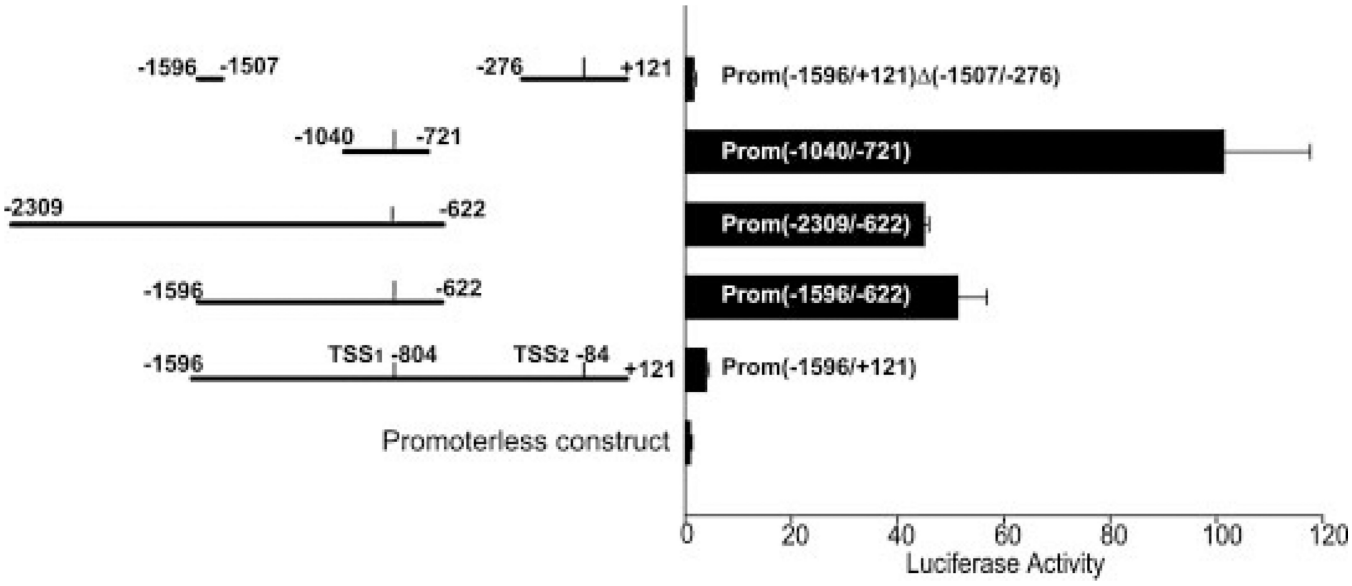

Fig. 2.

Analysis of hZIP1 promoter with a luciferase reporter system. PC-3 cells were transfected with constructs containing the Firefly luciferase reporter gene under the regulation of different regions of the hZIP1 promoter. Forty-eight hours after transfection, luciferase activity was measured. The background activity obtained with the promoterless construct is set as I. Each value represents the mean ± SE of two independent experiments with N = 3 within each group for each experiment.

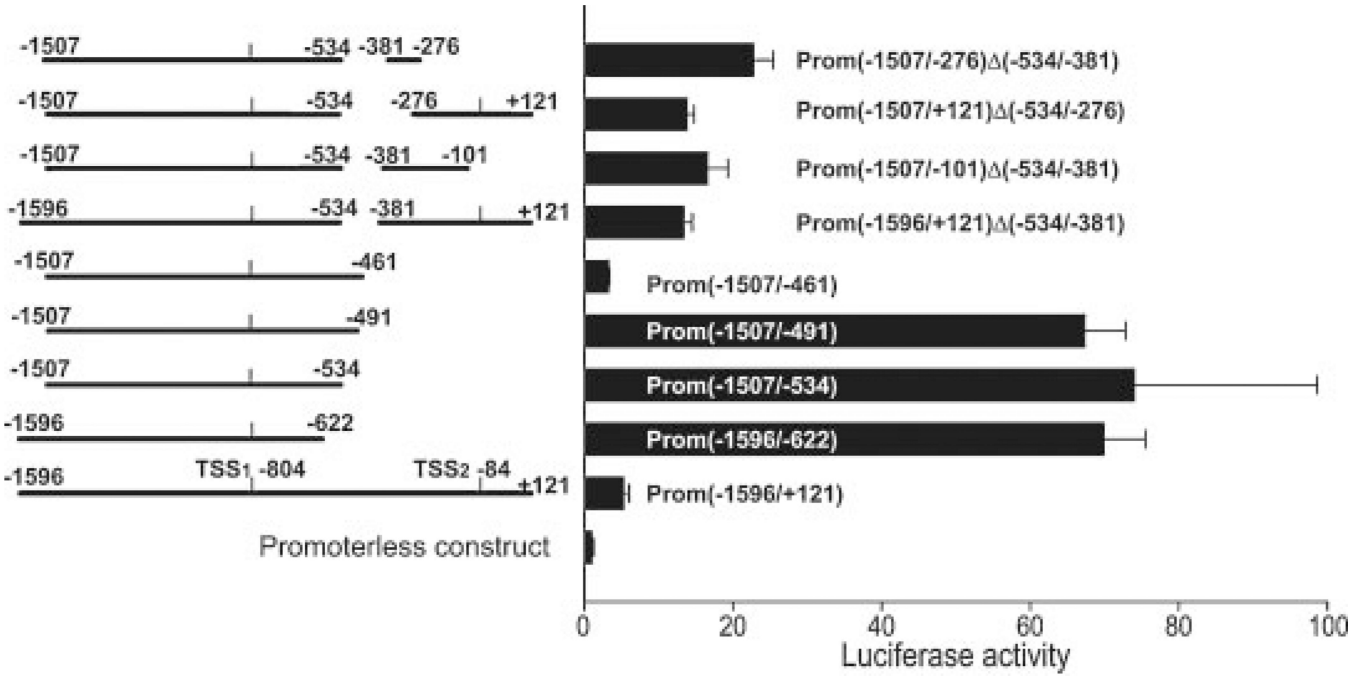

Fig. 3.

Location of the inhibitory regions in hZIP1 promoter. PC-3 cells were transfected with luciferase constructs containing several deletions in the 3′ region of the hZIP1 promoter. Forty-eight hours after transfection, luciferase activity was measured. The background activity obtained with the promoterless construct is set as I. Each value represents the mean ± SE of two independent experiments with N = 3within each group for each experiment.

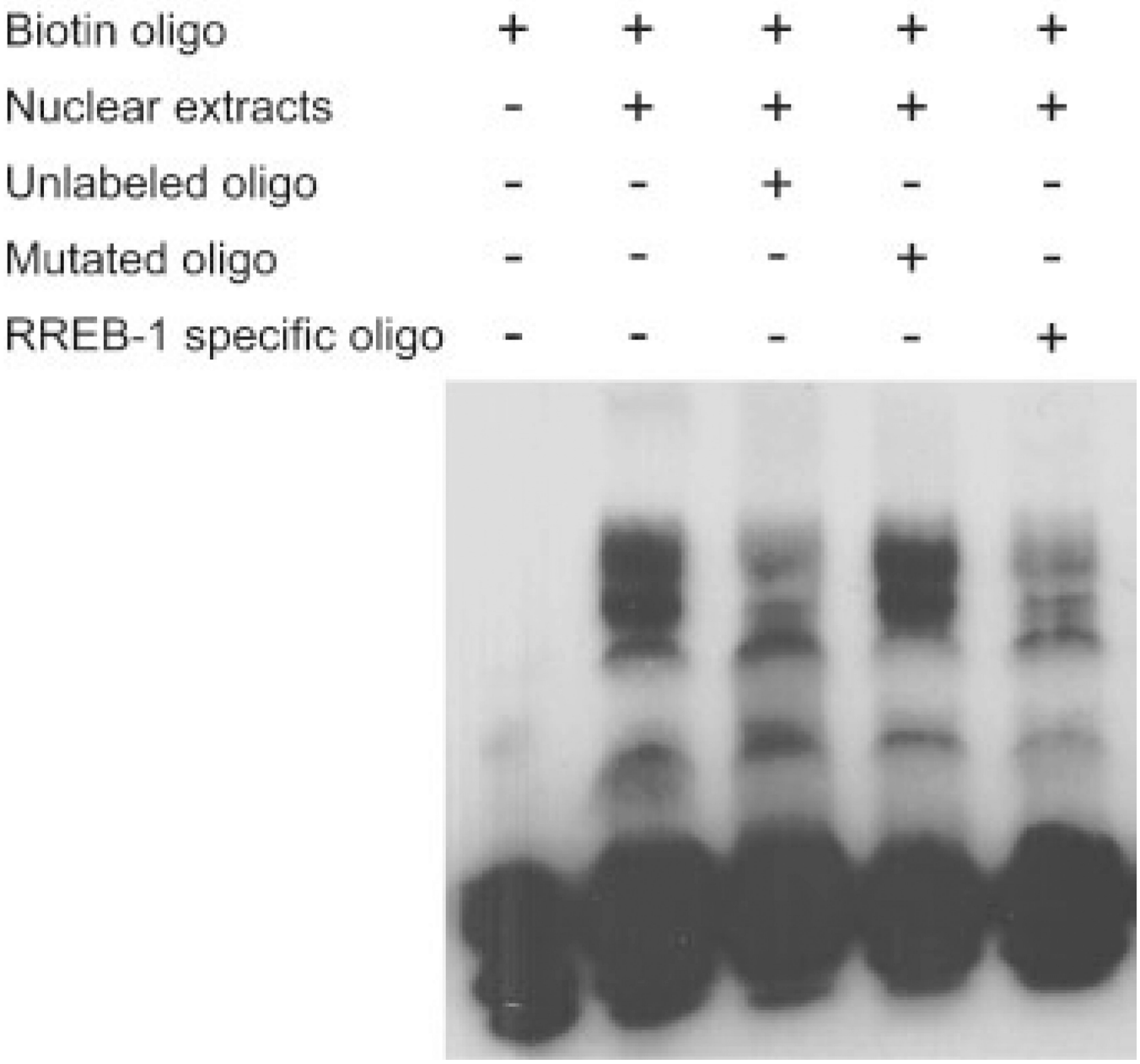

Fig. 4.

Electrophoretic Mobility Shift Assay with nuclear extracts from PC-3 cells. Gel shift experiment using PC-3 cell nuclear extracts and a biotin-labeled oligonucleotide including the −491/−461 inhibitory sequence. The unlabeled oligonucleotide has the same sequence as the biotin-labeled one. The unlabeled RREB-1 oligonucleotide contains a specific binding site for the transcription factor. The mutated oligo correspond to the −491/−461 sequence with mutations in the potential RREB-1 site. The competition was performed by adding 200× excess of unlabeled oligonucleotides.

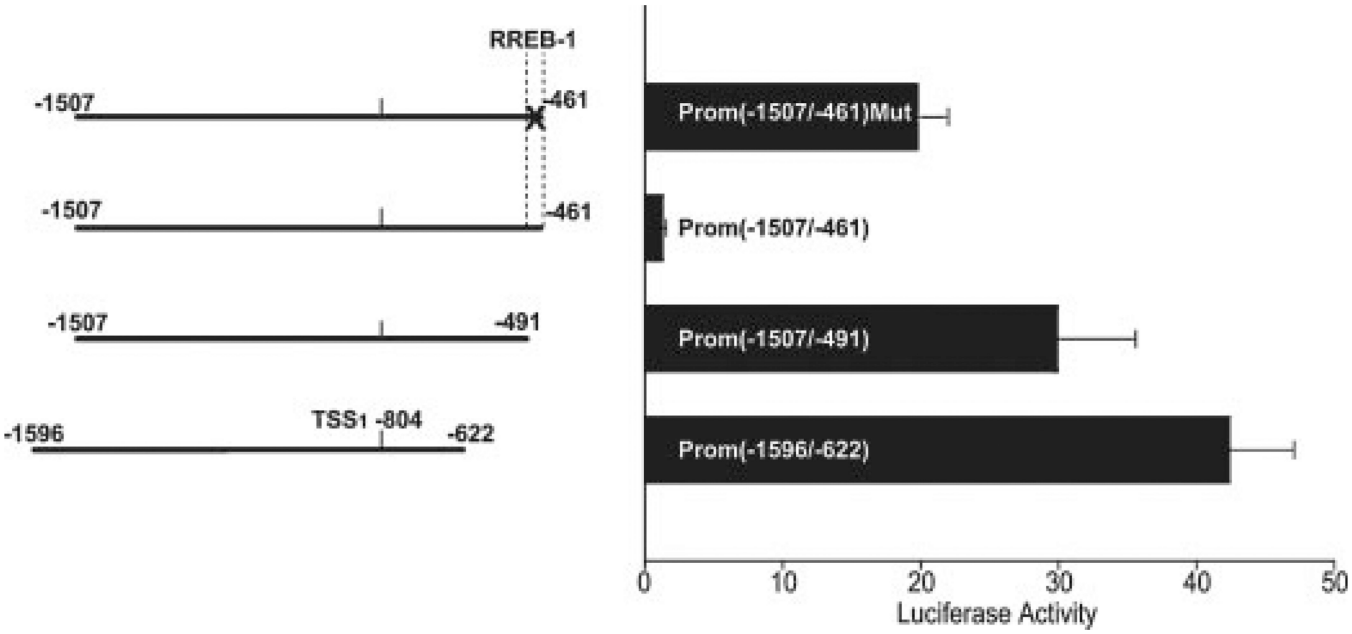

Fig. 5.

Effect of the potential RREB-1 site mutation on luciferase activity. PC-3 cells were transfected with the construct Prom(−1507/−461) containing the potential RREB-1 site and the construct Prom(−1507/−461)Mut in which the RREB-1 site is mutated. The cross represents the mutation. The luciferase assays were performed 48 h after transfection. The background activity obtained with the promoterless construct is set as I. Each value represents the mean ± SE of one representative experiment (N = 3).

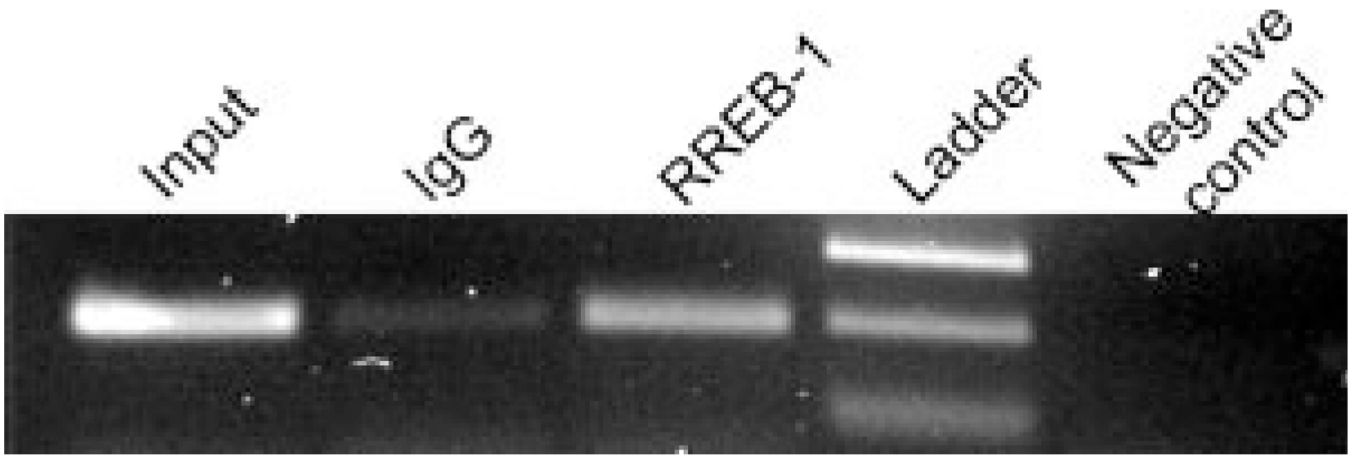

Fig. 6.

Binding of RREB-1 on hZIP1 promoter in vivo. PC-3 cells were cross-linked and chromatin/protein complexes were immunoprecipitated with a RREB-1 antibody or IgG antibody. The purified DNA before immunoprecipitation (input) and after immunoprecipitation (IgG and RREB-1) was subjected to a PCR specific for the region −491/−461 of the hZIP1 promoter. For the negative control, the PCR was performed without DNA.

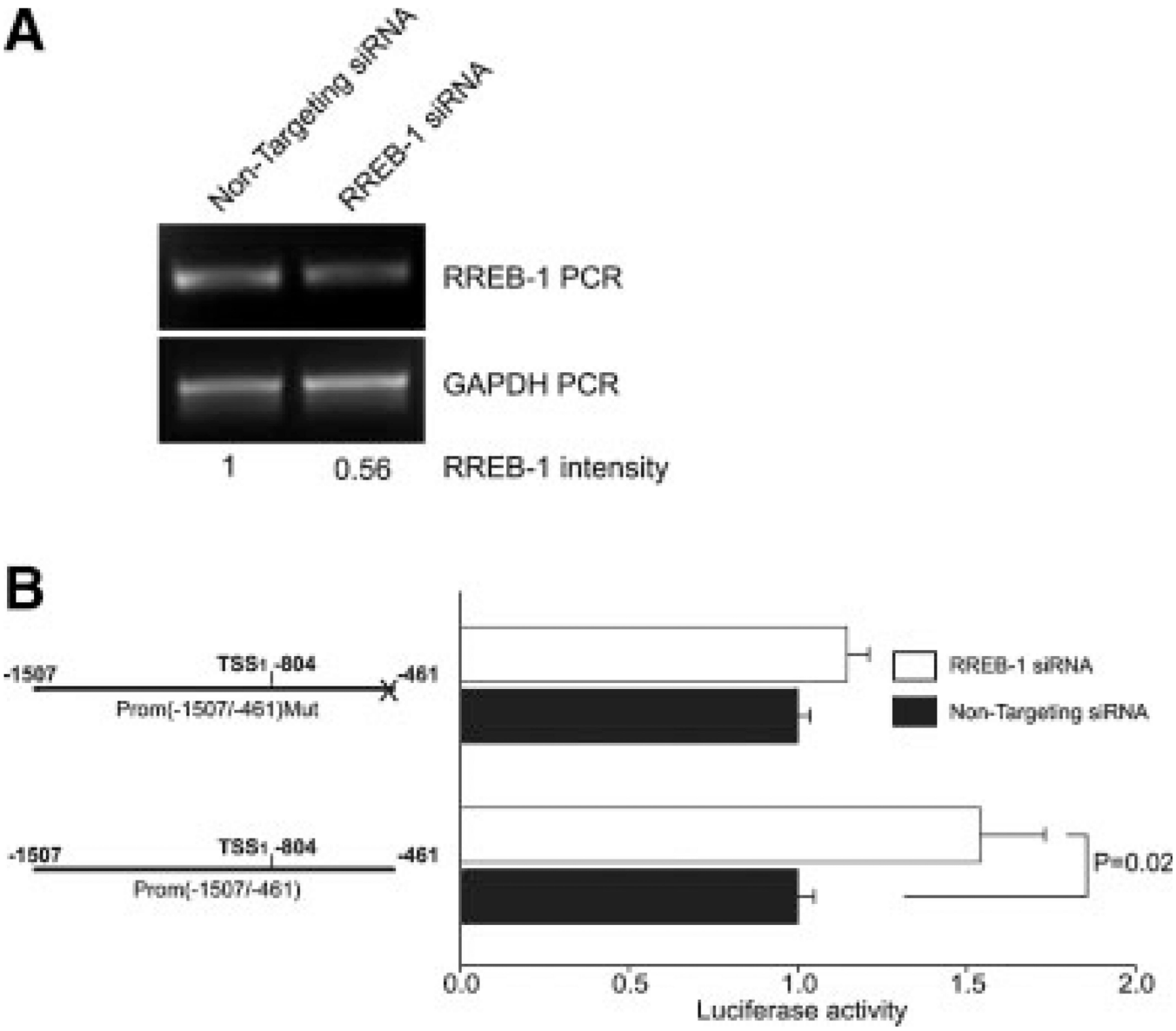

Fig. 7.

Effect of RREB-1 knock-down on luciferase activity. **A**:RT-PCRfromPC-3 cells transfected with Non-Targeting siRNA or RREB-1 siRNA. RNA was extracted 48 h after transfection. The band intensities were measured with the spot denso function of the AlphaEase v. 5.5 software. The intensities for RREB-1 were corrected with the GAPDH values. The intensities obtained with the Non-targeting RT-PCR were set as one. **B**: PC-3 cells were co-transfected with siRNA and the luciferase constructs. The Firefly luciferase activities were corrected with their respective background (promoterless construct + RREB-1 siRNA or promoterless construct + Non-Targeting siRNA) and the values obtained with Non-Targeting siRNA were set as I. Each value represents the mean ± SE of two independent experiments with N = 3 within each group for each experiment. Statistical analyses were performed using a Student's *t*-test with a risk level (*P*) set at 0.05.

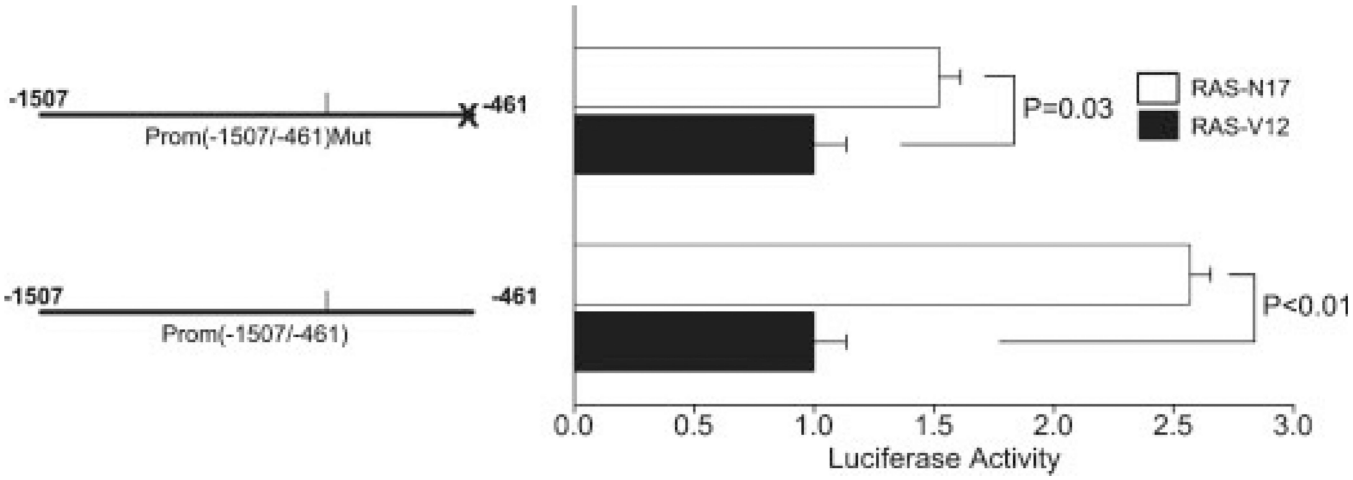

Fig. 8.

Effect of the RAS dominant-negative protein on luciferase activity. PC-3 cells were co-transfected with the luciferase constructs and a plasmid expressing a constitutively active RAS (RAS-VI2) or a plasmid expressing a dominant-negative form of RAS (RAS-NI7). The luciferase assays were performed 48 h after transfection. The Firefly luciferase activities were corrected with their respective background (promoterless construct + RAS-VI2 or promoterless construct + RAS-NI7) and the values obtained with RAS-VI2 were set as one. Each value represents the mean ± SE of two independent experiments with N = 3 within each group for each experiment. Statistical analyses were performed using a Student's *t*-test with a risk level (*P*) set at 0.05.