A member of the nuclear factor-1 family is involved in the pituitary repression of the human placental growth hormone genes

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The human growth hormone (GH) gene family consists of five tandemly arranged and highly related genes, including the chorionic somatomammotropins (CSs), at a single locus on chromosome 17. Despite striking homologies in promoter and flanking DNA sequences, the genes within this locus have different tissue-specific patterns of expression: GH-N is expressed almost exclusively in the somatotrophs of the anterior pituitary; the remaining genes, including CS-A, are expressed in placental syncytiotrophoblast. Previously we proposed that active repression of the placental gene promoters in pituitary GC cells is mediated by upstream 'P' sequences and, specifically, a 263 bp region containing two 'P' sequence elements (PSE-A and PSE-B) and corresponding factors (PSF-A and PSF-B). We have now examined the possibility that PSF-A and PSF-B are members of the nuclear factor (NF)-1 family. Transcripts of NF-1A, NF-1C and NF-1X, but not of NF-1B, were readily detected in GC cells.

High-affinity binding of NF-1 to PSE-B, but not to PSE-A, was confirmed by competition of DNA–protein interactions by using NF-1 DNA elements and antibodies. Functionally, a NF-1 element was able to substitute for PSE-B as a promoter-specific repressor in GC cells after gene transfer. However, there was a difference in the magnitude of repression exerted by the NF-1 and PSF-B elements on the CS-A promoter and, with the use of mutations, this difference was shown to be consistent with variations in NF-1-binding sequences. These results indicate that PSF-B, but not PSF-A, is a member of the NF-1 family, which participates in the PSF complex and in the repression of the CS-A promoter in pituitary GC cells.

Key words: chorionic somatomammotropins, NF-1, placenta, placental lactogen.

INTRODUCTION

The human growth hormone (GH) gene family consists of five tandemly arranged and highly related genes, including pituitary GH (GH-N), placental GH variant (GH-V) and the chorionic somatomammotropins (CSs) CS-A, CS-B and CS-L. These genes are located within a single 47 kb locus on chromosome 17 [1]. The members of this family are believed to have evolved by gene duplication and are therefore similar in structure, transcriptional orientation and flanking DNA sequences [1]. Despite these similarities, the genes within this locus have different tissue-specific patterns of expression: GH-N is expressed almost exclusively in the somatotrophs of the anterior pituitary; the remaining genes (CS-L, CS-A, GH-V and CS-B) are expressed in the placental syncytiotrophoblast [1]. Efficient expression of GH-N in the somatotrophs is dependent on binding of the transcription factor Pit-1 (also known as GHF-1) [2]. Binding sites for Pit-1 are located remotely upstream of the GH/CS gene locus as well as within the GH-N promoter [2–6]. The placental genes encoding GH/CS also possess Pit-1-binding sites in their promoters but, despite an open chromatin structure and the presence of abundant Pit-1 protein within pituitary cells [7], these genes are not expressed in the pituitary *in vivo* [1]. However, Pit-1 is capable of binding to the CS-A, CS-B and GH-V promoters *in vitro* [8,9] and gene transfer experiments have shown that the CS-

A promoter, when used alone to drive a reporter gene, is as active as the GH-N promoter in rat pituitary GC cells [10]. This evidence suggests that a mechanism exists for the active repression of the placental CS/GH promoters in pituitary cells.

On the basis of alignment and comparison of sequences in the human GH/CS locus, regions referred to as 'P' sequences were identified upstream of each of the four placental genes encoding GH/CS, but not the pituitary-expressed gene encoding GH-N [1]. Subsequently, we identified a highly conserved 263 bp domain within these 'P' sequences (263P) located approx. 2 kb upstream of the transcription initiation site of each placental gene [11]. The 263P fragment repressed the CS-A promoter significantly in pituitary cells but not in placental tumour cells [11]. Two 'P' sequence elements (now referred to as PSE-A and PSE-B) were identified by virtue of their protection ('footprint') by nuclear proteins, referred to simply as 'P' sequence factor (PSF)-A and PSF-B, from nuclease digestion. PSF-A and PSF-B, or the PSF complex, were shown to be expressed in cells derived from multiple tissues, including rat and human pituitary, but with the possible exception of placenta [11]. More recently we were able to detect PSF complexes in human placental cells (R. M. Surabhi, L. D. Norquay and P. A. Cattini, unpublished work). The lack of tissue or cell specificity for PSF proteins suggested the involvement of a ubiquitously expressed transcription factor(s) in the PSF complex; however, PSF-A and PSF-B were not

Abbreviations used: CAT, chloramphenicol acetyltransferase; CS, chorionic somatomammotropin; EMSA, electrophoretic mobility-shift assay; GH, growth hormone; NF-1, nuclear factor 1; PSE, 'P' sequence element; PSF, 'P' sequence factor; RSV, Rous sarcoma virus; TKp, viral thymidine kinase promoter.

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identified. Indeed, it was not clear whether they represented one or two separate factors, because PSE-A and PSE-B individually could compete for both PSF-A and PSF-B footprints [11].

A re-examination of PSE-A and PSE-B reveals some similarity with the consensus nuclear factor (NF)-1 DNA element (5'-TGGN₆GCCAAT-3') [12,13]. In vertebrates there are four different genes that comprise the NF-1 family: NF-1A, NF-1B, NF-1C (also referred to as CTF-1) and NF-1X [14]. Classification of the different NF-1 genes into this family is based on a highly conserved N-terminal region of approx. 200–220 residues. This region is essential for dimerization and DNA binding; all members therefore recognize the same consensus DNA binding sequence [15–17]. NF-1 was identified originally as a protein essential for the replication of adenoviral DNA in human cervical tumour HeLa cells [18,19]. The NF-1 family has since been shown to be involved in the transcriptional activation and repression of both ubiquitous and tissue-specific genes. Variations in the C-terminal region, alternative splicing events and the ability of NF-1 proteins to form both homodimers and heterodimers have resulted in the wide variety of activities that can be attributed to different members of this family ([20–22]; reviewed in [23]). Here we provide evidence that one or more members of the NF-1 family participate in the PSF complex through binding to PSE-B.

MATERIALS AND METHODS

Oligonucleotide sequences

Double-stranded DNA elements were generated by synthesizing and annealing sense and anti-sense oligonucleotides (Gibco-BRL, Life Technologies, Burlington, Ontario, Canada). The sense strands for the elements were as follows: PSE-B, 5'-GATGGCAGGGCCCCAGCA-3'; PSE-B3, 5'-GATCGATGGCAGGGCCCCAGCA-3'; PSE-B Cm, 5'-GATGGCCGGGCCCCAGCA-3'; PSE-B Gm, 5'-GATGGCAGGGCGCCAGCA-3'; PSE-B CGm, 5'-GATGGCCGGGCGCCAGCA-3'; PSE-B CTAm, 5'-GATCGCAGGGTACCAGCA-3'; high-affinity NF-1, 5'-CTAGCTATTTTGGCATCATGCCAATATG-3'. A consensus NF-1 element, 5'-TTTTGGATTGAAGCCAATATGATAA-3', and NF-1 mutant (NF-1m), 5'-TTTTGGATTGAATAAAATATGATAA-3', were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.).

Plasmid constructions

The immediate 5' flanking region of the gene encoding (–492 to +6) was isolated as an *Eco*R1/*Bam*H1 fragment (blunted) and inserted into the *Hind*III site (blunted) upstream of the gene for firefly luciferase (Luc) in pXP1 [24] to generate CSp.Luc. Synthetic double-stranded DNA elements were phosphorylated and inserted upstream of the CS-A promoter in the *Sma*I site of CSp.Luc or pT81luc [24] to generate PSE-B/CSp.Luc, PSE-B Cm/CSp.Luc, PSE-B Gm/CSp.Luc, PSE-B CGm/CSp.Luc, CTAm/CSp.Luc, NF-1/CSp.Luc, PSE-B/TKp.Luc and NF-1/TKp.Luc (where TKp stands for viral thymidine kinase promoter). All constructs were sequenced to confirm orientation and insert copy number. The 263P fragment, corresponding to a region of the 'P' sequences upstream of the gene encoding CS-A, had previously been subcloned into pUC19 [11]. To construct 263P/TKp.Luc, the 263P fragment was released from 263P/pUC19 by using *Sac*I/*Hind*III and subcloned into pT81luc [24]. To generate 263P/CSp.Luc, 263P was released from 263P/TKp.Luc as a *Bam*HI/*Xho*I fragment and subcloned into CSp.Luc. The chloramphenicol acetyltransferase (CAT)

gene directed by the Rous sarcoma virus (RSV) promoter (RSVp.CAT) was as described elsewhere [25].

Cell culture and gene transfer

Rat anterior pituitary tumour (GC) cells were maintained at 37 °C as monolayers in Dulbecco's modified Eagle's medium supplemented with 8 % (v/v) fetal bovine serum and antibiotic in a humidified air/CO₂ (19:1) atmosphere. Gene transfer was performed with the calcium phosphate/DNA precipitation method, essentially as described previously [10]. In brief, cells were plated at a density of 10⁶ cells per 100 mm plate; after 24 h they were transfected with 10 µg of test (luciferase) plasmid and 1 µg of RSVp.CAT on each plate as a control for DNA uptake. Cells were washed thoroughly with calcium-free, magnesium-free PBS 20–24 h after transfection and refed with growth medium. Cells were harvested 48 h after transfection. Lysis of the cells was performed as described previously [26] and luciferase activity was determined with the Luciferase Assay System (Promega Corp., Madison, WI, U.S.A.) with a photon-counting luminometer (Lumat LB9507; EG&G Berthold) in accordance with the manufacturer's instructions. CAT activity was measured with a two-phase fluor diffusion assay [27], modified as described previously [26]. Values for CAT activity were determined by regression analysis to obtain c.p.m./min. Luciferase values were then normalized with CAT activity (v/v) and the resulting luciferase activity was expressed as mean luciferase/CAT units. Values are expressed as means for at least three separate precipitations.

Preparation and fractionation of nuclear extracts

Nuclear protein extracts from GC cells were made in accordance with published protocols [28] and dialysed as described previously [29]. Protein concentration of the extracts was assessed by using the Bradford [30] protein assay, with BSA as a standard. Extracts were stored as aliquots at –70 °C.

DNase I protection assays

DNase assays were performed as previously described [2]. Briefly, 25 µg of GC cell nuclear protein was preincubated on ice with double-stranded competitor oligonucleotides (1000–5000 pmol excess of the probe) for 15 min. Subsequently, 0.25–0.5 ng of 263P fragment probe, radiolabelled at the 3' end, was added to each reaction and incubated on ice for an additional 15 min. Digestion with DNase I proceeded at 26 °C for 1 min and the samples were subjected to electrophoresis in a 6 % (w/v) polyacrylamide gel.

EMSA

EMSA was performed essentially as described previously [2]. Competitor oligonucleotides (2.5-fold, 5-fold, 10-fold, 25-fold, 50-fold, 100-fold and 1000-fold mass excess of the probe), anti-(NF-1) antibodies (1 µl) [15] or normal rabbit serum (1 µl) were preincubated with 2–5 µg of GC cell nuclear protein and 1–2 µg of poly(dI-dC) in reaction buffer [10 mM Tris/HCl (pH 7.5)/50 mM NaCl/1 mM dithiothreitol/1 mM EDTA/5 % (v/v) glycerol] for 10 min at room temperature in a 20 µl final volume. Radiolabelled oligonucleotide probes were added, and the reactions were incubated for a further 10 min at room temperature before electrophoresis in a 5 % (w/v) polyacrylamide gel in Tris/borate buffer [31].

RNA isolation and blotting

RNA was isolated from GC cells with guanidinium thiocyanate and then enriched for poly(A)⁺ RNA with a cellulose oligo(dT) slurry [31]. Poly(A)⁺-enriched RNA (25 µg per lane) was run in a formaldehyde/1.5% (w/v) agarose gel and transferred to nitrocellulose before hybridization with radiolabelled specific probes. Rat cDNA species were kindly provided by Dr M. Imagawa [32]. Inserts were released initially as *Eco*RI/*Xho*I fragments and gel-purified. Specific probes for each of the rat NF-1 genes were made by restriction digests of the cDNA species and isolation of C-terminal fragments (NF-1A/*Xcm*I, 918 bp; NF-1B/*Pvu*II, 599 bp; NF-1C/*Dde*I, 207 bp; NF-1X/*Bgl*II, 590 bp). Probes were radiolabelled by random-priming (Prime-A-Gene; Promega, Madison, WI, U.S.A.). Each probe was assessed for cross-reactivity or specificity against 1 or 0.1 ng of rat NF-1 cDNA species (NF-1A, NF-1B, NF-1C and NF-1X) immobilized on nitrocellulose, with a slot-blot apparatus.

Statistical analysis

Statistical analysis was performed with a two-tailed, unpaired Student *t* test. A value of *P* < 0.05 was considered statistically significant.

RESULTS

PSE-B contains the core consensus binding site for the NF-1 family

A comparison of the sequences of PSE-A and PSE-B [11] with the consensus binding site for members of the NF-1 transcription factor family is shown in Figure 1. The essential core sequence for NF-1 binding, as identified by Gronostajski [12,13], is boxed within the consensus sequence; the presence of mismatches in PSEs are indicated by lower-case letters. In contrast with PSE-A, there is no variation in PSE-B from the NF-1 core sequence.

Detection of NF-1A, NF-1C and NF-1X transcripts in rat pituitary GC cells

Specific probes for NF-1A, NF-1B, NF-1C and NF-1X transcripts were isolated from the C-terminal regions of their respective cDNA species. The specificity of these probes was tested against 1 and 0.1 ng of plasmid containing each of the four NF-1 cDNA species, by slot-blot analysis. Specific binding with

Figure 1 Identification of NF-1-like DNA elements within the PSF-A and PSF-B 'footprint' regions (PSE-A and PSE-B)

Sequences corresponding to nt 139–228 within the 263P fragment (nt 1–263) are shown. The sequences of PSE-A (nt 192–228) and PSE-B (nt 139–165) and regions protected by PSF-A and PSF-B are underlined. The presence of the consensus NF-1 recognition sequence (5'-TGGNNNNNGCCAA-3') indicates the location of potential NF-1-binding sites within PSE-A and PSE-B. The two essential core sequences for NF-1 binding within the consensus sequence are boxed; mismatches with PSE sequences are indicated by lower-case letters.

Figure 2 Detection of NF-1 transcripts in pituitary GC cells by RNA blotting

(A) Samples (1 and 0.1 ng) of plasmids containing rat NF-1 cDNA species (NF-1A, NF-1B, NF-1C and NF-1X) immobilized in quadruplicate on nitrocellulose with a slot-blot apparatus. Each of the four was then assessed with one of the radiolabelled specific probes for NF-1A, NF-1B, NF-1C and NF-1X. Results were detected by autoradiography for each of the probes as indicated. (B) Poly(A)⁺-enriched RNA (25 µg per lane) from GC cells was separated by formaldehyde-gel electrophoresis, transferred to nitrocellulose and probed with specific probes for NF-1A, NF-1B, NF-1C and NF-1X. The locations of 28 S and 18 S, corresponding to 4.7 and 1.9 kb respectively, are indicated; they were used to estimate the size of the NF-1 transcripts after autoradiography.

little evidence of cross-reactivity between NF-1 gene products was observed (Figure 2A). The same probes were then used to assess poly(A)⁺-enriched RNA isolated from GC cells after electrophoresis and blotting to nitrocellulose (Figure 2). NF-1 gene expression was confirmed in GC cells; transcripts for NF-1A (5.1 and 4.4 kb), NF-1C (6.0 and 4.0 kb) and NF-1X (5.7 and 4.6 kb), but not NF-1B, were detected under the conditions used.

Competition with a NF-1 element disrupts protein binding to the 263P fragment

The 263P fragment was radiolabelled and incubated with pituitary GC cell nuclear extract in the absence or presence of competitor double-stranded oligonucleotides/DNA elements and subjected to DNase I protection assay (Figure 3). The characteristic PSF-A and PSF-B footprint patterns were observed and competed for by PSE-B, as described previously (Figure 3; compare lanes b and c). Two NF-1 elements were also used as competitors to determine whether members of the NF-1 family were involved in the formation of the PSF complex. The first was a commercially available consensus NF-1 element and the second was reported as a high-affinity NF-1 element [17]. As a control, a commercially available mutation of one of the core regions (GCC to TAA) of the consensus NF-1 oligonucleotide was also tested as a competitor. Both intact NF-1 elements were capable

Figure 3 Competition of PSF-A and PSF-B nuclease-protected regions with NF-1 DNA elements

The 263P fragment was radiolabelled and incubated (lane a) without or (lanes b–f) with pituitary GC cell nuclear extract in the absence (lane b) or presence (lane c) of a 5000 pmol excess of PSE-B, or a 1000 pmol excess of consensus NF-1 (lane d), high-affinity (h.aff.) NF-1 (lane e) or mutated NF-1 (NF-1m) (lane f) competitor DNA elements, then subjected to a DNase I protection assay. The characteristic PSF-A and PSF-B 'footprints' are indicated.

of competing with the PSF-A and PSF-B protected regions at a 1000-fold mass excess of the 263 P fragment probe (Figure 3, lanes d and e). In contrast, efficient competition was not seen under identical conditions with the NF-1 oligonucleotide competitor with the mutated DNA element (Figure 3, lane f).

NF-1 proteins can bind to PSE-B but not to PSE-A *in vitro*

EMSA, combined with competition with specific double-stranded oligonucleotides, was performed to assess whether members of the NF-1 family were capable of binding directly to either PSE-A or PSE-B (Figure 4). Both low-mobility and high-mobility complexes were observed when PSE-A was used as a probe in EMSA experiments with pituitary GC cell nuclear extract (Figure 4A). The lower-mobility complexes (open arrowheads) reflect specific high-affinity complexes because they competed efficiently (5–50-fold mass excess) with excess unlabelled PSF-A-binding sites. In contrast, neither the NF-1 consensus nor the NF-1 mutant oligonucleotide competitors were able to compete for specific or low-affinity PSF-A complexes (Figure 4A). In addition, we were unable to either compete or supershift any of the complexes formed on PSE-A by using a NF-1 antiserum (Figure 4A).

Low-mobility and high-mobility complexes were also seen by EMSA by using PSE-B as a probe with GC cell nuclear protein. Specific high-affinity complexes, defined as those that competed

Figure 4 Direct *in vitro* binding of NF-1 proteins to PSE-B, but not to PSE-A

EMSA was performed with nuclear extract from pituitary GC cells and radiolabelled PSE-A (**A**) and PSE-B3 (PSE-B) (**B**) as probes, combined with competition with specific double-stranded oligonucleotides (PSE-A, PSE-B, NF-1 and NF-1m) or NF-1 antibodies (Ab) and normal rabbit serum (NRS) as indicated. Competitor oligonucleotides were used in 2.5–50-fold mass excess compared with probe. For (**A**) the excesses of PSE-A and NF-1 were 5-fold, 25-fold and 50-fold and those of NF-1m were 25-fold and 50-fold. For (**B**) the excesses of NF-1 and NF-1m were 2.5-fold, 5-fold and 25-fold and those of PSE-B were 2.5-fold, 5-fold, 25-fold and 50-fold. Specific high-affinity complexes and supershifted bands of lower mobility are indicated by open and filled arrowheads respectively.

efficiently with excess unlabelled PSF-B binding sites, were observed (Figure 4B, open arrowheads). The NF-1 consensus site, but not the mutant NF-1 site, was able to compete efficiently for these specific 'PSF-B' complexes. Direct binding of NF-1 protein to PSE-B was confirmed by using NF-1 antisera. Addition of antibodies against NF-1 to the EMSA resulted in both a loss of specific 'PSF-B' complexes, and the appearance of supershifted bands of lower mobility (Figure 4B).

Figure 5 Assessment of potential low-affinity PSE–PSF interactions

EMSA was performed with nuclear extract from pituitary GC cells and radiolabelled PSE-A (**A**) and PSE-B4 (PSE-B) (**B**) as probes, combined with competition with specific double-stranded oligonucleotides (PSE-A, PSE-B and NF-1) as indicated. A mutated PSE-B, PSE-B CTAm, was used as a non-specific competitor. Competitor oligonucleotides were used in 10–1000-fold mass excess compared with probe. For (**A**) the excesses of PSE-A, PSE-B, NF-1 and PSE-B CTAm were 100-fold and 1000-fold. For (**B**) the excesses of PSE-B and PSE-B CTAm were 100-fold and 1000-fold and those for NF-1 and PSE-A were 10-fold, 100-fold and 1000-fold.

We also used GC-cell nuclear protein and EMSA with higher levels of competitor (100–1000-fold mass excess) to assess possible lower-affinity interactions between PSF-A and PSF-B–NF-1 complexes (Figure 5). Both PSE-B and NF-1 were able to compete partially with complexes formed on PSE-A with similar affinity; however, competition was much less than observed with

Figure 6 NF-1 and PSE-B repress CS-A promoter but not TKp activities in pituitary GC cells

Hybrid luciferase (Luc) genes were used to assess the effect of 263P, PSE-B or the NF-1 element on $-492/+6$ CS-A promoter (CS-Ap) and $-81/+54$ TKp activities in GC cells. DNA uptake was controlled through co-transfection with RSVp.CAT. Corrected results are expressed as percentages of the activity of CSp.Luc or Tkp.Luc, which were arbitrarily set at 100%. The basal Luc (light units/ μ l of cell lysate) per CAT (c.p.m./min per μ l of cell lysate) values (Luc/CAT) for CSp.Luc and Tkp.Luc were 1683.1 ± 343 and 129.8 ± 10.4 respectively. Bars represent S.E.M. * $P < 0.05$; ** $P < 0.005$.

PSE-A (Figure 5A). Consistent with the above was our observation, in the reciprocal experiment using PSE-B as a probe, that PSE-A was a less efficient competitor of specific PSF-B complexes (compare the intensity of the complex with 10-fold competitor) than the NF-1 element (Figure 5B).

Both NF-1 consensus and PSE-B repress CS-A but not TKp promoter activities in GC cells

PSE-B was described previously as a repressor of CS-A promoter activity in rat pituitary GC cells after gene transfer; [11] we therefore tested the consensus NF-1 element for similar activity. A single copy of either the NF-1 consensus or PSE-B double-stranded oligonucleotides was inserted immediately upstream of the CS-A promoter (-492 to $+6$) or a viral TKp (-81 to $+54$) linked to the firefly luciferase reporter gene (CSp.Luc or Tkp.Luc) to generate NF-1/CSp.Luc or NF-1/TKp.Luc and PSE-B/CSp.Luc or PSE-B/TKp.Luc respectively. Constructs containing the 263P fragment (263P/CSp.Luc and 263P/TKp.Luc) were also tested, for comparison. The effects of adding these DNA elements were assessed by comparing the relative luciferase activity of these constructs with that of CSp.Luc or Tkp.Luc (arbitrarily set at 100%) in transfected GC cells (Figure 6). The presence of the consensus NF-1 sequences, PSE-B and 263P fragment resulted in significant repression of CS-A promoter activity by 38% ($P < 0.05$, $n = 12$), 72% ($P < 0.001$, $n =$

Figure 7 Point mutations of PSE-B that do not disrupt NF-1 binding can affect functional activity

(A) Sequence of the single (PSE-B Cm, PSE-B Gm), double (PSE-B CGm) and triple (PSE-B CTAm) base-pair mutants of PSE-B and comparison with the NF-1 consensus element. The two essential core sequences for NF-1 binding are boxed within the consensus sequence; the presence of identical matching sequences in PSE-B are indicated with lines. (B) EMSA assays were performed with pituitary GC-cell nuclear extract and PSE-B mutant (Cm, Gm, CGm, CTAm) probes as indicated, combined with competition with a 25-fold mass excess of specific double-stranded oligonucleotides (NF-1 and NF-1m) or NF-1 antibodies (Ab) and normal rabbit serum (NRS). Specific high-affinity complexes and supershifted bands of lower mobility are indicated by open and filled arrowheads respectively. (C) Hybrid luciferase (Luc) genes were used to assess the effect of PSE-B mutations on -492/+6CS-A promoter (CS-Ap) activity in GC cells. DNA uptake was controlled through co-transfection with RSVp.CAT. Corrected results are expressed as percentages of the activity of CSp.Luc, which was arbitrarily set at 100. The basal Luc (light units/ μ l of cell lysate) per CAT (c.p.m./min per μ l of cell lysate) values (Luc/CAT) for CSp.Luc was 1683.1 ± 343 . Bars represent S.E.M. * $P < 0.05$; ** $P < 0.005$.

12) and 60% ($P < 0.05$, $n = 5$) respectively. Although a decrease in CS-A promoter activity was observed with both NF-1 consensus and PSE-B, the difference in the level of repression between these elements was also significant ($P < 0.001$, $n = 12$). In contrast, there was no significant effect of NF-1 and PSE-B sequences on TKp activity but a significant 3.5-fold stimulation of promoter activity was observed in the presence of the 263P fragment ($P < 0.005$, $n = 6$).

Minor variations outside the consensus NF-1 core binding sequence in PSE-B affect functional activity

A comparison of PSE-B and the consensus NF-1 element reveals that their sequences differ outside the essential core NF-1-binding regions (boxed, Figure 1). Mutants of PSE-B were constructed to investigate the possibility that the differences in the degree of repression exerted by the consensus NF-1 element and PSE-B on CS-A promoter activity were the result of minor sequence variations (Figure 7A). A single base-pair change was made to the PSE-B outside the essential NF-1 binding core to

convert a cytosine (C) into the guanine (G) found in the NF-1 consensus. This mutation was named PSE-B Gm. An additional single base-pair change was made to PSE-B in the spacer region of the NF-1 consensus site (or N) to convert the adenosine (A) of PSE-B into a cytosine (C). This mutation was named PSE-B Cm. A double mutant with both changes was also constructed and called PSE-B CGm. As a control, a triple mutant (PSE-B CTAm) was also generated in which one of the essential core binding regions was modified.

PSE-B Gm, Cm and CGm, which contain mutations outside the essential core binding sequence, were assessed by EMSA to confirm that they had retained valid NF-1-binding sites (Figure 7B). All of the mutants were able to form specific complexes when incubated with GC-cell nuclear extract. This specific pattern could be competed through the addition of a competitor NF-1 wild-type consensus oligonucleotide, but not with the addition of mutant NF-1 (NF-1m). A supershifted band of lower mobility and the loss of the specific pattern was achieved by the addition of polyclonal antibody against NF-1 to the reactions, confirming that the mutant PSE-B elements retained the ability

to bind to NF-1 proteins. In contrast, no supershifted band (Figure 7B, filled arrowhead) was obtained, with the triple mutant containing a modified core binding site. There was also no complex with a mobility corresponding to that which competed with a NF-1 DNA element (Figure 7B, open arrowhead).

Single copies of the PSF-B mutant elements were inserted upstream of the CSp.Luc gene and tested for effects on CS-A promoter activity in GC cells after gene transfer. These effects were assessed by comparing the relative luciferase activities of these constructs with that of CSp.Luc, which was arbitrarily set at 100% (Figure 7C). Modest (23%) but significant repression of the CS-A promoter was observed with PSE-B Gm ($P < 0.05$, $n = 6$); this was significantly different from the repression exerted by both the wild-type PSE-B (72%) and consensus NF-1 (38%) elements. Both PSE-B Cm and PSE-B CGm, the single and double mutants respectively, repressed the CS-A promoter significantly by 39% ($P < 0.005$, $n = 6$). This level of repression was significantly different from the effect of wild-type PSE-B on the CS-A promoter but, in contrast with PSE-B Gm, not significantly different from the effect seen with the NF-1 consensus element. Consistent with the lack of detectable NF-1 binding was our observation that the PSE-B CTAm 'core region' mutant was unable to repress CS-A promoter activity. Indeed, a slight (1.3-fold, $n = 9$) but not significant ($P = 0.06$) increase in expression was observed.

DISCUSSION

We reported previously that the 263P fragment or its component elements, PSE-A and PSE-B, can repress CS-A promoter in pituitary GC cells after gene transfer [11]. The proteins or protein complex that bound these elements was called PSF-A/B or PSF-1 but remained uncharacterized beyond its ability to bind to these sequences. The results of our present study indicate that one or more members of the NF-1 family of transcription factors participate in the PSF complex and are responsible for PSF-B but not PSF-A. This is based on the following observations: (1) the high level of similarity between the PSF-B and the NF-1 essential core binding sequences (Figure 1), (2) the competition of the PSF-B–DNA interaction with excess NF-1-binding sites and antibodies (Figures 3–5), (3) the ability of a NF-1 element to substitute for PSE-B functionally as a repressor of the CS-A promoter (Figure 6), and (4) the loss of PSE-B repressor function by a mutant (PSE-B CTAm) that is unable to bind NF-1 proteins (Figure 7).

Although PSE-B and the consensus NF-1 sites were able to mediate repression of the CS-A promoter in transfected GC cells, there was a significant (34%) difference in the magnitude of response observed with the PSE-B and consensus NF-1 element (72% and 38% respectively) (Figure 6). A possible explanation is that a protein that is not a member of the NF-1 family is responsible for the repressor activity exerted on the CS-A promoter in culture but is not seen under the conditions used for nuclease protection or EMSAs, possibly because it is excluded by NF-1. Certainly histone H1 is capable of binding weakly to NF-1-binding sites and this has been proposed as a mechanism through which these elements might mediate repression [33,34]. However, the specificity of histone binding to this sequence is controversial [35] and we were able to show a highly specific interaction between PSF-B protein and NF-1 elements (Figure 4). Proteins that are not members of the NF-1 family were also implicated in the binding of NF-1-like sites in both the rat GH and human cellular retinol-binding protein 1 genes [36]. However, there are important differences between their results and ours. Unlike in their study, we were able to compete our

specific PSF-B complexes with excess unlabelled NF-1 element and see a supershift with specific antibodies against NF-1 (Figure 4). In addition, we did not see the appearance of additional 'footprints' or shifted complexes after competition with NF-1-binding sites in nuclease protection assays and EMSAs, which would have suggested the presence of a binding event that had been largely excluded by the presence of NF-1 binding (Figures 3–5). Thus, although we cannot rule out the possibility that other proteins are involved in PSE-B binding, our results strongly support the participation of NF-1 in the PSF complex and, more specifically, that NF-1 is PSF-B.

Despite the ability of the NF-1 consensus site to repress the CS-A promoter in GC cells, the level of repression was significantly less than that exerted by PSE-B. Given that PSE-B contains an intact NF-1 essential core binding sequence (Figure 1), we pursued the possibility that differences in activity might be related to subtle sequence variations lying outside this region. Although all members of the NF-1 family possess the same DNA-binding site specificity [17], there is evidence to support the concept that the affinity between NF-1 family members for different binding sites can vary. Whereas all NF-1 family members bind the adenovirus replication origin with higher affinity than the GST-P site, the individual family members with the highest affinity for each of these sites are NF-1A and NF-1X respectively [32]. Although changes in the affinity profiles have been shown with binding assays *in vitro*, the functional effects of minor variations in NF-1 sequences had not to our knowledge been demonstrated. We were able to alter the effect of PSE-B on the activity of the CS-A promoter in GC cells significantly by introducing single base-pair changes, even though these changes were outside the core regions and each mutant DNA element remained a valid NF-1-binding site (Figure 7). Conversion of a G residue into a C in the spacer region of PSE-B effectively decreased the level of repression to that seen with the consensus NF-1 site. The change in activity was consistent with an alteration of the profile of affinities of the different members of the NF-1 family. We were able to demonstrate the expression of NF-1A, NF-1C and NF-1X in pituitary GC cells (Figure 2). NF-1B was shown by others to be expressed efficiently in rat lung [32]. Using a NF-1B-specific probe (Figure 2A), we were able to detect NF-1B transcripts in 30 µg of poly(A⁺)-enriched mouse lung but not GC cell RNA (results not shown). Thus if NF-1B expression occurs in GC cells, it is at very low levels or possibly through an alternatively spliced transcript that is undetectable with our probe [22]. Regardless, the detection of NF-1A, NF-1C and NF-1X suggest that the effect of PSE-B on the CS-A promoter might represent the activity of one or more NF-1 family members favoured in the context of PSE-B, whereas the effect of the consensus NF-1 element on the CS-A promoter is representative of the actions of one or more different members of the NF-1 family.

NF-1 family members are known to act as both activators and repressors of transcription ([22,37–39]; reviewed in [23]). Diversity between individual members occurs in the C-terminal regions of the proteins, where transcriptional activation and repression domains can be found [15,39]. The C-terminal region is also known to mediate the ability of NF-1 to interact with other proteins, as in the interaction between human NF-1C with TFIIB [40] and yeast TATA-binding protein [41]. The lack of repression seen with PSE-B (and NF-1) on TKp in comparison with the CS-A promoter is consistent with the participation of additional factors required to modulate the direction and/or degree of response. Previously we provided evidence for an interaction between the PSF complex and the pituitary-specific factor Pit-1 [11], for which a binding site is present in the CS-A

promoter but not TKp. Our results also support the idea that PSF-A and PSF-B are distinct but interacting factors. Although it was possible to use a NF-1 element to compete with PSF-A in the context of the PSF complex formed on the 263P fragment used for nuclease protection (Figure 3), competition of PSF-A with a NF-1 element was not seen under conditions of high specificity/affinity in an EMSA (Figures 4). This is consistent with the differences between the PSE-A and essential core sequences in an NF-1 DNA element (Figure 1) and is also supported by the failure to see a supershift with the use of a PSE-A probe with antibodies against NF-1 (Figure 4).

Given the differential effect of NF-1/PSF-B on the activity of different promoters (Figure 6), it seems likely that the participation of additional factors that can influence/constitute the PSF complex, including PSF-A and Pit-1, contributes to the repression of the CS-A promoter observed in pituitary GC cells after gene transfer. However, in a recent review of the NF-1 family [23] it was noted that because individual members of the family both activate and repress transcription, the context of NF-1 sites is likely to affect the direction in which transcription is affected. As a result, a switch from repressor to enhancer function can be envisaged through modification of the interactions or participants in the PSF complex. This potential was demonstrated by the complete reversal of 263P activity, which repressed CS-A promoter activity by 60% but increased TKp function 3.5-fold in GC cells.

In summary, we have shown that the PSF complex is able to recognize a consensus NF-1-binding site with high affinity and that members of the NF-1 family are able to recognize PSE-B specifically. The role of NF-1/PSF-B within the PSF complex in the pituitary seems to be as a repressor; however, the ability of NF-1 family members to act as both repressors and activators offers a mechanism for both repression and stimulation of placental members of the genes encoding the human GH family. With regard to NF-1 DNA elements, we were also able to demonstrate that minor sequence variations, even in the spacer region, can have a significant effect on the way in which these sites are able to influence transcription.

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