Thimet oligopeptidase expression is differentially regulated in neuroendocrine and spermatid cell lines by transcription factor binding to SRY (sex-determining region Y), CAAT and CREB (cAMP-response-element-binding protein) promoter consensus sequences

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The zinc metalloprotease thimet oligopeptidase (EP24.15) is found predominantly in the neuroendocrine–gonadal axis where it is implicated in the processing of bioactive peptides, including GnRH (gonadotropin-releasing hormone), β-neoendorphin, α-neoendorphin and dynorphin$_{(1–8)}$, the progression of spermatogenesis and the normal clearance of β-amyloid in brain cells. Regulation of the enzyme's activity may occur in part by phosphorylation and redox disruption of intermolecular disulphide bridges. The elevated levels of both EP24.15 activity and mRNA within testicular and neuroendocrine tissues indicate that EP24.15 gene expression is differentially regulated. In the present paper, we present a detailed analysis of the rat EP24.15 promoter region previously isolated and partially characterized in this laboratory. Employing site-directed mutagenesis to create a series of promoter deletions and full-length promoter mutants, and measuring their activity in luciferase reporter gene and electrophoretic mobility-shift assays, we have shown that the transcription of the EP24.15 gene is differentially regulated in neuroendocrine and spermatid cell lines by transcription factor binding to SRY (sex-determining region Y), CAAT and CREB (cAMP-response-element-binding protein) promoter consensus sequences. The key to identifying the *in vivo* role of thimet oligopeptidase is likely to be found within the mechanisms by which it is regulated, and it is therefore of particular significance that EP24.15 expression is regulated by SRY and CREB/CREM (cAMP-response element modulator), the principle testes-determining protein and the major orchestrator of spermatogenesis respectively.

Key words: luciferase, metalloprotease, thimet oligopeptidase. (EP24.15).

INTRODUCTION

Thimet oligopeptidase (EC 3.4.24.15; EP24.15) is a thiol-sensitive zinc metalloprotease that cleaves peptides of 5–18 amino acids, three residues from their C-terminus preferentially where they have hydrophobic residues in the P1 and P3′ positions [1–3]. EP24.15 has been cloned and sequenced from a wide range of species, and displays the highest specific activity in cytosolic extracts from testes, pituitary and brain, although it is also found in many peripheral tissues and blood [4].

EP 24.15 is the prototype member of the M3 family of zinc metallopeptidases [5] with neurolysin (EC 3.4.24.16; EP 24.16) having 60 % identity with its closest relative [6]. Both enzymes have been implicated in the degradation of neuropeptides, but differ in cellular distribution; EP 24.15 being located primarily in testicular and endocrine tissue, whereas neurolysin has high activity in both the liver and kidneys [7,8]. The crystal structure of EP 24.16 has been resolved, revealing that the enzyme has a deep, narrow channel that runs the length of the molecule and midway encompasses the active site [9]. Given the degree of sequence identity between EP 24.16 and EP 24.15, it is likely that this channel is also found in EP 24.15 where it restricts the access of peptides >18 amino acids to the active site [10].

As rat EP24.15 cDNA contains no signal or secretory sequence, the enzyme was originally described as cytosolic [4]. However, 20 % of its activity is associated with membranes [11], and activity assays have located the enzyme to the nuclei of a broad range of hypothalamic and extrahypothalamic brain nuclei, where EP24.15 mRNA has also been detected [12]. A number of putative nuclear localization sequences have been predicted, but not demonstrated to be functional. It is not yet understood how the enzyme achieves its subcellular localization and membrane association, but one proposed mechanism is through the mediation of 14-3-3 binding proteins acting on a predicted consensus sequence near the C-terminus of the protein [13]. EP24.15 is secreted from AtT20 mouse pituitary [14], C6 glioma [15] and Madin–Darby canine kidney cells [16]. In the rat hypophyseal median eminence, the enzyme is present in the perivascular space and is secreted into portal blood [17,18]. Recently, immunohistochemistry has monitored the anterograde axoplasmic transport of EP24.15 from the cell body to the synaptic cleft of sciatic nerves where its secretion from nerve terminals is proposed [19]. The transport is relatively slow and is likely to involve the cytoskeleton, which is consistent with the enzyme's co-localization with microtubules and neurofilaments in the dendrites and axons of neuronal cells [20].

Being located primarily in tissues rich in neuropeptides and hormones, several roles have been postulated for EP24.15 in the processing of bioactive peptides, including GnRH (gonadotropin-releasing hormone), β-neoendorphin, α-neoendorphin and dynorphin$_{(1–8)}$ [7,18]. The enzyme has also been implicated in the progression of spermatogenesis [21] and the normal breakdown of the β-amyloid plaques that accumulate in Alzheimer's disease [22]. The key to identifying the *in vivo* role of thimet oligopeptidase is likely to be found within the mechanisms by which it is regulated and targeted to subcellular compartments.

Abbreviations used: CBP, CREB-binding protein; CRE, cAMP-response element; CREB, CRE-binding protein; CREM, CRE modulator; EMSA, electrophoretic mobility-shift assay; EP24.15, thimet oligopeptidase; Gfi1, growth factor independence 1; GnRH, gonadotropin-releasing hormone; HMG, high-mobility group; NF-Y, nuclear factor Y; RORα, retinoid-related orphan receptor α; SRY, sex-determining region Y; TF, transcription factor.

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EP24.15 has no known endogenous inhibitors [8]. Regulation of the enzyme's activity may occur in part by phosphorylation, which has been demonstrated to alter its kinetic parameters and substrate specificity for GnRH/$GnRH_{(1–9)}$ [13]. Other evidence suggests that EP24.15 activity may be regulated by conversion from an inactive multimer, with access to the active site blocked, into active monomers by disruption of disulphide bridges [23]. However, predictions of the structure of the dimer suggest that the active site would be accessible in multimers, and their loss of substrate affinity must be due to factors other than steric restriction [10].

Enzyme availability within a cell depends on both its rate of synthesis and rate of degradation. The half-life of EP24.15 is unknown; however, the enzyme appears to be stable in all the species and tissues studied [24], and it is likely that its availability is dependent on gene expression. The elevated levels of both EP24.15 activity and mRNA within testicular and neuroendocrine tissues indicates that gene expression is regulated differentially between these and peripheral tissues.

Previous work in this laboratory has led to the isolation and partial characterization of the rat EP24.15 promoter region −901/+120, which, coupled with transfection and DNA-binding assays performed in a variety of cells lines, identified two fragments −901/−219 and −219/−102 that appeared to confer positive and negative control respectively over EP24.15 transcription, although no differential regulation was observed between the cell types analysed [25]. In the present paper, we present a more detailed analysis of the rat EP24.15 promoter region that clearly demonstrates cell-specific promoter activity, indicating that EP24.15 proteolytic activity is regulated, at least in part, by the rate of gene transcription.

MATERIALS AND METHODS

Synthesis of rat EP24.15 serial deletions in a pGL3 basic luciferase vector

The complete rat EP24.15 promoter fragment −901/+120 had previously been cloned into the pGL3 basic luciferase reporter vector (Promega) so that the *Kpn*I site in the vector polylinker was intact [25]. The QuikChange™ Site-Directed Mutagenesis Kit (Stratagene) was used to create *Kpn*I restriction sites in the −901/+120 EP24.15 promoter fragment to generate, by *Kpn*I digestion and re-ligation, a series of deletion mutants that progressively removed regions of potential TF (transcription factor) binding as identified by MatInspector v2.2 [26] and the TRANSFAC data base version 4.0 (http://www.genomatix.de/). Primer pairs were synthesized to create mutations (the sequence of the forward primer is given): rTOP797mf (5′-GCCATCATGTGGTACCTGGGAATTGAACTCAGG-3′), rTOP595mf (5′-GCATTACCAGAAACTAGGTACCTGGGGAAGGAGGATTGC-3′), rTOP380mf (5′-CGAACACGAGGGAACGAGGGTACCCTAGAGCTCAAGGTCAAGGTCATCC-3′), rTOP354mf (5′-GCTCAAGGTCATCCTTGGGTACCTGAGGAGAGTGAGGCCC-3′), rTOP138mf (5′-GGGCTGTGCTGGTACCGCGCATGCGCAAAATCTCACCATTGGC-3′) and rTOP76mf (5′-GGATGACGTGGGCGGACGGTACCACTGGCGCCAGACTG-3′). For each reaction, 5 μl of reaction buffer, 5 μl of template DNA pLRPF-901 [25] (10 ng/μl), 1.25 μl each of forward and reverse primers (100 ng/μl), 1 μl of dNTP mix and 36.5 μl of double-distilled water were mixed with 1 μl of *Pfu* Turbo DNA polymerase (2.5 units/μl). PCR was carried out according to the manufacturer's (Stratagene) instructions (95 °C for 30 s, before 18 cycles of 95 °C for 30 s, 55 °C for 1 min and 68 °C at 14 min). Reactions were finally held at 4 °C.

After digestion of template DNA with *Dpn*I, remaining mutated DNA was transformed into Epicurian Coli XL1-Blue supercompetent cells. DNA from the resultant colonies was digested with *Kpn*I to remove the required deletion, and the vector and remaining promoter insert re-ligated. Re-ligated fragments were transformed into DH5α competent bacteria, and an endotoxin-free DNA Maxi Prep (Machery-Nagel, Düren, Germany) was used to prepare DNA for transfections. All deletions were sequenced in both directions using ThermoSequenase (Amersham Biosciences) and reactions were visualized and analysed on a Li-COR 4200-1 automated sequencer.

Synthesis of full-length rat EP24.15 promoter mutations in pGL3 luciferase basic vector

Full-length promoter mutations were prepared using the QuikChange™ Site-Directed Mutagenesis Kit, as above. The primers used to create the mutations were rTOPSRY-1mutf (5′-GCACACTAAGCTTACAAAAGTAAAACAAACAAGCAAAAACTCGTGGAGG-3′), rTOPSRY-2mutf (5′-GCACACTAAGCTTACAAAAACAAAACATACGAGCAAAAACTCGTGGAGG-3′), rTOPCREBmutf (5′-GGCAACCTCAGGAAGACGTGGGCGGACGG-3′) and rTOPCAATmutf (5′-CCCACTCACCCTTGACAAGCAATCGTGGGCTGTGC-3′). All mutations were prepared and sequenced as described above.

Cell culture

GC-2spd(ts) cells, accession number CRL-2196, were obtained from the American Type Culture Collection (Manassas, VA, U.S.A.). GH3, Mat-Lu and PC12 cells, accession numbers ECACC 87012603, ECACC 94102735 and ECACC 88022401 respectively, were obtained from the European Collection of Cell Cultures (Porton Down, Salisbury, Wilts., U.K.). All cell lines were grown at 37 °C in an atmosphere of 5 % CO_2 and in the presence of penicillin (10 000 units/ml) and streptomycin (10 000 μg/ml). GC-2 mouse spermatocyte cells were grown in Dulbecco's modified Eagle's medium supplemented with 4.5 g/l glucose, 10 % (v/v) foetal bovine serum, 2 mM L-glutamine and 100 mM non-essential amino acids. GH3 rat pituitary cells were grown in Ham's F10 medium supplemented with 10 % (v/v) foetal bovine serum and 2 mM L-glutamine. Mat-Lu rat dorsal prostate adenocarcinoma cells were grown in RPMI 1640 medium supplemented with 10 % (v/v) foetal bovine serum, 2 mM L-glutamine and 250 nM dexamethasone. PC12 rat adrenal carcinoma cells were grown in RPMI 1640 medium supplemented with 10 % (v/v) foetal bovine serum and 2 mM L-glutamine. PC12 cells were differentiated by addition of dexamethasone to the growth medium (250 nM final concentration).

Transfection assays

Mat-Lu, GH3 and GC2 cell lines were transfected using Tfx-20™ (Promega) at a 3:1 Tfx-20™ reagent to DNA charge ratio. At 24 h before transfection, 100 μl of cells (at 10^5 cells/ml) was plated per well in a tissue-culture-treated 96-well plate. Each construct to be tested was transfected six times and a mastermix, sufficient for seven wells, comprising 280 μl of appropriate serum-free medium, 4.5 μl of Tfx-20™, 1 μg of test plasmid and 20 ng of *Renilla* control plasmid was prepared and incubated at room temperature (20 °C) for 15 min. Medium was aspirated from the 96-well plate, 40 μl of transfection mix was added to each well and plates were incubated for 2 h under normal growth conditions (see above). The transfection mix was then removed and 200 μl of appropriate complete medium was added to each well. Cells

were incubated for a further 48 h under normal growth conditions (see above) before the luciferase assay.

PC12 cells were transfected using TransFast™ (Promega) at a 3:1 TransFast™ reagent to DNA charge ratio. Immediately before transfection, cells were counted and 25 μl of cells (at 10^6 cells/ml) was transferred into each well of a tissue-culture-treated 96-well plate. Each construct to be tested was transfected six times and a mastermix, sufficient for seven wells, comprising 175 μl of appropriate complete medium, 4.5 μl of TransFast™, 1 μg of test plasmid and 20 ng of *Renilla* control plasmid was incubated at room temperature for 15 min. Transfection mix (25 μl) was added to each well and plates were incubated for 2 h under normal growth conditions (see above). Appropriate complete medium (50 μl) was added to each well without removal of the transfection mix. Cells were incubated, under normal growth conditions (see above), for a further 48 h before the luciferase assay. Differentiated, adherent PC12 cells were treated as for PC12 cells, but were plated at 100 μl of cells (at 10^5 cells/ml) per well 24 h before transfection.

Growth medium was aspirated from 96-well plates and cells were washed in PBS. Cells growing in suspension were subjected to centrifugation at 2000 ***g*** in a Beckman plate rotor, for 5 min, before removal of both medium and PBS. Passive lysis buffer (50 μl; 1×) (Promega) was added to each well and plates were incubated on a shaking platform at room temperature for 30 min or until the cells had lysed. Following lysis, 20 μl of the lysate was transferred to a white flat-bottomed microplate (Microlyte) and luminescence was read in an Anthos Lucy I luminometer. Luciferase reagent II (20 μl) (Promega) was dispensed into a well and, after a 2.5 s delay, the luminescence was measured at 10 ms intervals for 4 s. Each well of the plate was measured before the procedure was repeated, but injecting 20 μl of Stop & Glo Reagent (Promega) to quench the luciferase luminescence, and the luminescence of the control *Renilla* plasmid was measured.

EMSAs (electrophoretic mobility-shift assays)

Nuclear protein extracts were prepared as previously described [25]. HPSF® (high-purity salt-free)-purified oligonucleotide pairs (MWG Biotech) (the forward oligonucleotide sequence is shown): rTOPSRYf (5′-GCTTACAAAAACAAAACAAACAAGCA-3′), rTOPSRY-1 (5′-AAAAGTAAAACAAACAAGC-3′), rTOPSRY-2 (5′-AAAAACAAAACATACGAGC-3′), rTOPGfi1f (5′-GCATGCGCAAAATCTCACCATTGGCAAGG-3′) and rTOPCAATGFi (5′-CTTGACAACCAATCGTGGGCTGTGCTG-3′) were annealed by incubating 8.75 μl of both forward and reverse oligonucleotides (10 pmol/μl) with 20 μl of 600 mM NaCl and 162.5 μl of distilled water at 65 °C for 5 min in a heat block and then cooling to room temperature.

Annealed oligonucleotides were 5′ end-labelled with [γ-^{32}P] ATP by incubating 4 μl of annealed oligonucleotide with 3 μl of [γ-^{32}P]ATP and 1 μl each of distilled water, 10× kinase buffer and polynucleotide kinase at 37 °C for 40 min. The reaction was stopped by the addition of 1 μl of 0.5 M EDTA. Unincorporated oligonucleotides were removed by chromatography through Sephadex G-25 in 20 mM Hepes, pH 7.9.

Nuclear extract (2.5 μg), 10 μl of 2× DNA Binding Assay Buffer, 1 μl of poly(dI-dC)·(dI-dC) carrier DNA (1 mg/ml) and 2 μl of unlabelled double-stranded competing oligonucleotide, where appropriate, were mixed with an appropriate volume of 0.5 M NaCl to give a final salt concentration of 60 mM and water to bring total reaction volume to 20 μl after the addition of label. Reactions were incubated at 4 °C for 20 min before the addition of 40000 c.p.m. of γ-^{32}P-labelled oligonucleotide probe and then for a further 40 min before separation on a 5% (w/v) polyacrylamide gel in 0.5× Tris/borate/EDTA at 4 °C and 200 V for 2 h. Complexes were visualized by autoradiography.

RESULTS

EP24.15 promoter serial deletions

By creating a series of deletion mutations of the rat EP24.15 promoter and investigating the effects of these deletions on promoter activity *in vivo* using a dual luciferase reporter assay, regions of the rEP24.15 promoter that display dissimilar activities in prostate (Mat-Lu), pituitary (GH3), spermatid (GC2), immature adrenal (PC12) and mature adrenal (PC12diff) cells have been identified. The activities of the deletion constructs are expressed as test plasmid luciferase firefly to control plasmid *Renilla* luminescence ratios and are shown in Figure 1(A). Figure 1(B) schematically depicts the serial promoter deletions and indicates the possible TF-binding sites removed by each construct as identified by MatInspector [26]. To reflect the progressive nature of the deletions, statistical significance has been calculated for each construct with reference to the preceding construct in the series rather than the full-length promoter fragment. Significance therefore relates to the promoter deletion introduced by each construct and to the potential TF-binding sites concurrently depicted in Figure 1(B).

rTOP797 produced a statistically significant effect in PC12diff cells, where it increased promoter activity by 27%. rTOP595 reduced promoter activity in Mat-Lu, PC12 and PC12diff cells by 26, 49 and 72% respectively, although the reduction was only statistically significant in PC12diff cells. Deletions rTOP380 and rTOP354 produced no significant effect in any cell line studied; however, in Mat-Lu cells, the removal of bases −379 to −354 by rTOP354 resulted in a reduction of 39% in promoter activity.

rTOP219 had no effect on promoter activity in Mat-Lu cells, but in GH3, GC2, PC12 and PC12diff, it reduced promoter activity by a further 57, 48, 67 and 38% respectively, although this reduction was only statistically significant in GC2 cells. rTOP138 increased promoter activity significantly by 264% in PC12 cells, and by 113 and 54% in GH3 and PC12 cells, but had no effect in Mat-Lu or GC2 cells. The gross deletion rTOP76 resulted in a massive loss of promoter activity in all the cell lines analysed.

EMSAs

EMSAs had been used previously to identify three regions of the rat EP24.15 promoter that bind TFs: −658 to −642 [SRY (sex-determining region Y)/SRY]), −376 to −363 [RORα (retinoid-related orphan receptor α)] and −128 to −114 [growth factor independence 1 (Gfi1)], as illustrated in Figure 1(B). Two regions of the promoter fragment were selected for further EMSA analysis, on the basis of previous studies and the results obtained from the serial promoter deletions.

The double SRY site −658 to −641 containing two adjacent SRY consensus sequences (SRY/SRY) was examined using the oligonucleotide previously shown to bind nuclear extracts, but here it was competed for with a further two oligonucleotides. One designed so that the 5′ SRY site (SRY-1) at −658 to −646 and the other so that the 3′ SRY (SRY-2) site at −653 to −641 was knocked out. In confirmation of our previous observations, Mat-Lu and GC2 nuclear proteins all bound to the SRY/SRY oligonucleotide and were reproducibly competed for by its unlabelled counterpart (Figure 2). This finding was also observed with PC12 nuclear extracts that had not been analysed previously. In addition, the SRY-1 oligonucleotide was able to compete with the highest bands at positions A, B and C, but not with the most distinct band,

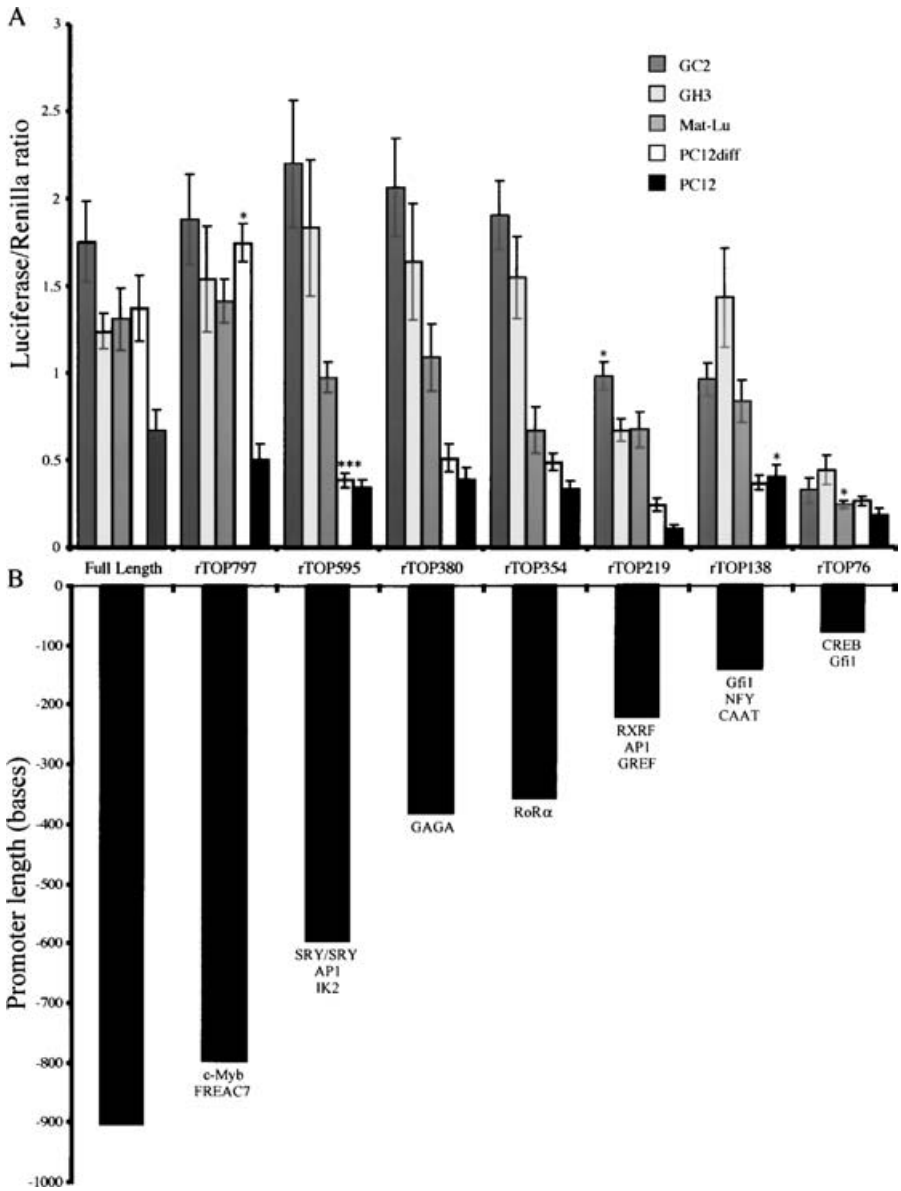


Figure 1 Dual luciferase reporter assay of rat EP24.15 promoter serial deletions

(**A**) Luciferase firefly/*Renilla* ratio for rat EP24.15 promoter constructs as determined by co-transfection in GC2, GH3 and Mat-Lu cells using Tfx20 and PC12 cells using TransFast™ transfection reagents. Results are means ± S.E.M. for six separate assays, and are expressed as the ratio of firefly to *Renilla* luciferases. Asterisks indicate where a deletion results in a luciferase ratio significantly different from its predecessor as determined by a one-way ANOVA with Bonferroni multiple comparison post-test where the null hypothesis can be rejected at $^{*}P < 0.05$ and $^{***}P < 0.001$ respectively. (**B**) Schematic representation of the rat EP24.15 promoter serial deletion constructs. TFs listed beneath each bar are deleted from the construct compared with the previous construct and correspond directly to the luciferase measurements shown in (**A**).

visualized at D in all cell types. The SRY-2 oligonucleotide was able to compete with all the bands visualized, including the major band D, but with GC2 nuclear extracts, the competition was incomplete.

Deletion rTOP138 removed the overlapping CAAT/NF-Y/Gfi1 sites (where NF-Y stands for nuclear factor Y), indicating a potential transcriptional repressor binding site in this region. An oligonucleotide corresponding to this area of the promoter −171/−146 was labelled and incubated with nuclear extracts without and in the presence of an identical unlabelled oligonucleotide (Figure 3A). This oligonucleotide was also incubated in the presence of an unlabelled Gfi1 oligonucleotide (Figure 3B). PC12, GC2 and, to a far lesser extent, Mat-Lu and GH3 cell extracts produced a band at A that was effectively competed by unlabelled oligonucleotide CAAT/NFY/Gfi1 (Figure 3A), but was not competed for by the unlabelled Gfi1 oligonucleotide (Figure 3B). The probe alone produces a band at B, suggesting that this is likely to be due to the formation of probe aggregates. Bands observed at C are not competed by the consensus oligonucleotide or the Gfi1 oligonucleotide, indicating that this is not a specific complex.

EP24.15 promoter mutations

To identify more specifically which TFs were involved in differential rat EP24.15 promoter regulation, a set of full-length promoter mutant constructs were synthesized. These constructs

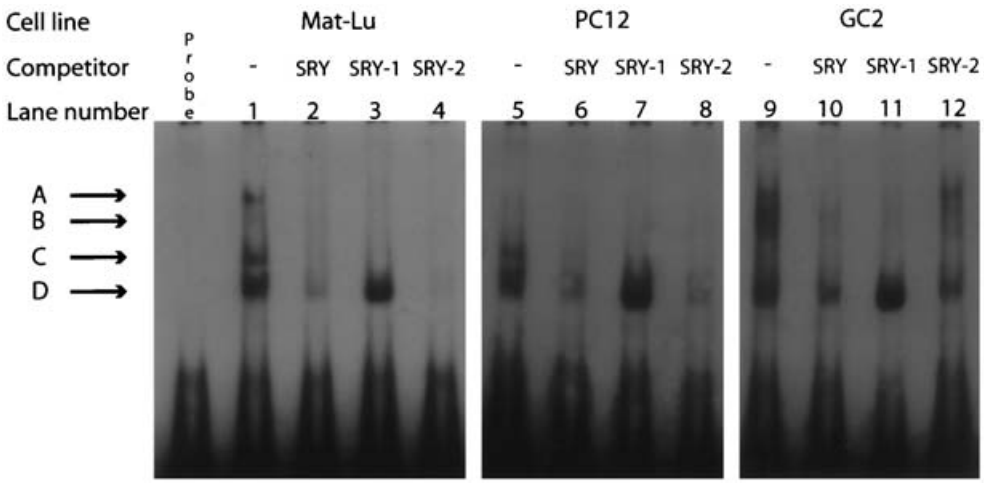


Figure 2 EMSA for the SRY/SRY site at position −665/−640 in the rat EP24.15 promoter using PC12, GC2 and Mat-Lu nuclear extracts

Nuclear proteins (2.5 μg) from Mat-Lu (lanes 1–4), PC12 (lanes 5–8) and GC2 cells (lanes 9–12) were incubated with labelled SRY/SRY double-stranded oligonucleotide (probe). In lanes 2, 6 and 10, the probe was competed with an unlabelled identical SRY/SRY oligonucleotide (SRY). In lanes 3, 7 and 11, the probe was competed with an unlabelled oligonucleotide with the 5′ SRY site removed (SRY-1). In lanes 4, 8 and 12, the probe was competed with an unlabelled oligonucleotide with the 3′ SRY site removed (SRY-2). Protein–DNA complexes formed were run on a 5 % (w/v) native polyacrylamide gel for 2 h and were visualized after autoradiography. Arrows indicate the positions of protein–DNA complexes A–D. The EMSA was performed in triplicate and the gel shown are representative of all three assays.

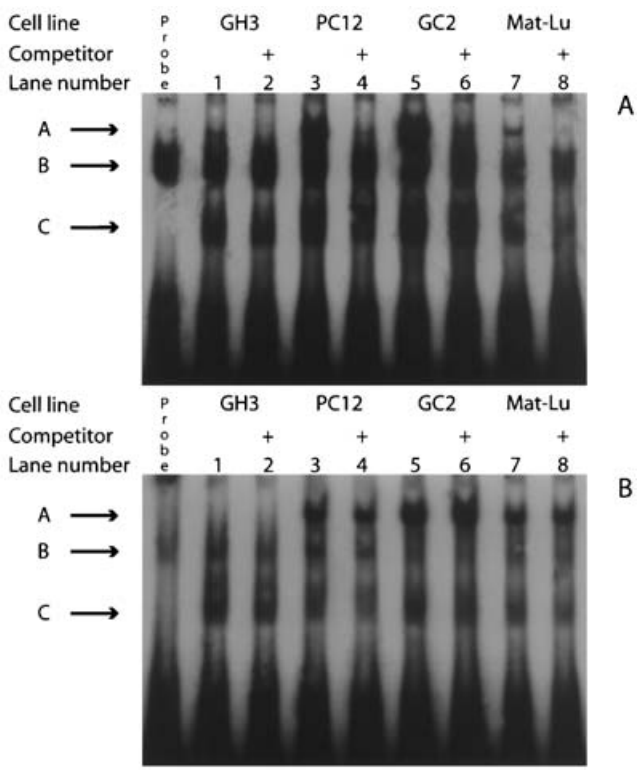


Figure 3 EMSA for the CAAT/NFY/Gfi1 at position −171/−146 in the rat EP24.15 promoter using Mat-Lu, PC12, GC2 and GH3 nuclear extracts

Nuclear proteins (2.5 μg) from GH3 (lanes 1 and 2), PC12 (lanes 3–4), GC2 (lanes 5 and 6) and Mat-Lu (lanes 7 and 8) cells were incubated with labelled CAAT/NFY/Gfi1 probe. The left-hand lane (probe) shows probe only. In lanes 2, 4, 6 and 8, the probe was competed with an unlabelled identical CAAT/NFY/Gfi1 oligonucleotide (**A**) and unlabelled dsGfi1 oligonucleotide (**B**) indicated by +. Protein–DNA complexes formed were run on a 5 % (w/v) native polyacrylamide gel for 2 h and were visualized after autoradiography. Arrows indicate the positions of protein–DNA complexes A–C. The EMSA was performed in triplicate and the gels are representative of all three assays.

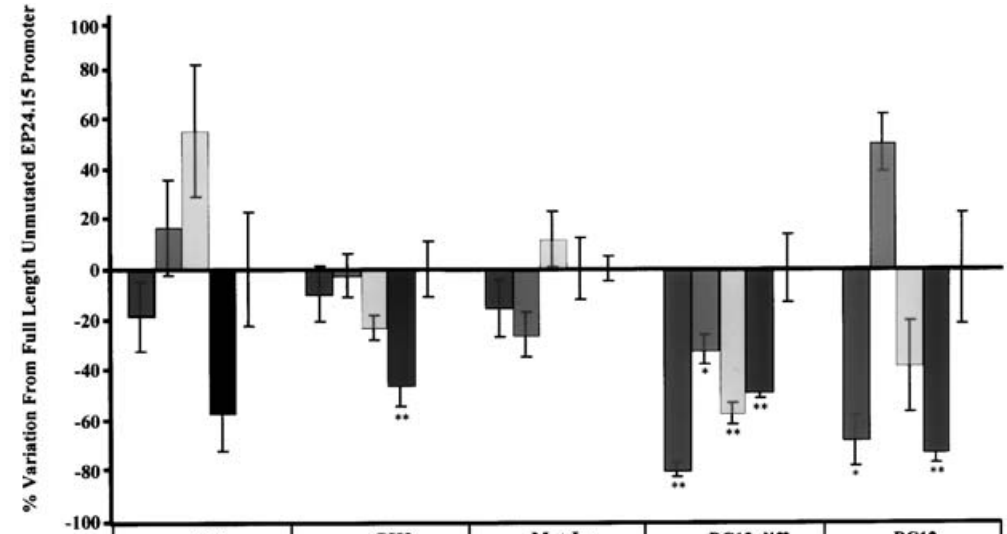
all contained the complete rat EP24.15 promoter fragment −901/+120 in the pGL3basic vector but, in each construct, site-directed mutagenesis was used to knock out the core sequence of a specific TF-binding site. Four mutants were made to knock out predicted SRY, CAAT and CREB (cAMP-response element-binding protein) consensus sequences at positions −658 (ΔSRY-1), −653 (ΔSRY-2), −165 (ΔCAAT) and −99 (ΔCREB). In each case, the mutated fragment was analysed using MatInspector [26] to ensure that the mutation created would specifically eliminate the intended TF-binding consensus sequence, without inadvertently creating a novel one.

The full-length mutated constructs were transiently transfected into the same cell lines as used for the serial deletions, and promoter activity was measured using the dual luciferase reporter assay as above. Luciferase firefly/*Renilla* ratios obtained for the constructs are expressed as percentages (± those of the full-length EP24.15 promoter construct) and are shown in Figure 4.

None of the full-length mutations produced a statistically significant difference in Mat-Lu cells, although the SRY-1 and SRY-2 mutants reduced promoter activity by 16 % and 26 % respectively. In GH3 cells, neither of the SRY mutants altered promoter activity, but removal of the CAAT and CREB sites resulted in reductions in promoter activity of 23 % and 46 % respectively.

PC12 and PC12diff cells displayed dissimilar activity profiles. Mutation of the SRY1 site resulted in large reductions in promoter activity in both cell lines of 69 % and 80 %; however, the SRY2 mutation had a contrasting effect whereby promoter activity increased by 51 % in immature PC12 cells, but decreased by 32 % in the mature cells. The CREB mutation produced reductions of 74 % and 49 % in the immature and differentiated cells respectively. The CAAT site mutation reduced activity in PC12diff cells by 58 %.

In common with GH3 cells, the GC2 cells displayed no significant variations in promoter activity on mutation of either of the SRY sites, but in contrast to all the other cell lines, where the CAAT mutation resulted in a loss of activity, in GC2 cells, when this binding site was mutated, there was an increase in promoter activity of 56 %. The CREB mutation resulted in a 49 % loss of promoter activity.



	GC2	GH3	Mat-Lu	PC12 diff	PC12
ΔSRY-1	-18.6	-10	-15.6	-80.2	-68.3
ΔSRY-2	16.6	-2.6	-26.2	-32.4	49.8
ΔCAAT	55.2	-23.2	11.6	-57.7	-38.9
ΔCREB	-57.2	-45.8	0	-49	-73.2

Figure 4 Dual luciferase reporter assay of rat EP24.15 promoter full-length mutants

Percentage variation in luciferase firefly/*Renilla* ratio ± percentage of the un-mutated full-length promoter fragment for full-length rat mutant EP24.15 promoter constructs, as determined by co-transfection in GC2, GH3 and Mat-Lu cells using Tfx20 and PC12 cells using TransFast™ transfection reagents. Results are means ± S.E.M. for six separate assays, and are expressed as the percentage variation from the un-mutated control. Asterisks indicate where a deletion results in a luciferase ratio significantly different from its predecessor as determined by a one-way ANOVA with Dunnett's post-test where the null hypothesis can be rejected at $^{*}P < 0.05$ and $^{**}P < 0.01$ respectively.

DISCUSSION

Transcription occupies a central role in gene expression rendering it an effective control point for regulating protein production in particular cell types or in response to specific stimuli. Where a protein is only present to a significant degree in certain tissues (for EP24.15, neuroendocrine and testicular), control processes that ensure its corresponding gene is only transcribed in these specific tissues generally regulate protein production [27].

In eukaryotic promoters, there are two distinct types of regulatory elements. Those found in the basal process of transcription itself that complex with RNA polymerase II and those that are only found in genes transcribed in response to a signal or in a tissue that produces a specific TF. Specific factors can be either enhancers that act by increasing the activity of a promoter or inhibitors that act to reduce promoter activity, and it is the balance of these positively and negatively acting TFs binding to the regulatory sites on the promoter that dictate the rate of gene transcription and consequent protein production [27].

The rat EP24.15 promoter serial deletions progressively removed sections of the 5′ untranslated region and were assayed for promoter activity in several cell types. Mat-Lu (rat prostate), GH3 (rat pituitary) and GC2 (mouse spermatid) cell lines were selected as they have been shown to express EP24.15 [25]. PC12, immature adrenal chromaffin cells [28], were also included as they have EP24.15 activity [29] and can be differentiated by dexamethasone to acquire the features of mature adrenal cortical cells [30].

The serial deletions revealed several promoter regulatory regions that varied with the cell lines analysed. The cell lines GH3 and GC2, which express the lowest levels of EP24.15, [25] appear only to have regions of positive transcriptional control immediately upstream of the transcriptional start site at positions −354 to −219 and −138 to −76 of the promoter. GH3 cells have an additional negatively acting region from −219 to −138. Mat-Lu cells have medium levels of EP24.15 expression and exhibit a promoter profile distinct from all the other cell lines analysed. They are the only cell line studied that display enhancer activity in the region −380 to −354 that contains a RORα site previously shown by EMSA to bind nuclear protein from Mat-Lu cells [25], and unlike the other cell lines, deletion of region −354 to −219 has no effect. In contrast with GH3 and GC2 cells, the EP24.15 promoter in Mat-Lu cells additionally responds positively to factor(s) acting on the −797 to −595 region. This positive response is also found in PC12/PC12diff cells, which display high levels of EP24.15 activity [29].

The initial promoter study identified a perfect $(GA)_{20}$ dinucleotide repeat located between −406 and −445 on the EP24.15 promoter as a potential site for transcriptional regulation. This motif has been shown to play a critical role in the expression of several *Drosophila* genes [31]. It was thought that it could have a role in EP24.15 expression; however, the only deletion mutant in this study that did not alter promoter activity in any of the cell lines analysed was rTOP380 that removed the potential GAGA protein-binding site, making the involvement of this factor in EP24.15 regulation unlikely.

The RORα site at position −376 to −363 was removed by deletion rTOP354 resulting in a loss of activity and therefore potential enhancer binding only in Mat-Lu cells. rTOP354 removed a relatively short region of DNA, 26 bp, and it is therefore

possible that it is significant in the regulation of the EP24.15 promoter in prostate cells. RORα is a nuclear orphan hormone response, DNA-binding element, found in a broad range of tissues, including testes and brain, and has several putative target genes including oxytocin [32] that, like EP24.15 is found in the neuroendocrine system. The RORα-binding site in the EP24.15 promoter has not been analysed further in the present study, but its potential as an EP24.15 transcriptional regulator is worthy of further consideration.

The effect of each deletion is cumulative in that several different TF sites could be removed by a single deletion, and it is therefore impossible to predict from a large deletion which specific factors are exerting effects on promoter activity. However, we were able to conclude that the EP24.15 promoter is differentially regulated in the cell lines analysed and use this information to perform a more detailed analysis of three regions of the promoter.

The EP24.15 promoter contains a CAAT box TF [NF-Y (nuclear factor-Y)/CTF (CCAAT TF)/NFI (nuclear factor I)] binding consensus adjacent to a Gfi1-binding site at -171 to -146 that binds nuclear proteins from all the cell lines that were analysed (Figure 3A). As the binding was not competed by a Gfi1 oligonucleotide (Figure 3B), it was likely that the CAAT/NFY site was the negatively acting factor responsible for the net increase in activity, upon its removal by deletion rTOP138, in GH3 and PC12 cells.

The CAAT box TF family of site-specific DNA-binding proteins contains four members that can be expressed via alternative splicing in a variety of isoforms [33]. They combine as heterotrimers to form NF-Y that binds to CAAT boxes in gene promoters, causing either transcriptional repression or enhancement dependant on the specific isoforms involved [34]. Multiple mechanisms are involved in the modulation of promoter activity, including direct interaction with the TATA binding protein, recruitment of enhancer/repressor proteins, synergistic interactions with adjacent TFs and disruption of histones [33].

In GH3 and PC12diff cells transfected with ΔCAAT, there were decreases in promoter activity of 23% and 58% respectively, which were surprising given that deletion rTOP138, which removed the CAAT site, had resulted in a rise in promoter activity in these cells. The EP24.15 promoter lacks a TATA box [25], and therefore the enhancer activity afforded by binding to the CAAT box cannot be due to interaction with the TATA protein. Nuclear extracts from GH3 cells have previously been shown to bind specifically to a Gfi1 oligonucleotide [25], and therefore this or a closely related transcriptional repressor is expressed in GH3 cells. Given that the CAAT/NFY/Gfi1 EMSAs performed in this study (Figures 3A and B) show that CAAT, but not Gfi1, specific factors bind to their adjacent consensus sequences, it is possible that NF-Y, binding to the CAAT site, represses Gfi1 binding. When the CAAT site is mutated, Gfi1 is able to interact with its adjacent consensus sequence exerting a negative effect resulting in the reduction seen in promoter activity in GH3 and PC12diff cells on CAAT site mutation. An increase in activity is seen when the entire region is removed because this also removes the Gfi1 site that is never entirely repressed by NF-Y competitive binding.

Although in GC2 cells, no alteration in promoter activity was seen with the cumulative rTOP138 deletion, the CAAT mutation resulted in a 56% increase in promoter activity, signifying that NF-Y binding to the EP24.15 promoter CAAT box in spermatid cells exerts an inhibitory effect on EP24.15 expression. The complexity of these results may be a direct reflection of the equilibrium between activation and repression achieved by the balance of tissue specific NF-Y isoforms. This can result in the repression of a promoter in one cell line, but its enhancement in another [33]. It is possible that the mutation altered the composition of NF-Y dimers able to bind to the CAAT site rather than simply knocking the site out. MatInspector [26] analysis of the mutation would refute this and, consequently, it is more likely that the CAAT site at -237 in the rat EP24.15 promoter is a key factor in the tissue-specific regulation of EP24.15 expression.

The *SRY* and related *Sox* genes encode TFs which all contain the HMG (high-mobility group) domain that binds to the A/TA/TCAA/T consensus sequence in promoter DNA, causing it to bend [35], and on forming complexes with co-repressor/enhancer proteins, alters target-gene expression [36]. Although SRY is normally taken to be a testis-specific TF [37], 38% of prostate tumours are characterized by the absence of SRY [38], and it is also transcribed in adult mouse brain [39] and human foetal and adult Sertoli and germ cells [40]. There are at least 20 Sox proteins that are expressed in a broad range of tissues [36].

As deletion rTOP595 had reduced EP24.15 promoter activity in Mat-Lu, PC12 and, significantly, PC12diff cells, the SRY/SRY site removed by this deletion was analysed further. The EMSAs performed using nuclear cell extracts from these and GC2 cells indicated that the major site for protein binding was the 5′ SRY site, as an oligonucleotide with this site mutated (SRY-1) was unable to compete with the major binding complex formed at D on Figure 2. The SRY-1 oligonucleotide was, however, able to compete with higher cell-specific bands. These bands, rather than being representative of alternative SRY/Sox protein binding are probably due to the presence of co-proteins. This is likely because the mutation used knocked out the HMG binding consensus that is essential to the DNA binding of all the SRY/Sox factors. The higher bands resulting from the binding of a Sox family protein complexed with a co-factor could still be competed because a complexed SRY/Sox protein can more stably bind DNA than its solo counterpart [36], and can therefore compete more effectively for the un-mutated, labelled 5′ SRY binding sites.

It is also possible that the higher bands are competed, in spite of the ΔSRY-1 mutation because they are caused by protein binding outside the mutated site. If this is the case, given the brevity of the oligonucleotides used, this site would almost certainly have to be the 3′ SRY site. Although mutation of this site did not alter the ability of an otherwise identical oligonucleotide to effectively compete all the protein complexes formed, it may become the binding site of choice for some SRY/Sox proteins in the absence of the 5′ SRY site or perhaps even in co-operation with it.

The full-length mutations of the SRY sites highlight a further tissue-specific region of differential EP24.15 promoter activity. Neither of the individual SRY mutations caused a significant reduction in promoter activity in GC2 nor Mat-Lu cells. However, if the combined effect of both mutations is cumulative and indicative of co-operation between the adjacent sites, then the effect of a double deletion would be significant in Mat-Lu cells and may represent a Sox-dimer-binding site. The SRY/Sox proteins have been shown to form dimers with several co-factors, but Sox5/Sox6 dimers have also been reported to co-operatively activate the collagen type II gene [41], and may have a similar role in regulating the EP24.15 promoter in Mat-Lu cells.

The ΔSRY-1 mutation causes a drastic reduction in promoter activity in both immature and differentiated PC12 cells; however, the ΔSRY-2 mutation, while inhibiting EP24.15 promoter activity in mature cells, increased it in the immature undifferentiated cells. Therefore, whereas binding to the 5′ SRY site enhances promoter activity in both cell types, TF binding to the second site inhibits activity in immature cells, but enhances it in the mature differentiated cells. When the SRY/SRY site is removed by deletion rTOP595, both PC12 cell types display identical promoter-activity profiles and luciferase firefly/*Renilla* ratios with the remaining

serial deletions. This demonstrates that the EP24.15 SRY/SRY promoter-binding consensus plays an important role in tissue-specific EP24.15 expression and again is indicative that both the sites, either individually or in co-operation, are required for gene regulation.

The SRY and CAAT full-length mutants identified sites in the EP24.15 promoter that were responsive to TFs in a tissue-specific manner, but neither were involved in interactions with the DNA polymerase II initiation complex. We therefore decided to create a full-length CREB mutation at −99 in an attempt to find a site of basal activation. This site was selected because the cAMP responsive nuclear factors have been shown to play important physiological and developmental roles in the hypothalamic–pituitary–gonadal axis [42], particularly during spermatogenesis [43,44], a process in which EP24.15 has also been implicated [21].

CREs (cAMP-response elements) generated by the alternative splicing of *creb* and *crem* genes translated from multiple transcription-start sites, bind as dimers to the CRE consensus sequence TGAGCTCA in gene promoters [42]. They act either as transcriptional activators or repressors dependant on the presence of a kinase-inducible domain that contains a serine residue, which, when phosphorylated, promotes association of CRE with the CBP (CREB-binding protein). CBP then interacts positively with the basal machinery associated with RNA polymerase II transcription.

The ΔCREB promoter mutant displayed significant loss of activity when assayed in PC12, PC12diff, GH3 and GC2 cell lines. This indicates that the CRE-binding site in the rat EP24.15 promoter plays an important role in the basal transcription of the enzyme in neuroendocrine tissues and provides further evidence of its involvement, by virtue of its specific enhanced expression within these tissues, in bioactive-peptide processing and the progression of spermatogenesis.

The present study has identified TF-binding sites that are responsible for the basal and tissue-specific expression of the EP24.15 gene. Additional analysis in the form of EMSAs utilizing mutations of the broader matrix, rather than the core binding-consensus sequences, combined with supershift assays will permit identification of the specific SRY, CRE and NF-Y family members involved in EP24.15 transcriptional regulation. Concomitant analysis of EP24.15 mRNA and protein levels in cells treated with reagents such as cAMP, which are known to modulate CRE-dependent promoters, will provide a valuable insight into the genetic regulation of EP24.15 and, as a result, its role in the hypothalamic–pituitary–gonadal axis.

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