NRSF/REST confers transcriptional repression of the GPR10 gene via a putative NRSE/RE-1 located in the 5′ promoter region

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Abstract The G protein-coupled receptor GPR10 is highly localized to areas of the brain. In an effort to reveal transcriptional determinants of this tissue specificity, we recognized a putative NRSE (neuron-restrictive silencer element) located in the 5′ promoter region of the gene. The cognate NRSE binding protein NRSF (neuron-restrictive silencer factor) restricts gene expression to mature neurons and endocrine cells by repressing their transcription in non-neuronal/-endocrine cells. In cell lines where NRSF-mediated gene repression has been functionally established, the activity of the GPR10 promoter was repressed in a manner consistent with NRSE-dependent regulation. A specific point mutation to confer non-functionality of the NRSE revealed a 10-fold de-repression of reporter gene expression. In contrast, in the GPR10-expressing cell line GH3, mRNA transcripts of NRSF were undetectable and suppression of promoter activity was not observed. However, transfection of a rat NRSF expression vector resulted in significant repression of transcription, which was reversed by mutation of the NRSE. In conclusion, we demonstrate that the GPR10 gene is specifically regulated by NRSF, and suggest this to be a contributory factor in the tissue-specific distribution of GPR10 in vivo.

Key words: Neuron-restrictive silencer factor; Neuron-restrictive silencer element; GPR10

1. Introduction

GPR10 (prolactin-releasing peptide receptor, hGR3) is a member of the seven-transmembrane, G protein-coupled receptor superfamily [1], and is the human ortholog of rat unknown hypothalamic receptor-1 [2]. Initially isolated from hypothalamus, GPR10 was subsequently identified in various other brain regions, with highest expression levels localized to the reticular nucleus of the thalamus, the area postrema, and the anterior pituitary [3–6]. Discovery of a specific peptide ligand using a method of reverse pharmacology [7] facilitated the functional and pharmacological characterization of the receptor [8–10], which led to the observation that GPR10 stimulates prolactin release in the anterior pituitary [7]. However, subsequent reports have led to the assertion that its function in prolactin regulation may not be the primary role of GPR10, as conflicting data have emerged in the literature regarding temporal, spatial and environmental regulation of this signaling pathway [11–13]. Intuitively, the distribution profile of GPR10 alone suggests alternative functions such as a role in maintaining neuroendocrine and autonomic homeostasis [4,5], and enhanced receptor levels in the dorsomedial hypothalamus substantiates this implication with the possible involvement of GPR10 in mechanisms of feeding. This was confirmed by Lawrence et al., who reported that a single injection of prolactin-releasing peptide into rats resulted in a significant reduction in food intake with no obvious change in behavior [11]. It remains to be seen whether GPR10 may be a candidate target for therapeutic intervention in the treatment of obesity. While the physiological roles of GPR10 continue to be addressed, so the transcriptional regulation of the gene also is under investigation. Putative binding sites for transcription factors including Pit-1, Ptx-1, AP-1 and Sp1 have been identified in the cloned 5′ flanking region of the gene [14], and regulation of the promoter activity by forskolin and bromocryptine was shown to be indirectly mediated by CREB [15]. However, no regulatory evidence has been reported to justify its tissue-specific distribution. We therefore examined whether the restricted expression profile of GPR10 may be conferred by transcriptional regulation of the gene via specific elements in the 5′ promoter. One particular *cis*-regulatory element that directs mature neuronal and endocrine cell-specific expression is NRSE (neuron-restrictive silencer element [16]), also commonly referred to as RE-1 (repressor element-1 [17]). This 21-bp sequence confers transcriptional repression of genes in many non-neuronal cell types by binding the zinc finger transcriptional repressor NRSF (neuron-restrictive silencer factor [18]) or REST (RE-1 silencing transcription factor [19]). When bound, NRSF recruits the co-repressor mSin3 and/or coREST causing repression of gene expression, possibly by induction of hypoacetylation of histone and the remodeling of the chromatin structure [20–22]. However, in mature neuronal and endocrine cells repression is alleviated [18,19,23]. This de-repression is achieved either through attenuation of NRSF expression, or by the relative increase in alternatively spliced NRSF mRNA, generating truncated dominant negative isoforms such as REST4 that interfere with NRSF function [24–26]. An elegant example of this repressor mechanism was reported by Chen et al. who targeted the disruption of NRSF in embryonic mice, causing a de-repression of neuron-specific tubulin expression in non-neuronal tissue [27].

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Abbreviations: NRSE, neuron-restrictive silencer element; NRSF, neuron-restrictive silencer factor; RE-1, repressor element-1; REST, RE-1 silencing transcription factor

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Thus, transcriptional regulation by NRSE/NRSF confers an established mechanism of tissue-specific gene expression.

Here, we demonstrate that the GPR10 gene promoter exhibits a functional NRSE in the 5′ flanking region, and propose that the observed tissue distribution of this receptor may be influenced by NRSF activity.

2. Materials and methods

2.1. Cell lines

All cell culture reagents were purchased from Life Technologies (Grand Island, NY, USA) except fetal bovine serum (FBS) (Omega Scientific, Tarzana, CA, USA). GH3 cells were cultured in Ham's F12 medium with 2 mM L-glutamine, and supplemented with 15% horse serum (HS) and 2.5% FBS. C6 cells were cultured in RPMI with 10% FBS and 5% HS. Both 3T3 and HeLa cells were cultured in Dulbecco's modified Eagle's medium, supplemented with 10% FBS. All media contained 100 U/ml penicillin and 100 μg/ml streptomycin.

2.2. Gel-shift assays

Nuclear extracts from 3T3 cells were prepared essentially as described previously [28], and protein concentrations were determined using the BCA kit (Pierce, Rockford, IL, USA). Double-stranded oligonucleotides containing two tandem copies of the GPR10-NRSE (5′-CCAGC GGAAG CTGTC CGTGG TGCTG ACTCC TGCCT G-3′) were synthesized, and end-labeled using the Klenow fragment of DNA polymerase I in the presence of [α-^{32}P]dATP. Free nucleotides were separated by centrifugation through a G-50 column. DNA–protein binding reactions were carried out in 20 μl final volume of reaction buffer containing 10 mM Tris (pH 7.6), 50 mM NaCl, 0.5 mM dithiothreitol, 0.5 mM EDTA, 1 mM $MgCl_2$, 5% glycerol and 250 μg of poly(dI-dC) per ml. The nuclear extract (10 μg of protein) was

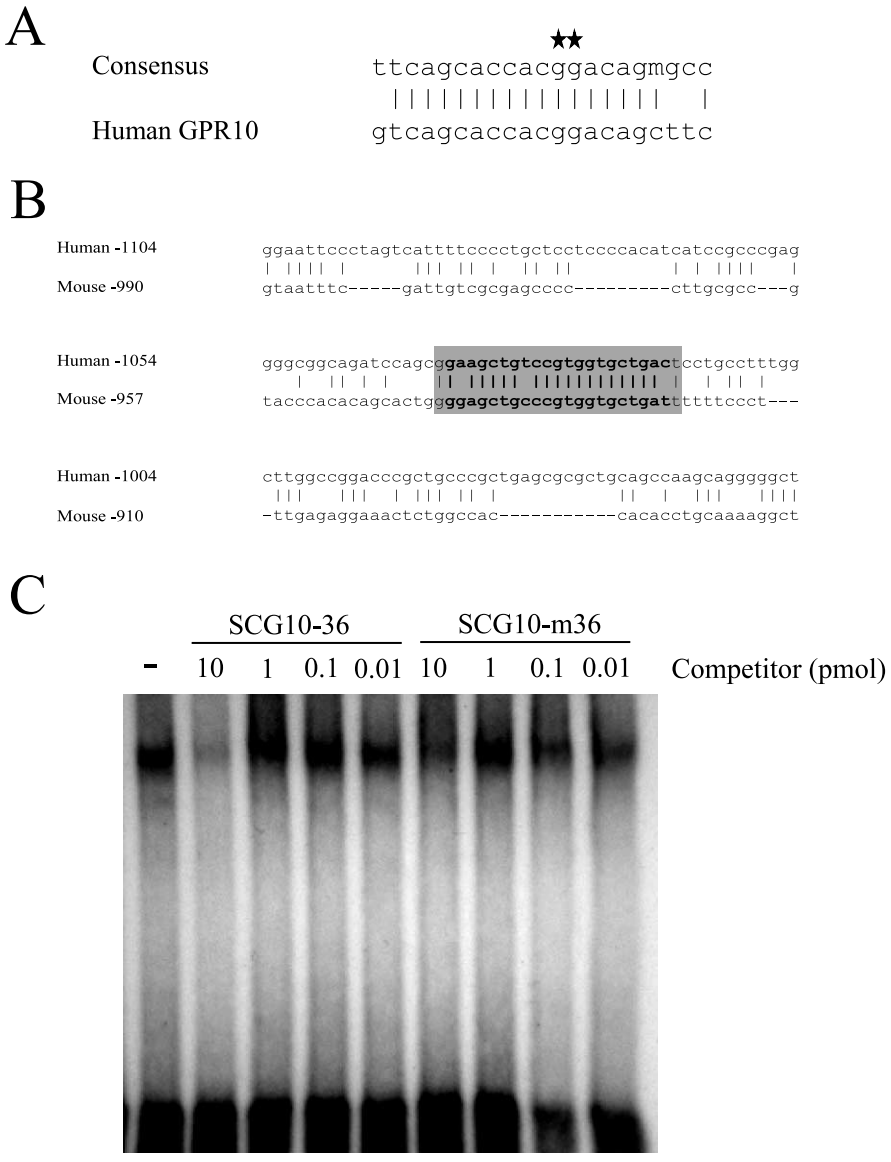

Fig. 1. Identification of a complex-forming NRSE in the 5′ flanking region of the GPR10 gene. A: Alignment of the consensus sequence derived from 29 functional NRSEs with the putative NRSE in the human GPR10 promoter. The nucleotides marked by asterisks were mutated to thymidine residues in the mutant competitor oligonucleotide shown in C. B: The partial nucleotide sequences of the cloned human gene aligned with the corresponding sequence from the mouse genome. The putative NRSF binding site is highlighted in the shaded box. C: Gel shift to show sequence-specific binding activity of NRSF to the human GPR10 NRSE. A ^{32}P-labeled probe consisting of two tandem repeats of the GPR10 NRSE was incubated with 3T3 cell nuclear extracts.

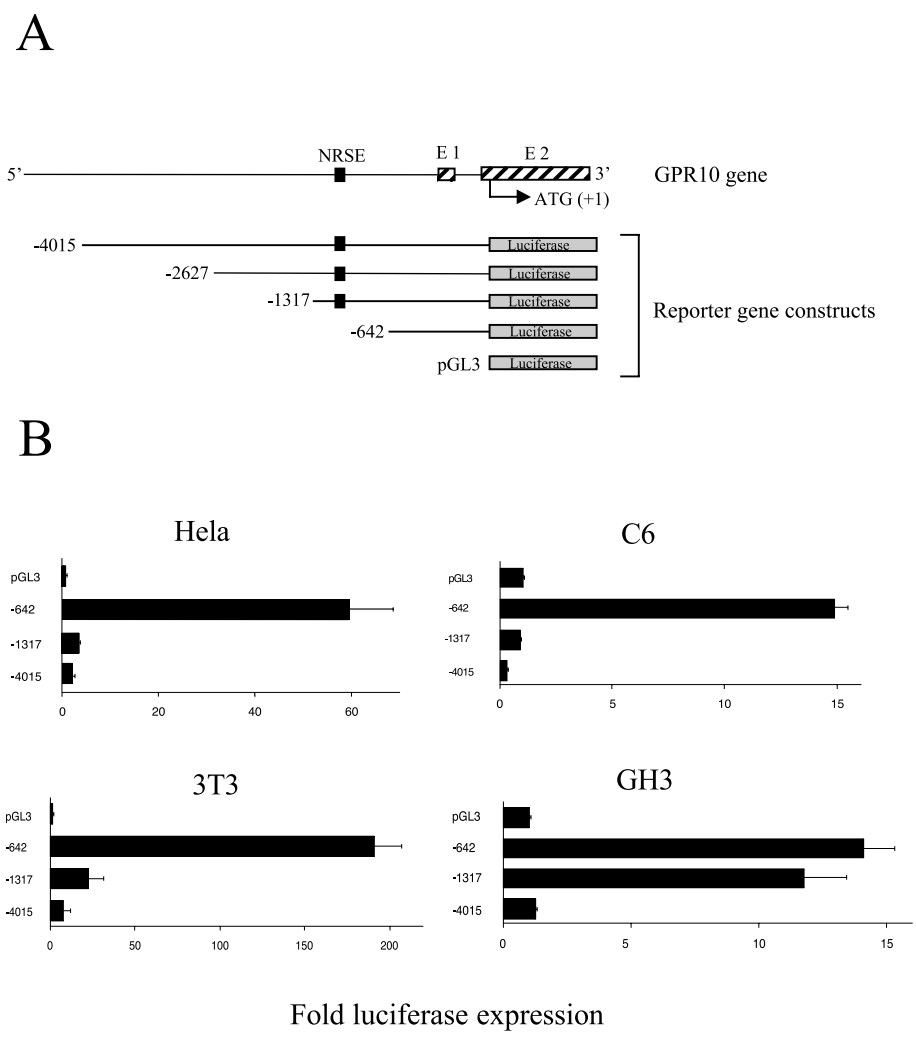

Fig. 2. Cell-specific expression pattern of GPR10 promoter constructs. A: Schematic representation of the reporter gene constructs used for transient transfections from fragments of the cloned human GPR10 gene promoter region. B: Cells were transiently transfected with the corresponding reporter gene constructs represented above. Firefly luciferase reporter activity was normalized to *Renilla* luciferase activity from a co-transfected internal control plasmid (pRL-CMV). Each experiment was performed at least three times in quadruplicate. Results are expressed as mean ± S.E.M.

added to the reaction buffer in the absence or presence of unlabeled competitor DNA (SCG10–36 5′-GCAAA GCCAT TTCAG CACCA CGGAG AGTGC CTCTG C-3′ and SCG10–m36 5′-GCAAA GCCAT TTCAG CACCA CTTAG AGTGC CTCTG C-3′) and pre-incubated for 10 min on ice. Radiolabeled probe (20 000 cpm) was then added and the mixture was incubated for a further 20 min on ice. Electrophoresis to resolve DNA–protein complexes was performed in 4% non-denaturing polyacrylamide gels in 0.25× Tris-borate–EDTA buffer at 150 V for 2–3 h.

2.3. Transient transfections and reporter gene assays

The cloned reporter gene constructs −4015, −2627, −1317 and −642 containing various lengths of the 5′ flanking region of the GPR10 gene were generously provided by Dr. Kazuo Chihara, Kobe University School of Medicine, Japan. Site-directed mutagenesis of the −2627 construct to introduce two juxtaposed mutations within the NRSE was generated using the Stratagene QuikChange, site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA). Mutation was confirmed by DNA sequence analysis. Rat NRSF cDNA cloned into pcDNA3 was a kind gift from Dr. Tonis Timmusk, University of Helsinki, Finland.

Cells were plated in 24-well plates and grown overnight or until 75–80% confluent and then transfected using Lipofectamine 2000 according to the manufacturer's procedure (Invitrogen, Carlsbad, CA, USA). 400 ng of reporter gene construct or pGL3 basic vector alone was co-transfected into the cells along with 1 ng of pRL-CMV containing the cDNA encoding *Renilla* luciferase (Promega, Madison, WI, USA) to normalize the luciferase activity. When necessary, 1 μg of rat NRSF expression vector was co-transfected, and controlled with 1 μg of pcDNA3. After transfection, cells were incubated for 5 h in serum- and antibiotic-free medium and then for a further 48 h in normal growth medium. Cells were then lysed and measurements of luciferase activity were performed using the dual luciferase assay system (Promega) in conjunction with a AutolumatPlus dual injection port luminometer (Berthold, Bad Wildbad, Germany). Firefly luciferase values were normalized with the *Renilla* luciferase activity of co-transfected pRL-CMV. Values are expressed as multiples of induction relative to the basic activity of pGL3.

2.4. RNA extraction and RT-PCR

Whole cell RNA was extracted by using the RNeasy Kit (Qiagen, Valencia, CA, USA) in accordance with the manufacturer's specifications. Total RNA (5 μg) was combined with 0.5 μg of oligo$(dT)_{16}$ and heated to 65°C for 10 min, then cooled on ice. RT buffer, dNTPs (50 μM each), dithiothreitol (5 mM), SuperScript II enzyme (50 units) (Life Technologies, Rockville, MD, USA) and H_2O were added for a total volume of 20 μl, and reactions were incubated at 42°C for 40 min. PCR reactions were carried out in 100 μl volume using 1 μl of cDNA template. Reactions contained 20 pmol each of forward and reverse primers, 0.2 mM each of dNTPs and 2.5 U of thermostable

Taq polymerase (TaKaRa Biomedical, Berkeley, CA, USA). Oligonucleotides: NRSF+: 5′-CGCTG TGACC GCTGT GGCTA CAATA C-3′, and NRSF−: 5′-GGGCA ATTAA GAGGT TTAGG-3′ were used to amplify rat NRSF. For rat prolactin, PRL+: 5′-GCCAA GTGTC AGCCC GGAAA-3′ and PRL−: 5′-GGCTT GTTCC TTGTC TTCAG G-3′ oligonucleotides were used. The rat insulin and β-actin primers have been reported previously [29].

Specific PCR amplification was confirmed by restriction digestion and direct sequencing after cloning the corresponding fragment into the pCRII TA cloning system (Invitrogen, San Diego, CA, USA).

3. Results and discussion

3.1. Identification of a putative NRSE in the 5′ regulatory region of the GPR10 gene

We used the on-line transcription factor database TRANSFAC to screen the 5′ flanking region (approx. 4.0 kb) of the human GPR10 gene [14]. This provided evidence for a highly conserved putative NRSE located at position −1018 to −1038 relative to the translational start site. Orientation of the NRSE has been confirmed as inconsequential to function by various investigators [30–32], and in the GPR10 promoter the sequence is located 3′ to 5′ on the reverse DNA strand. Alignment with the consensus sequence revealed 86% (18/21 bp) identity (Fig. 1A). To determine the probability that this site was indeed a functional binding site, and not simply a false positive return, we performed an inter-species comparative genomic sequence analysis. Inter-species conservation of non-coding sequences is a powerful approach to identifying regulatory elements, as specifically conserved sequences suggest relevance [33]. Alignment with the mouse genome showed that the sequence was conserved to 87% identity, whereas peripheral sequences were poorly conserved (40% identity) (Fig. 1B). Furthermore, the few mismatches within the NRSE were at non-essential nucleotides with respect to NRSE/NRSF function, as established by statistical analysis of nucleotide conservation in known functional NRSEs [30].

We then performed electromobility shift assays to determine whether the isolated sequence could bind NRSF (Fig. 1C). An oligonucleotide comprising two tandem 36-bp repeats from the GPR10 gene promoter was used as probe (see Fig. 1B). A single band was generated when 3T3 cell nuclear extract was incubated with the radiolabeled probe. This complex could be competed out by the unlabeled SCG10 gene NRSE sequence, but not by the same sequence containing a well-characterized double base pair mutation (see Fig. 1A). This indicated that the putative NRSE within the GPR10 gene promoter was suitable for specific binding by NRSF, further suggesting that this NRSE is functionally significant.

3.2. NRSF confers repression of promoter activity

We next addressed the functional significance of this element, based on a series of reporter gene studies. Constructs of increasing 5′ deleted portions of the GPR10 gene promoter, designed around the pGL3-basic reporter vector were employed to determine promoter activation levels (Fig. 2A) [14]. Using three cell lines known to impart transcriptional repression of genes via the NRSE, along with the anterior pituitary cell line GH3, which is known to express the GPR10 gene, we compared the expression levels of the reporter constructs (Fig. 2B). In HeLa, C6, and 3T3 cells, highest expression levels were observed with the −642 construct, whereas in all three cell lines, the −1317 construct displayed a significantly attenuated level of reporter gene activity. This was consistent with the fact that the NRSE is located at −1018–1038. In contrast, in GH3 cells there was no apparent attenuation of reporter gene expression.

RT-PCR was employed to ascertain whether NRSF was expressed in GH3 cells (Fig. 3A). C6 cells were used as a positive control and the INS-1 pancreatic β-cell line was used as a negative control. NRSF gene expression has been reported to be absent from INS-1 cells, allowing the expression of several genes in these cells that are suppressed in non-neuronal cells by NRSF [34,35]. As expected, only INS-1 cells expressed insulin, and only GH3 cells expressed prolactin. NRSF transcripts were detected in C6 cells but no product was observed from either GH3 cells or INS-1 cells. These data suggest therefore that the lack of repression of GPR10 promoter activity in GH3 cells, shown in Fig. 2B, is due to the absence of full-length NRSF in these cells.

To determine whether NRSF-mediated repression could be

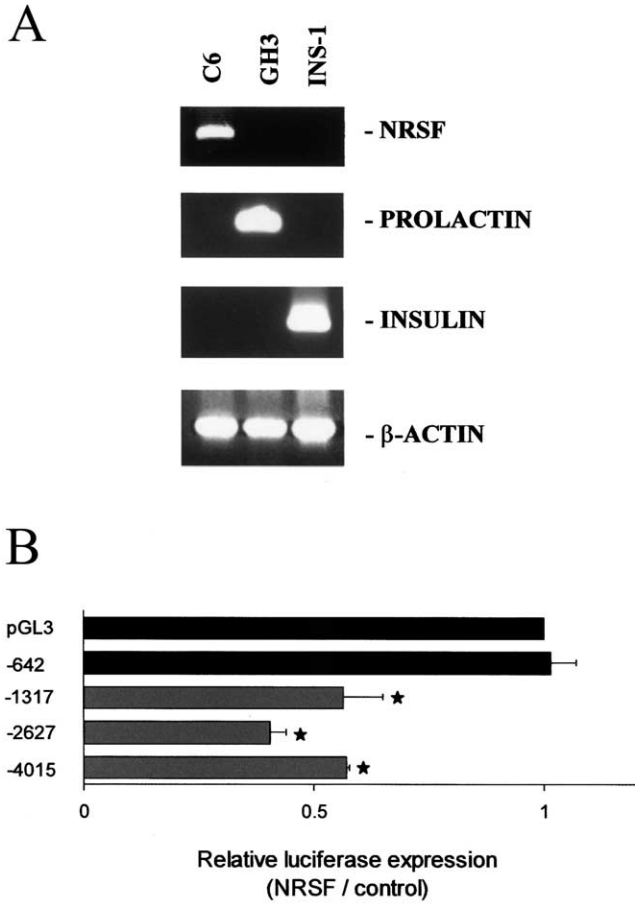

Fig. 3. Co-transfection of NRSF induces susceptibility of GH3 cells to NRSE-mediated repression of GPR10 promoter activity. A: Whole cell RNA from C6, GH3, and INS-1 cells was subjected to RT-PCR and the products separated by electrophoresis. B: Cells were co-transfected with the indicated reporter gene construct and either an NRSF expression vector or a control vector. Expression levels were then presented as the ratio of reporter gene activities between the two conditions. The hatched bars represent constructs that carry the putative NRSE. Luciferase activities were normalized using pRL-CMV. Each experiment was performed at least three times in quadruplicate. Results are expressed as mean ± S.E.M. (*$P < 0.05$).

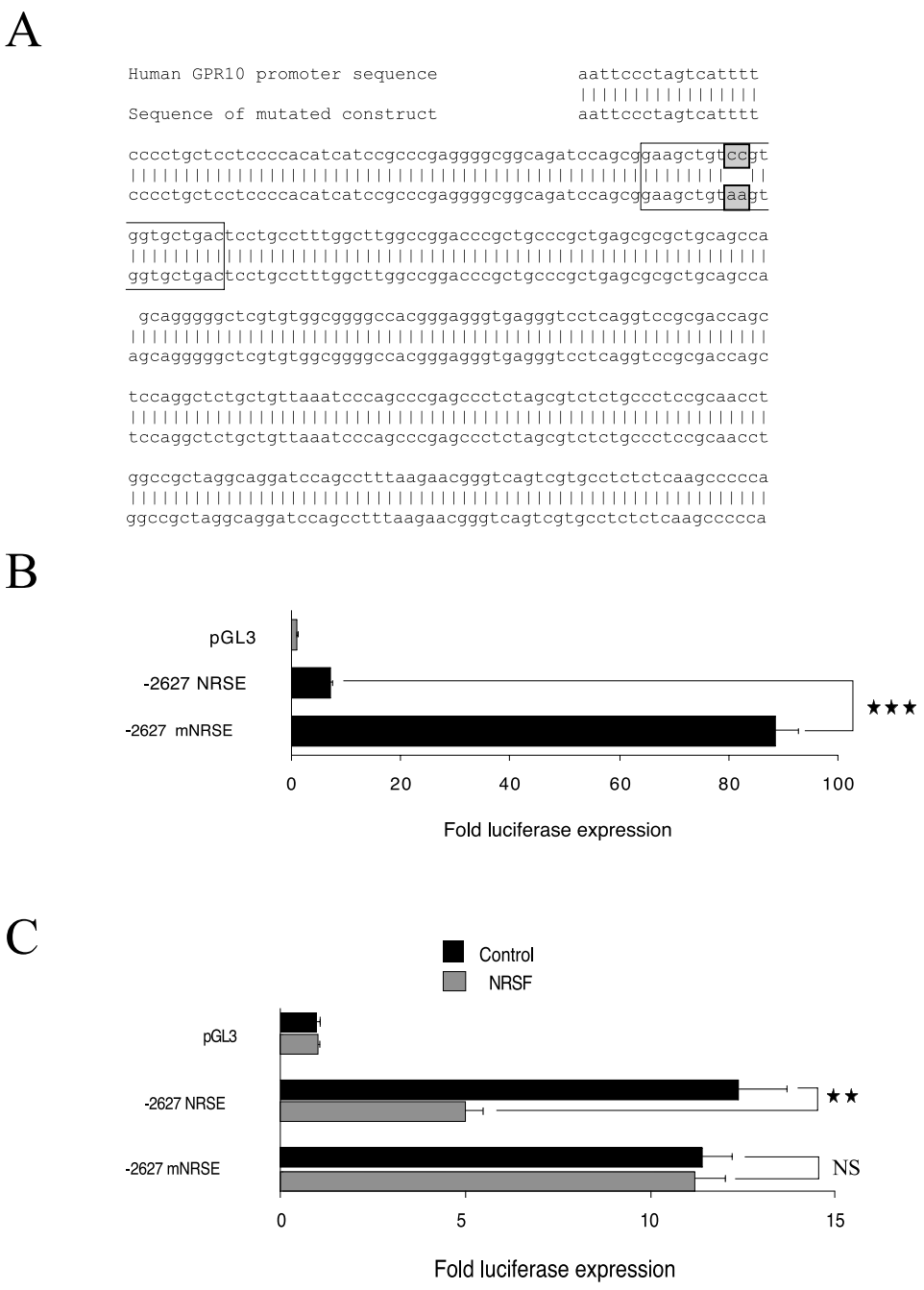

Fig. 4. Point mutation of the NRSE sequence alleviates repression. A: A site-specific mutation was introduced into the −2627 clone and subsequently sequenced. The alignment to the wild-type sequence is shown. The boxed region represents the NRSE, and the shaded areas highlight the specific mutations. B: The wild-type or mutant reporter genes were transiently transfected into C6 cells. C: Wild-type and mutant reporter vectors were transiently co-transfected into GH3 cells with either the NRSF expression plasmid or the control plasmid. Luciferase activities were normalized using pRL-CMV *Renilla*. Each experiment was performed at least three times in quadruplicate. Results are expressed as mean ± S.E.M. (**$P < 0.01$, ***$P < 0.001$, NS, not significant).

introduced into the GH3 cell context, we co-expressed either an NRSF expression vector or a null expression vector into these cells. Relative expression levels were analyzed to indicate whether each construct was susceptible to inhibition by NRSF. As expected, the −642 construct displayed no relative change in expression in the presence of NRSF (Fig. 3B). However, the longer constructs, containing the NRSE motif, showed a significant decrease in reporter gene expression level, suggesting that the promoter is indeed repressed by NRSF, and further supporting the idea that the putative NRSE in the GPR10 gene promoter is functionally significant.

Notably, the full-length −4015 construct displayed an attenuated expression level compared with the −1317 construct in GH3 cells (Fig. 2B). This circumstance is paradoxical given that GPR10 is expressed in GH3 cells [9,10,14]. The most plausible explanation for this discrepancy is that regulatory elements in other regions of the gene may play important functional roles in the endogenous expression of this receptor.

This does not affect or compromise the rationale for this study however, as even with the −4015 construct, co-expression of NRSF imparted a similar relative repression in reporter gene expression to that seen with the shorter constructs containing NRSE (Fig. 3).

3.3. Repression is specifically mediated by the NRSE

To confirm that NRSF-mediated repression was directed specifically through the NRSE, we introduced a site-directed mutation in the −2627 construct. By mutating the two juxtaposed cytosine residues to adenine residues in the NRSE motif, we created a loss of function mutation (Fig. 4A). This mutation has previously been shown to inactivate the NRSE of the SCG10 and IB1 genes among others by abolishing DNA binding of the repressor, and hence, its silencing activity [18,35]. When compared with the wild-type construct in C6 cells, the expression level of the mutant construct was enhanced over 10-fold (Fig. 4B). This marked de-repression in reporter gene expression offers strong evidence that this element is functional in the context of the GPR10 promoter.

In GH3 cells, both wild-type and mutant constructs expressed similar levels of reporter gene (Fig. 4C). However, when co-expressed with the NRSF expression vector, a significant difference was observed. Whereas activity of the wild-type construct was inhibited significantly, no attenuation in expression level was seen with the mutant construct, providing further evidence that NRSF confers repression through the NRSE in the GPR10 promoter.

We therefore propose that NRSF contributes to cell-specific expression of the human GPR10 gene. As this receptor may prove to be an important target for therapeutic intervention of obesity, it is important that the regulatory characteristics of this gene be clearly recognized.

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