Novel cell lines for the analysis of preprotachykinin A gene expression identify a repressor domain 3′ of the major transcriptional start site

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Until now, no clonal cells have been identified that support the expression of a marker gene expressed from the rat preprotachykinin A (rPPT) promoter. We have analysed recently available cell lines that are candidates for supporting reporter gene expression directed by the rPPT promoter. These are the neuronal-derived cell line NF2C and the pancreatic cell lines RINm5F and a derivative RIN-1027-B2. The NF2C line was derived from the brain homogenate of a transgenic animal in which a temperature-sensitive simian virus 40 large T antigen was expressed from a neurofilament promoter. All three lines are able to support expression of a reporter gene directed by a fragment of the 5′ rPPT promoter. Analysis of reporter gene expression supported by various fragments of the rPPT promoter demonstrated that although −865 to +92 bp supported expression, the addition of fragments between +92 and +447 bp led to repression of expression. Subsequent analysis of reporter gene constructs microinjected into primary cultures of dorsal root ganglion neurons (DRG) confirmed the existence of this repressor domain. This repression could be relieved totally in both RIN cell lines and partly in NF2C cells by mutating residues between +373 and +396 bp. This indicates that these cell lines support PPT promoter activity similar to that observed in DRG and determines a novel repressor domain within the promoter.

Key words: dorsal root ganglion neurons, NF2C cell lines, RIN cells, substance P, tachykinin.

INTRODUCTION

The gene for rat preprotachykinin A (rPPT) encodes the neuropeptides substance P, neurokinin A, neuropeptide K and neuropeptide γ, which are derived by alternative splicing of primary RNA transcripts and post-translational processing of the peptide precursors [1,2]. Analysis of rPPT promoter activity has been hindered by the lack of a clonal cell line that either expressed the endogenous gene or allowed the expression of a reporter gene supported by fragments of the rPPT promoter [3–6]. It has previously been shown that the proximal rPPT promoter, when linked to a reporter gene, directs high levels of expression in microinjected rat dorsal root ganglion neurons (DRG) grown in tissue culture [3,4]. Microinjection of constructs into DRG is, however, time consuming, difficult and permits the analysis of only a few cells. In addition, the heterogeneity of primary cell cultures and the fact that the rPPT gene is only expressed in a subset of these neurons add a further complexity to this analysis. It would be desirable to have a clonal cell line in which the rPPT promoter is active to complement these studies. Such a cell line would also provide sufficient protein extract for the biochemical analysis of the transcription factors interacting directly with the rPPT promoter and for the dissection of the signal transduction pathways regulating tachykinin gene expression. To this end we have been trying to develop clonal cell line models that support reporter gene expression directed by the rPPT promoter. These are the neuronal-derived cell line NF2C, and the pancreatic cell lines RINm5F and a derivative RIN-1027-B2. The NF2C line was derived from the brain homogenate of a transgenic animal in which a temperature-sensitive simian virus 40 (SV40) large T (LT) antigen is expressed from a neurofilament promoter. The RIN cells are believed to reflect a more embryonic feature of the pancrease because PPT gene expression is observed in this organ during development but not in the normal adult [7].

Initial analysis of rPPT promoter activity focused on the ability of a proximal promoter region spanning −3356 to +447 bp (sequencing has confirmed the exact positioning of the region previously designated broadly as −3500 to +500 [3]) to support reporter gene expression in the neurons of primary cultures of DRG but not in the non-neuronal cells of these heterogeneous cultures [3]. The fragment spanning −3356 to +447 had three times more activity than −3356 to +92. Further demonstration that fragments spanning −3356 to +92 and −865 to +92 supported equivalent levels of reporter gene expression in DRG concentrated our biochemical and functional dissection of the promoter on the region of the promoter spanning −865 to +447 [5,6]. We have previously defined potential transcription factor interactions with this region biochemically with the use of DNAase 1 footprint analysis and electrophoretic mobility-shift analysis, as reviewed in [6]. Until now we have been unable to demonstrate reporter gene expression in any cell line, including those of neuronal origin generated by the fusion of DRG and a neuroblastoma cell line [8]. This restricted tissue-specific expression is regulated by the combinatorial action of multiple positive and negative *cis*-acting regulatory domains [4,9–18]. A major determinant of this restriction of expression to

Abbreviations used: rPPT, rat preprotachykinin A; DRG, dorsal root ganglion neurons; SV40, simian virus 40; LT, large T; CMV, cytomegalovirus; CAT, chloramphenicol acetyltransferase.

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DRG in transient reporter gene analysis is a dominant repressor protein that binds adjacent to the major transcriptional start site [4]. Fragments spanning the region −865 to +447 have now been used in reporter gene analysis of the above candidate cell lines that might support PPT promoter activity.

MATERIALS AND METHODS

Reporter gene constructs

rPPT fragments spanning nt −47 to +92 (MINPPT), −865 to +92 (5′PPT) and −865 to +447 (LPPT) were cloned into the *Xho*I site of the pGL3 basic vector (pGL3b; Promega) upstream of a cloned luciferase reporter gene. In addition, a mutated form of LPPT (LPPTmu), in which residues +373 to +378 and +391 to +396 were altered to *Bgl*II and *Bam*HI restriction sites respectively by means of PCR (Figure 1), was also cloned into pGL3b. All final constructs were confirmed by restriction-enzyme analysis and sequencing. The pGL3b vector lacks eukaryotic promoter or enhancer sequences, so the insertion of a functional promoter at the *Xho*I/*Hin*dIII site will direct the expression of the reporter gene. The first ATG codon encountered after transcriptional initiation is within the luciferase gene, as the first ATG in the rPPT gene is after +447 and within exon 2 at position +611.

Culture of cell lines

NF2C cells (TCS Biologicals) were incubated at 34 °C with air/CO_2 (19:1) in Dulbecco's modified Eagle's medium supplemented with 50 ng/ml insulin, 2 mM glutamine, penicillin/streptomycin, 10% (v/v) fetal calf serum and 10% (w/v) neuronal cell growth supplement (TCS Biologicals). Cells were maintained at 60–80% confluence, being passaged approx. every 3 days. At the appropriate time the cells were transferred to 39 °C and incubated for 14 days to inactivate the LT antigen and allow differentiation to a neuronal phenotype.

RINm5F and a derivative RIN-1027-B2 cells were incubated at 37 °C as described by McGregor et al. [7]. The RINm5F cells are derived from a rat pancreatic tumour and express the rPPT gene endogenously, therefore being known as RIN$^+$. The RIN-1027-B2 cell line is derived from the same rat insulinoma tumour as the RINm5F but does not express substance P on the basis of radioimmunoassay and PPT Northern blot analyses, therefore being known as RIN$^-$.

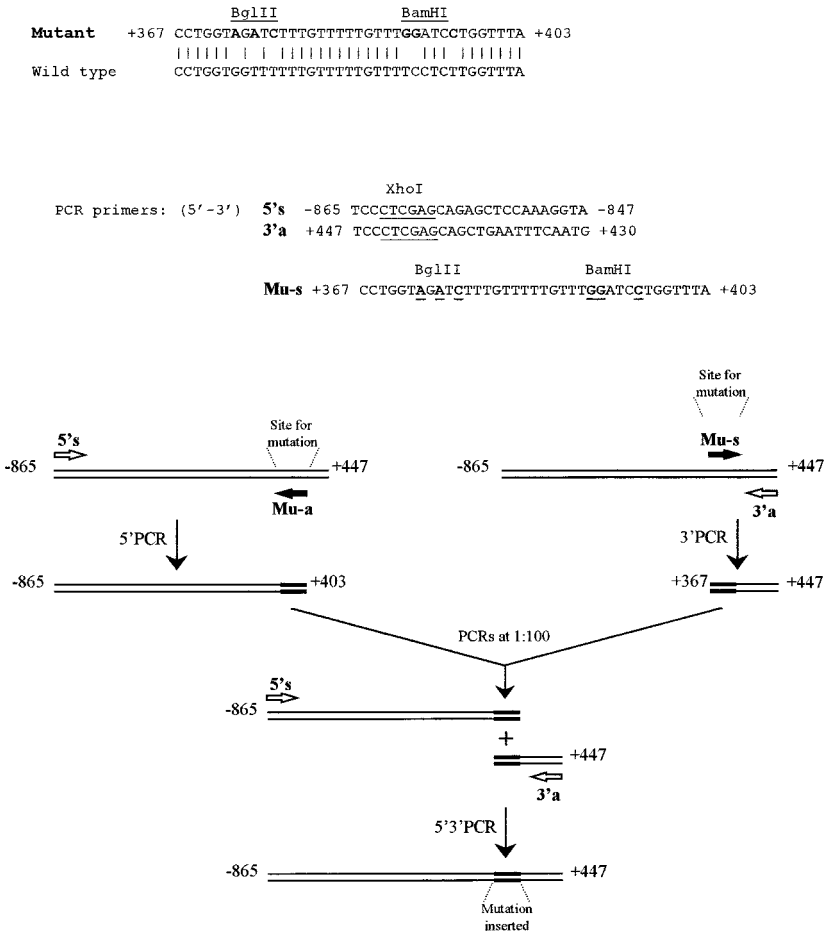

Figure 1 PCR-directed site-specific mutagenesis of LPPT

(**A**) The mutated region of LPPT is shown spanning nt +367 to +403. Mutation of LPPT gives rise to new *Bgl*II and *Bam*HI restriction-enzyme sites shown in bold in the mutant LPPTmu sequence. Unaltered nucleotides match in the two sequences. (**B**) Nucleotide positions of the PCR primers used for creating the mutations. 5′s and 3′a correspond to the ends of LPPT with *Xho*I restriction-enzyme sites added for cloning purposes. Mu-s and its anti-sense strand Mu-a (not shown) are the PCR primers containing *Bgl*II and *Bam*HI sites. (**C**) Scheme illustrating the PCR steps required for the creation of muLPPT. Initial PCR reactions created two short products each incorporating one of the mutant primer sequences. When mixed together in very dilute solution these products anneal, giving rise to LPPTmu after PCR with the flanking 5′s and 3′a primers.

Primary cultures of DRG

Dorsal root ganglia were excised from Wistar albino female rats (aged 4–5 weeks). Finely chopped ganglia in Ham's F14 medium plus $NaHCO_3$ (2 g/l, pH 6.7), were digested for 2 h with 0.125 % (v/v) collagenase/dispase; the digested tissue was then centrifuged for 1 min at 1000 rev./min (150 ***g***). Cells were resuspended in 1 ml of Saito's medium diluted in Ham's F14, then incubated overnight on plates pretreated with poly-(D-ornithine). Neurons, which adhered only loosely, were finally replated on poly-(D-ornithine)/laminin (20 μg/ml) coated plates in fresh Saito's medium supplemented with recombinant nerve growth factor (2 ng/ml).

Transfection by electroporation of NF2C and RIN cells

Four transfection experiments were performed for each cell line, with quadruplicate samples being transfected on each occasion. Specific rPPT-pGL3b construct (20 μg) plus 2 μg of cytomegalovirus (CMV)–chloramphenicol acetyltransferase (CAT) reporter gene were used per transfection. Flasks of NF2C cells maintained at 34 °C or alternatively after 14 days at 39 °C were used for transfection. Cells were transferred to medium with 0.5 % (v/v) serum for 16 h before electroporation. Cells were trypsin-treated and resuspended at 2×10^5 cells in 800 μl of medium containing 0.5 % (v/v) serum. Electroporation was performed in 4 mm electroporation cuvettes with the use of the EquiBio EasyJect. An electric pulse of 270 V and a capacitance of 1500 μF were employed (established as optimum). The cells were then resuspended immediately in fresh complete medium in new flasks.

Microinjection of DRG

Reporter plasmid DNA was diluted to 150 μg/ml in 10 mM Tris/HCl buffer, pH 7.5, and loaded into Eppendorf femtotip injection pipettes, tip diameter 0.5 ± 0.2 μm. Microinjection was performed with an Eppendorf microinjector 5242 and micromanipulator 5170 system. Reporter gene constructs were microinjected 1–4 days after plating, with a standard injection time of 0.5 s. Micro-injection was performed as described previously [3] except that cultures were plated on shallow 10 mm diameter polystyrene wells instead of on glass coverslips. The constructs were microinjected into 100 DRG per well.

Reporter gene assay

After 48 h at the appropriate temperature, cells were washed twice with PBS, then lysed with Promega reporter lysis buffer. After 15 min at room temperature the cell lysate was vortex-mixed and centrifuged briefly at 10000 ***g***. Supernatants were stored at −70 °C. rPPT-directed reporter gene expression was measured by using the Promega luciferase assay system. Supernatant (20 μl) was added to 100 μl of luciferase assay reagent in opaque 96-well plates and the light emission was measured over a given time interval with the Life Sciences Labsystems Luminoskan, model RT. The linearity of luciferase detection was established from standard curves of luciferase activity with the use of 2-fold serial dilutions of luciferase enzyme from 1 μg/ml. CAT activity was measured with the Boehringer Mannheim kit in accordance with the manufacturer's instructions. Luciferase results were normalized to CAT values to control for transfection efficiencies. Results are means ± S.D. for quadruplicate determinations.

PCR

mRNA was isolated from equivalent numbers of cells of each type using RNAzol (Biogenesis) and cDNA was synthesized with Boehringer Moloney-murine-leukaemia virus reverse transcriptase under standard conditions. cDNA was amplified with the primers of Harmar et al. [19] from exon 2 and exon 7 sequences, which include the rPPT mRNA initiation and termination codons respectively, i.e. sense, 5′-AGAATTCAACATGAAAATCCTCGTG-3′ (with an *Eco*RI site introduced at bases 2–7); anti-sense 5′-TGGATCCTCGCGGACACACGCTGGAGAT-3′ (with a *Bam*HI site introduced at bases 2–7). PCR parameters were: 3 min at 94 °C; 40 cycles of amplification (45 s at 94 °C, 45 s at 55 °C, 90 s at 72 °C); followed by 5 min at 72 °C.

RESULTS

Novel cell lines that support rPPT promoter activity

We obtained a novel neuronal cell line, NF2C, from TCS Biologicals. This NF2C line was generated from a transgenic rat in which a temperature-sensitive SV40 LT antigen is expressed under the control of the neurofilament light-chain promoter. SV40 LT antigen expressed as a transgene has previously been demonstrated to immortalize cells that had previously proved very difficult to culture *in vitro* and is the basis of the technology in the 'immortomouse' [20]. NF2C cells will divide in culture at 34 °C when LT antigen is expressed; however, when moved to a higher temperature the LT antigen will be inactivated and the cells will not divide. At the higher temperature the cells undergo a marked change in morphology and now resemble the DRG that we grow in culture, i.e. large and round with a light-refractive cell membrane. The morphological changes take at least 5 days to be observed, with a stable morphological change by 14 days. Cell death is observed in some of the population when the cells are shifted to 39 °C; however, the remaining cells are then stable for several weeks. The NF2C cells should always be used at low passage number because continued passage leads to loss of the morphological changes observed when the cells are shifted to 39 °C. The cells do not grow well if shifted back to 34 °C after several days at 39 °C.

Attempts have previously been made to produce clonal cells with the properties of neurons, such as the fusion of primary culture neurons with neuroblastoma cell lines [8]. Although some of such cells exhibit some neuronal properties, in our hands all fail to express the endogenous rPPT gene or support the expression of reporter gene constructs driven by rPPT promoter fragments. The RIN^+ cells are derived form a rat pancreatic tumour and express the rPPT gene endogenously [7]. It is believed that this line reflects a more embryonic feature of the pancreas because PPT gene expression is observed in this organ in development but not in the normal adult [21]. The RIN^- cell line is derived from the same rat insulinoma tumour as the RIN^+ but was found not to express substance P on the basis of radioimmunoassay and PPT Northern blot analyses. Both RIN cell lines are predominantly homogeneous populations, whereas the NF2C line that was made from a brain homogenate represents a number of different cell types. The NF2C cells, both at 34 °C and at 39 °C, and both RIN cell lines express the endogenous rPPT gene when analysed by PCR (Figure 2). Under the conditions used, two splice variants were detected in the RIN cells, whereas only one was detectable in NF2C and DRG. This is a reflection of the relative amounts of specific mRNA in the different cell samples and confirms the potential for the cell lines to support rPPT transcripts *in vitro*.

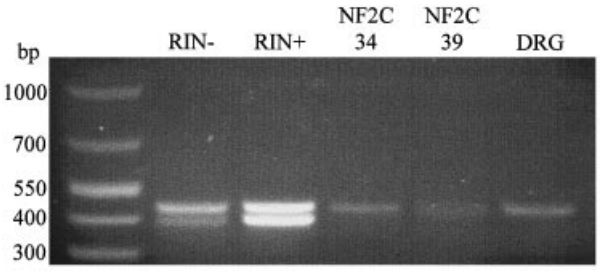

Figure 2 PPT transcripts in rat RIN, NF2C and DRG

rPPT was amplified by PCR with the primers of Harmar et al. [19]. Shown are cDNA from RINm5F^{-} (lane 2), RINm5F^{+} (lane 3), NF2C/34 °C (lane 4), NF2C/39 °C (lane 5) and normal rat DRG (lane 6). Various bands are seen corresponding to the major splice products in each tissue type. The sizes of molecular mass standards (lane 1) are shown at the left.

Table 1 Luciferase expression from MINPPT in NF2C and RIN cell lines

Cells were electroporated with vectors pGL3b, pGL3p or the construct MINPPT plus CMV–CAT in four separate experiments. Each experiment comprised quadruplicate wells for each construct. Combined data are shown as mean units of luciferase per experiment (means ± S.D.) expressed from the reporter gene in the different cell types after 48 h of incubation. All results have been normalized to CAT expression to correct for transfection efficiencies.

	Normalized luciferase activity (units)			
Vector	NF2C/34	NF2C/39	RIN^{+}	RIN^{-}
pGL3b	17.40 ± 0.86	1.01 ± 0.07	0.12 ± 0.04	1.47 ± 0.91
MINPPT	39.12 ± 0.21	2.55 ± 0.09	1.18 ± 0.05	33.43 ± 1.85
pGL3p	110.00 ± 28.86	6.79 ± 1.03	0.49 ± 0.01	5.10 ± 0.95

Table 2 Reporter gene expression from 5′PPT, LPPT and LPPTmu constructs in NF2C, RIN and DRG

Results are expressed as fold luciferase units relative to that from MINPPT in each cell line. NF2C and RIN cells were electroporated in quadruplicate for each construct and DRG were microinjected with constructs plus CMV–CAT. Electroporation data are means ± S.D. for four separate experiments except DRG results, which are per 100 cells injected. All results are normalized to CAT expression. Abbreviation: n.d., not determined.

	Normalized luciferase units (fold)				
Construct	NF2C/34	NF2C/39	RIN^{+}	RIN^{-}	DRG (100 cells)
MINPPT	1	1	1	1	1
5′PPT	5.80 ± 0.31	6.90 ± 1.45	3.56 ± 1.25	5.15 ± 1.47	12.2
LPPT	2.24 ± 0.73	2.67 ± 1.12	0.48 ± 0.02	0.44 ± 0.03	2.74
LPPTmu	4.23 ± 1.11	4.75 ± 0.98	3.80 ± 0.76	5.64 ± 1.01	n.d.

The rat tachykinin promoter fragment spanning −865 to +92 has supported reporter gene expression in primary cultures of DRG but not in any cell line previously tested [4,22], with the exception of PC12 cells exposed to the synergistic action of forskolin and potassium-evoked depolarization [9]. In all three lines tested here, and at both temperatures in the NF2C model, this promoter fragment supported high levels of expression (Tables 1 and 2). The minimal promoter, −47 to +92, supported levels of expression significantly higher than backgrounds obtained from transfection of the pGL3b vector, suggesting that this element has the ability to support expression in these cells (Table 1). This is distinct from the effects of the minimal promoter in cell lines tested previously, in which the minimal promoter appears as no higher than background levels of reporter gene expression [4].

Although all cells supported expression directed by the rPPT promoter, the absolute level of reporter gene expression varied between the different cell types. NF2C that were transfected 14 days after being shifted to 39 °C exhibited a tenfold lower level of reporter gene expression than that seen with cells left at 34 °C. Similarly, the level of expression in the RIN^{+} and RIN^{-} cells differed by an order of magnitude irrespective of the reporter construct used. We believe this reflects a difference in transfection efficiency because the Promega promoter vector (pGL3p), in which luciferase expression is under the control of the SV40 promoter, was affected similarly, as seen in Table 1.

Presence of a repressor domain 3′ of the major transcriptional start site

To address the role of the previously hypothesized regulatory elements present 3′ of the major transcriptional start site [15], the LPPT reporter gene construct was used in all three cell lines. Expression directed by the three constructs, MINPPT (−47 to +92), 5′PPT (−865 to +92) and LPPT (−865 to +447), was compared. Although all the cells supported the expression of each of the constructs, addition of the region spanning +92 to +447 acted as a repressor of transcription in all cells tested (Table 2). This repression was most marked in both RIN cell derivatives in which reporter gene expression supported by LPPT construct was less than that of the MINPPT construct. In the neuronal cell line, although the longer fragment still supported significant reporter expression compared with that of the MINPPT construct, it was decreased by at least 50 % relative to the truncated construct, 5′PPT. This was surprising because it had previously been demonstrated by us and others that the addition of sequences from +92 to +500 acted as an enhancer in primary cultures of DRG in the context of a promoter fragment spanning −3500 to +500 [3]. We therefore addressed the ability of the LPPT construct to drive expression in primary cultures of rat DRG. All three PPT reporter constructs were microinjected and the levels of luciferase activity were measured 48 h after injection. The results are similar to those observed in the NF2C cell line, in that a 3′ repressor was present between +92 and +447, although the LPPT construct still supported reporter gene expression.

Mutation of nucleotides between +373 and +396 relieves the repression of LPPT

We have initiated an analysis of the function of previously published transcription factor binding sites [6] in the NF2C and RIN cell lines. One region in particular, between +373 and +396 of LPPT, which has previously been identified by DNAase I footprint analysis, is of interest in the current context [15]. The sequence of this region has been demonstrated to bind several proteins including Oct1 and nuclear factor κB. We made several mutations in this region that not only might affect transcription factor binding to this region but would also allow us, if required, to make further modifications by using the newly generated restriction enzyme sites. The resultant LPPTmu was used for transfection analyses in NF2C and RIN cells. In all cases, mutation of residues +373 to +378 and +391 to +396 led to increased reporter gene expression relative to the wild-type construct, the effect again being most marked in the two RIN cell lines (Table 2). In NF2C cells some measure of repression was still present, whereas in both RINs the repression was completely

abolished. The fact that LPPTmu and 5′PPT were able to support equivalent levels of expression shows that the addition of the 355 nt from +92 to +447 is not a factor that in itself decreases reporter gene expression. It is therefore apparent that the region between +373 and +396 contains residues responsible for the strong repression of the LPPT construct. We have not determined the specific repressor molecule because several complexes bind to this region.

DISCUSSION

We have generated clonal cell line models in which to study rPPT gene expression. The cell lines express the endogenous gene and support transient gene expression directed by rPPT promoter fragments in reporter gene constructs. The NF2C cell line in particular might be a useful model delineating regulatory mechanisms that modulate rPPT promoter activity in neurons. The RIN cell lines might represent the dysregulation of rPPT promoter activity seen in tumours that exhibit a neuroendocrine phenotype [23–26] or they might reflect some developmental regulatory mechanisms operating on the rPPT promoter [21]. It is likely that the NF2C cells will be of more general use for analysis of neuronal gene expression because they have been reported to up-regulate the expression of neuronal markers on differentiation at 39 °C. Markers observed are neuronal specific enolase, synaptophysin, mitogen-activated protein 2, tau and neurofilament (TCS product information). These cell lines will be an invaluable source for biochemical characterization of the *cis*-acting domains regulating rPPT promoter activity and the subsequent identification of the factors binding to these elements.

Although all cells supported expression directed by the rPPT promoter, the absolute level of reporter gene expression varied between the different cell types. NF2C that were transfected 14 days after being shifted to 39 °C exhibited a log lower level of reporter gene expression than that of cells left at 34 °C. Although not extensively analysed, this could be related to the morphological changes in the cells, reflecting the same resistance to transfection observed with primary cultures of neurons ([4,22]; J. P. Quinn, unpublished work) or alternatively being due to altered expression at the higher temperature. The RIN^+ and RIN^- cells also showed levels of expression that differed by an order of magnitude irrespective of the reporter construct used. We believe that this reflects a difference in transfection efficiency because the Promega promoter vector (pGL3p), which uses the SV40 promoter, was similarly affected.

Microinjection of rPPT reporter gene constructs into DRG has previously established the expression patterns elicited by various rPPT fragments between −3356 and +447 [3]. The current study has shown the presence of a previously unidentified repressor domain that is 3′ of the major transcriptional start site and is apparent with the NF2C and both RIN cell lines when transfected with the −865 to +447 LPPT construct. This repressor function was also observed when the same constructs were microinjected into primary cultures of adult rat DRG. It should be noted that this repressor only affects the relative levels of reporter gene expression because the LPPT construct (−865 to +447) still shows a 3-fold increase over the minimum PPT construct (−47 to +92) in DRG. The ability of this domain to repress reporter gene expression also varied between the rat DRG and the cell lines. Specifically this was highlighted in both of the RIN cell lines in which addition of the 3′ domain completely negated reporter gene expression. Use of the mutant LPPTmu construct demonstrated that a major determinant of this repression lies within a short region between +373 and +396 of the rPPT promoter. The repressor therefore seems to exert a much greater effect in the non-neuronal RIN^+ and RIN^- cells than in the neuronal NF2C cells and DRG, which show respectively only 61.4% and 77.5% repression of the LPPT construct relative to the 5′PPT (−865 to +92) construct (Table 2). This result suggests a tissue-specific determinant regulating this transcriptional mechanism. Despite these differences in levels of repression, the overall trends and patterns of expression in all the cells tested here are remarkably similar.

It was of some surprise that analysis of a reporter gene construct spanning −865 to +447 in these lines revealed a repressor domain that is 3′ of the major transcriptional start site. Previous microinjection of reporter gene constructs into primary cultures of adult rat DRG demonstrated that the largest promoter fragments, spanning −3356 to +447, supported 3-fold greater expression than fragments spanning −865 to +92 or −3356 to +92. We therefore hypothesize that element(s) spanning −3356 to −865 are able to overcome the effects of the 3′ repressor but elucidating this mechanism is not within the scope of the present study.

Preliminary EMSA results with wild-type and mutated oligonucleotides designed to encompass the region +367 to +399 have shown the presence of a number of complexes with the various cell extracts. The wild-type and mutated oligonucleotides yield slightly different band patterns; the banding patterns also vary slightly between the cell types. This rPPT region has previously been characterized as containing multiple complexes and includes Oct1- and nuclear factor κB-binding sites [15]. The relevance of the different complexes to the proposed repressor binding protein is the subject of a separate and continuing study.

The results presented here validate the use of these cell-line model systems for studying rPPT expression because of the coincidence of patterns of receptor gene expression between the cell lines and DRG. Cell cultures, like the primary cultures, only offer a model for addressing potential transcriptional control mechanisms. These will still have to be further validated *in vivo*. These current results further our previous observations and the hypothesis that the rPPT promoter is regulated by the synergistic action of multiple positive and negative regulators that are likely to be regulated in both a tissue-specific and a stimulus-inducible manner. Having two different cell models, each of which can provide information about potential interactions with the rPPT promoter, will be an invaluable resource for dissecting out different aspects of the complex tissue-specific expression of rPPT. Further biochemical characterization of these transcription factors in the cell-line models will complement the biological significance of these regulators *in vivo*.

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