NEW PERSPECTIVES ON ENDOCRINE SIGNALLING

Proopiomelanocortin gene expression and DNA methylation: implications for Cushing's syndrome and beyond

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Abstract

Proopiomelanocortin gene (*POMC*) is recognised as playing an important role in the regulation of the hypothalamo–pituitary–adrenal axis, adrenal development and obesity. *POMC* is activated in ACTH-dependent Cushing's syndrome. The syndrome may occur when the highly tissue-specific 5' promoter of human *POMC* is activated in pituitary and non-pituitary sites. Whilst the factors involved in transcription in the corticotrophs of the anterior pituitary gland are becoming well delineated, the mechanism of activation in non-pituitary sites is not fully understood. This promoter is embedded within a defined CpG island, and, in contrast to somatically expressed CpG island promoters reported to date, is methylated in normal non-expressing tissues, but is specifically unmethylated in expressing tissues, tumours and the *POMC*-expressing DMS-79 small-cell lung cancer cell line. Methylation

in vitro is sufficient for silencing of expression. In particular, methylation near the response element for the tissue-specific *POMC* activator PTX1, diminishes *POMC* expression. Sites outside the PTX1 response element may be important for binding, and this may have implications for pituitary development. DMS-79 cells lack *POMC*-demethylating activity, implying that the methylation and expression patterns are likely to be set early or prior to neoplastic transformation, and that targeted *de novo* methylation might be a potential therapeutic strategy. It is conceivable that in *POMC* neurons of the hypothalamus the *POMC* promoter is subject to a variable density of methylation with clear implications for the signalling of satiety and obesity.

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Introduction

In recent years, interest in the biology of proopiomelanocortin (*POMC*) and *POMC*-derived peptides has seen a renaissance. *POMC* is a pivotal player in areas as diverse as obesity, depression, skin pigmentation, adrenal development, and, naturally, in the regulation of the hypothalamo–pituitary–adrenal axis. From a clinical endocrinology perspective increased expression of *POMC* is seen in adrenocorticotrophin (ACTH)-dependent Cushing's syndrome. In humans this is most commonly caused by an ACTH-secreting corticotroph adenoma of the anterior pituitary gland, but, far less commonly, *POMC* is also activated in a non-pituitary site. Much work has revealed that the regulation of tissue-specific expression of *POMC* depends on the co-ordinated action of a variety of transcription factors. Recently, we have shown that expression is also governed by the DNA methylation pattern of the *POMC* promoter.

Human *POMC*

The story of *POMC* started two decades ago. *POMC* was initially cloned in 1981 (Takahashi *et al.* 1981) and is located on chromosome 2p23. The organisation of the gene and regulatory 5' region was identified in 1982 (Cochet *et al.* 1982, Whitfeld *et al.* 1982), and the complete sequence reported in 1983 (Takahashi *et al.* 1983). The gene consists of three exons, interspersed by large introns, the first of which is untranslated, and spans 7665 bp (Fig. 1).

Exon 1 consists of 87 bp, is untranslated and is thought to act as a leader sequence that binds to ribosomes to facilitate the onset of translation. Exon 2 consists of 152 bp and corresponds to the initiation sequence in part of the 5' untranslated mRNA and codes for a 26 amino acid signal sequence required for transport across the rough endoplasmic reticulum, and the first amino acids of the N-terminal region. Exon 3 is of 833 bp and codes for the

Figure 1 Schematic representation of the human *POMC* gene. Translated regions in colour: green, the 26 amino acid signal sequence; blue, remainder of *POMC*; red represents the region corresponding to ACTH.

majority of the translated mRNA and for the 3' untranslated region.

POMC mRNA transcripts

POMC contains at least three distinct promoters, which differ in their tissue distribution of expression. In man, in corticotrophs of the anterior pituitary gland and POMC neurons in the hypothalamus, initiation of transcription occurs from the 5' region upstream of exon 1. The primary transcript is spliced to give a mRNA of 1150 nt. This mRNA has one primary translation product from the coding 801 nt, forming prePOMC. In contrast, very low levels of a mRNA of approximately 800 nt in length may be expressed from a promoter within intron B, upstream of exon 3, in a wide tissue distribution. POMC peptides are found in many peripheral tissues such as testes (Chen *et al.* 1984, Pintar *et al.* 1984, Gizang-Ginsberg & Wolgemuth 1985, Autelitano *et al.* 1989), ovary and placenta (Chen

et al. 1986), lymphoid cells (Lolait *et al.* 1986), adrenal medulla (Evans *et al.* 1983), and other normal and tumour tissue (Lacaze-Masmonteil *et al.* 1987, DeBold *et al.* 1988a, de Keyzer *et al.* 1989). Since the peptide translation product of this shorter mRNA species lacks a signal peptide it is unlikely that the translated proteins are normally secreted (Clark *et al.* 1990), and their role in peripheral tissues is unclear. A third longer 5' extended mRNA of approximately 1350 nt is commonly found in non-pituitary tumours that aberrantly express *POMC* and secrete ACTH (de Keyzer *et al.* 1985, 1989, DeBold *et al.* 1988b, Clark *et al.* 1989). This leads to the same POMC peptide as the 1150 nt message since there is only one translation initiation site. These tumours are usually small-cell lung cancers and carcinoid tumours, frequently of bronchial origin (Newell-Price *et al.* 1998). Although the major transcript in the pituitary is 1150 nt in length, very small quantities of the 1350 nt transcripts are also present. This switch of promoter usage is poorly understood, and

Figure 2 5' promoter of *POMC*. Schematic representation (not to scale) of positively trans-acting factors and positions of binding. PO-B is involved in basal activity and is expressed in a wide tissue distribution; PTX1 is a homeobox transcription factor that acts as the pan-pituitary activator; TPIT is a T box factor that acts in concert with PTX1 to drive corticotroph-specific *POMC* expression; NUR77 is an orphan nuclear receptor that antagonises the negative feedback of glucocorticoids on *POMC* expression; NeuroD1 acts with PTX1 to drive corticotroph-specific expression of *POMC*; E2F factors bind the distal region of *POMC* in small-cell lung cancer DMS-79 cells that express *POMC*.

the regulation of the promoter leading to the 1150 nt has been the subject of intensive study, whilst less is known of the control of expression of the 1350 nt transcript. Moreover, the tissue-specific response elements and binding partners responsible for hypothalamic *POMC* expression have yet to be characterised.

POMC 'pituitary' promoter

The 5' 'pituitary' promoter has been most extensively studied for rat *POMC*, and the mechanisms of activation appear to be very similar in man. Synergy exists between multiple *cis*-acting elements of the promoter to allow maximum expression (Therrien & Drouin 1991). The majority of studies utilise the mouse corticotroph AtT20 cell line and transgenic mice as models for assessing *POMC* expression, although some studies have also used human pituitary cells obtained at surgery and maintained in culture (Kraus *et al.* 1993). Regrettably, a human corticotroph cell line does not exist. For both the rat and human gene, promoter deletion data have demonstrated that the promoter region responsible for pituitary-specific expression is in the central and distal regions (Fig. 2).

The central region independently confers expression at levels one-fifth of the whole promoter, whilst the distal region has no independent activity. However, the distal and central regions can act synergistically to give expression levels comparable with the whole promoter. The distal region was originally reported as binding a cell-specific helix-loop-helix factor to a specific E box motif termed DE2C (Therrien & Drouin 1993). The proteins

that bound this region in gel-shift experiments were termed CUTE proteins for corticotroph upstream transcription element-binding proteins. Further analysis revealed that part of this complex was the transcription factor NeuroD/1A (Poulin *et al.* 1997).

The homeobox protein Ptx1 (for pituitary homeobox 1), cloned in the mouse in 1996, binds to a response element in this region (CE3) (Lamonerie *et al.* 1996). It has been shown to act in concert with NeuroD/1A to allow expression of the rat promoter in AtT20 cells (Poulin *et al.* 1997). *Ptx1* is expressed at embryonic day (E) 8 in the mouse and is widely expressed in most cells of Rathke's pouch at an early stage of pituitary development. Corticotrophs are the earliest cell type to appear in the anterior pituitary during development and Ptx1 is, therefore, thought to play an important part in the development of the anterior pituitary in general, and the corticotroph cells in particular (Drouin *et al.* 1998). Ptx1 acts in synergy with other transcription factors in the other cell types of the anterior pituitary to co-ordinate cell type-specific expression (Tremblay *et al.* 1998). Interestingly, *Ptx1*-null mice exhibit an increase in murine *POMC* transcripts in the pituitary, suggesting redundancy of action and that other factors such as Ptx2 are able to bind to response elements, presumably the same elements as Ptx1, to activate transcription (Szeto *et al.* 1999).

More recently, the T box factor, Tpit, was identified as a corticotroph-specific factor, acting in synergy with Ptx1 to drive murine *POMC* expression in corticotrophs (Lamolet *et al.* 2001). Tpit is expressed (as assessed by immunohistochemistry) at E12.5, and the importance of this factor for murine *POMC* expression is underscored by

experiments in which ectopic Tpit expression in transgenic mice resulted in POMC expression in those cells. Moreover, germline mutations of *TPIT* are found in some cases of isolated ACTH deficiency in man, in support of an important role in pituitary POMC lineage (Lamolet *et al.* 2001). This is an important advance in our understanding of POMC expression and corticotroph development. Intriguingly, however, the pituitary promoter of human *POMC* lacks an exact TPIT response element, and this, coupled with lack of mutations in some patients with isolated ACTH deficiency, suggests that more work is required to fully establish the role of this factor in *POMC* expression. In contrast, the human gene contains an exact PTX1-binding site and a CANNTG motif that is the CUTE in the rat gene. These sites lie in approximately the same relative position within the human promoter.

5' POMC promoter in ectopic ACTH secretion

In terms of assessing the regulation of the 5' human promoter in non-pituitary cells the *POMC*-expressing small-cell lung cancer cell line, DMS-79, has been most intensively studied. The regulation of *POMC* in DMS-79 cells has been reported to differ from that in AtT20 cells. In DMS-79 cells the same -417 to +21 bp region that is required for expression in AtT20 cells is active, but by use of 5' deletion constructs no synergy was observed between CUTE-binding proteins and Ptx1. Furthermore the sequence -376 to -417 was shown to be active only in DMS-79 cells, this being designated domain IVA (Picon *et al.* 1995). This region has been shown to bind factors of the E2F family of trans-acting factors (Picon *et al.* 1999) that play a role in the control of cell proliferation and differentiation (Lam & La Thangue 1994). Binding to the retinoblastoma gene product, Rb, usually sequesters E2F proteins. Rb is, however, absent in DMS-79 cells and thus E2F factors are freely available to bind response elements. Interestingly, the rat *POMC* sequence differs from the human in this region with no E2F-binding site present. Therefore, it has been proposed that the regulation of the human gene in DMS-79 cells differs from that of the rat gene in AtT20 cells. This is not to say that additional elements within the promoter are not important as the domain IVA element confers only an additional 1.3-fold induction of expression compared with the promoter fragment -376 to +21 (Picon *et al.* 1995).

Ectopic hormone secretion

Many tumours are capable of synthesising and secreting peptide hormones giving rise to clinical phenotypes. A frequent example of this is the syndrome of inappropriate antidiuretic hormone secretion (SIADH), as seen particularly in bronchogenic carcinoma. SIADH is also frequent

in suppurative lung disease. These examples suggest that there exists a reasonably easily activated mechanism allowing synthesis and secretion of vasopressin from either inflamed or tumour tissue. In contrast, it is surprising how infrequently the ectopic ACTH syndrome is seen in clinical practice, even in the context of known neuroendocrine tumours capable of secreting peptide hormones. Moreover, it is not seen in the context of inflamed non-tumour tissue. One interpretation of this is that the expression of *POMC* is tightly inhibited, whilst that of vasopressin is less so. A powerful means of silencing gene expression in a permanent fashion in normal tissues is DNA methylation, and this prompted an investigation as to whether this was playing a role in preventing *POMC* expression in non-pituitary sites.

DNA methylation

DNA methylation at CpG dinucleotides of gene promoters is associated with silencing of gene expression (Bird 1992). Patterns of methylation are set during development, in which processes of general demethylation are followed by generalised *de novo* methylation. This is subsequently followed by demethylation of specific genes in a temporal and spacial pattern. Methylated DNA is associated with transcriptionally incompetent condensed heterochromatin, whilst unmethylated DNA is associated with open transcriptionally competent chromatin. The density of methylation is important since a weak promoter will be silenced by only a few methylated CpGs whilst a higher density of methylation is required to repress a strong promoter (Boyce & Bird 1992). Although distant methylated sequences can contribute to repression (Hug *et al.* 1996), the repression is greater if the promoter itself is methylated (Nan *et al.* 1997).

The silencing mechanism is mediated by inhibition of binding of some transcription factors when their response elements contain CpG sites, and, more importantly, by the recruitment of methyl-binding proteins and histone deacetylases and methylases (Newell-Price *et al.* 2000). The methylation pattern is set *in utero* and maintained in the adult with extreme fidelity, although patterns may be changed prior to, or as a consequence of, oncogenic transformation.

CpG islands

CpG islands are dense clusters of CpGs usually extending over 1 kb and are frequently located in gene promoters and first exons (Bird 1992). They have been defined as being present where the G+C content is >60% and where the observed to expected ratio of CpG is more than 0.6 (Gardiner-Garden & Frommer 1987). In contrast to methylated sequences, CpG islands are associated with an open active chromatin formation. They have probably arisen

Figure 3 CpG islands in human *POMC*. Upper panel indicates observed to expected ratio of CpG, middle panel G+C content. CpG islands are defined where the G+C content is >60% and where the observed to expected ratio of CpG is more than 0.6 (horizontal lines in each panel respectively). Lower panel indicates position (to scale) of the exons of *POMC*. Note exons 1 and 3 and their promoters lie within CpG islands.

during evolution by depletion of methylated cytosines from the remainder of the genome due to the high level of mutability of 5-MeC, whilst unmethylated cytosines, resistant to this change, remain clustered as an island. House-keeping and many tissue-specific genes have CpG islands at their 5' end (Bird 1992), and it has been suggested that up to 60% of all gene promoters are embedded within CpG islands. The open chromatin formation of these regions fits with the necessity of these regions being capable of active gene transcription.

In contrast to the vast majority of CpGs in the genome which are methylated, CpG islands in somatic tissues are usually thought to be unmethylated in all tissues (Bird 1992). The exceptions to this include the inactive X chromosome in which the vast majority of CpG islands are methylated (Riggs & Pfeifer 1992), and genetic imprinting of genes such as seen for *H19* (Ferguson-Smith *et al.* 1993) and *Igf2r* (Stoger *et al.* 1993). In these circumstances CpG islands are densely methylated and exist in a condensed inactive heterochromatin configuration. As such this provides a powerful means to limit transcription from one allele even though the transcription factors needed for expression are present within the cell, since the other allele is expressed. To date variable methylation of somatically expressed tissue-specific CpG island-containing promoters has not been demonstrated. There is, therefore, a paradox since the normal somatic tissue-specificity of gene expression cannot be explained from these data at the level of

DNA methylation. Thus if CpG islands are free of such methylation, tissue-specific transcription from a CpG island promoter is likely to be directed solely by expression of the necessary tissue-specific transcription factors (Macleod *et al.* 1994).

However, differential methylation of germline CpG islands has been demonstrated. The human *MAGE* family of genes have CpG island promoters that are unmethylated in the germline but methylated in somatic tissues, and using transfection studies methylation of the promoter sequences resulted in inhibition of expression (De Smet *et al.* 1999). Similarly human *LDH-C* and rat *h2b* histone genes (Choi & Chae 1991, Choi *et al.* 1996), are unmethylated in testes but methylated in all other tissues. Thus methylation appears to be a powerful mechanism to maintain testes-specific genes in the inactive state in somatic cells.

POMC and DNA methylation

Human *POMC* contains two defined CpG islands (Gardiner-Garden & Frommer 1994) (Fig. 3). The *POMC* 3' promoter lies in the downstream CpG island and is weakly active in many tissues, whilst expression from the 5' CpG island promoter is highly tissue-restricted. Moreover, Southern analysis revealed that a single HpaII site at -300 bp in the 5' promoter is variably methylated in expressing and non-expressing tissues

Table 1 Tumours and tissues with methylated and unmethylated *POMC****POMC* unmethylated**

Tumours causing ACTH-dependent Cushing's syndrome
 Lung carcinoid tumours
 Pancreatic carcinoid tumour
 Corticotroph adenomas
 Cell lines expressing *POMC*
 DMS-79

***POMC* methylated**

Normal tissues
 Lung
 Kidney
 Spleen
 Leukocytes
 Pancreas
 Testes
 Cell lines not expressing *POMC*
 HeLa cells
 Neuroendocrine tumours not expressing *POMC*
 Non-functioning pituitary adenoma
 Pancreatic carcinoid (no Cushing's syndrome)
 Insulinoma

(Lavender *et al.* 1991). These data suggested that variable methylation may be a feature of this tissue-specific CpG island, and that this might have important implications for gene expression. Furthermore, the pattern of methylation in non-expressing normal tissues and non-expressing tumours may be different from those tumours actively expressing *POMC* and causing the ectopic ACTH syndrome.

To address these issues we used bisulphite sequencing to study the pattern of methylation across the entire 5' CpG island of *POMC* in human tissues and cell lines (Newell-Price *et al.* 2001). As expected, in ACTH-secreting tumours that were associated with Cushing's syndrome and the *POMC*-expressing DMS-79 cell line, these were fully unmethylated in this region. In contrast, in non-ACTH-secreting tumours, even of neuroendocrine origin, this region was methylated. Furthermore, in a range of normal tissues, including pancreas, spleen, lung, testes, and peripheral blood leukocytes, this region was methylated. This is the first report of a somatically expressed gene with a methylated CpG island in normal non-expressing tissue (Table 1).

Since the E2F-binding region of the promoter has been documented as being important for non-pituitary expression of *POMC*, and the fact that methylation of E2F response elements prevents binding (Di Fiore *et al.* 1999), we assessed the methylation status of this region in the promoter. The E2F response element was unmethylated in the DMS-79 cell line and in corticotroph tumours, whilst it was fully methylated in normal tissue and non-ACTH-secreting tumours. These data suggest that, even though E2F may drive expression of *POMC* (Picon *et al.* 1999),

the switch for expression is determined at the level of promoter methylation. To assess whether these patterns were specific to this region or simply a global change associated with some tumours, we assessed a region 6 kb 3' to the pituitary promoter and found, in all tissues, that this region was methylated whether or not these were ACTH-secreting. These data strongly suggest that the changes in the 5' promoter region are specific to *POMC* expression, rather than a random change in some tumours.

To test directly the effect of methylation on expression we used luciferase reporter constructs transfected into DMS-79 cells. Using both generalised and site-specific methylases prior to transfection to introduce a variable number of methylated CpG sites, we demonstrated that expression was inhibited by a high level of methylation, but that the introduction even of a few methylated CpG sites caused a substantial fall in the level of expression. This is consistent with existing data on the effect of methylated CpG density (Boyes & Bird 1992). Interestingly, the introduction of a single CpG moiety near the PTX1-binding region substantially reduced expression. This prompted us to examine the role of the PTX1-binding site in more detail. Using site-directed mutagenesis of this site in luciferase reporter constructs we demonstrated that this region was important for expression in DMS-79 cells. The PTX1-binding site itself, however, does not contain a CpG site, but is flanked by such sites. We therefore assessed whether the methylation of these flanking regions could influence binding by using gel-shift analysis and PTX1 protein made *in vitro*. These experiments revealed that methylation on the antisense strand of the flanking region completely abolished binding of PTX1. This has potential implications for pituitary development, since, for PTX1 to act as a driver for pituitary development it would clearly need to be able to bind DNA. If the DNA is methylated prior to the formation of Rathke's pouch, it seems unlikely that PTX1 would be able to have this effect, and suggests that the region on which PTX1 is acting may be demethylated at an earlier stage. This could, perhaps, be co-ordinated by an alternative factor determining the pituitary fate of this tissue at an earlier stage.

Finally, since the 5' *POMC* promoter appeared to be methylated in normal lung tissue we sought to assess whether DMS-79 cells were capable of actively demethylating this region. Using a combination of transfected luciferase reporter constructs and bisulphite sequencing we were unable to show evidence for such an effect. This suggests that either these cells have differentiated and lost the capacity to demethylate this region, or that the ectopic ACTH syndrome arises from a subset of cells that always possessed an unmethylated promoter.

Implications for *POMC* biology

Cushing's syndrome in general, and the ectopic ACTH syndrome in particular, are rare. These data do, however,

suggest that methylation patterns play an important role in *POMC* expression. *POMC* appears to be unique in having a methylated CpG island in normal tissue. Moreover, the experiments we have performed suggest that the overall level of expression can be titrated depending upon the density of methylated CpG sites in the promoter. This, coupled with the fact that demethylation of a methylated *POMC* promoter did not appear to occur in DMS-79 cells, suggests that targeted *de novo* methylation, with propagation of the inhibitory pattern in subsequent cell generations, could be a novel therapeutic strategy. This might be especially true of Cushing's disease, where no tumour is visible on imaging 50% of the time (Newell-Price *et al.* 1998), and the long-term cure following trans-sphenoidal surgery is only of the order of 50–60% (Newell-Price 2002). In this circumstance control of *POMC* expression, rather than attempts at tumour control, would lead to a favourable therapeutic outcome.

Increasing evidence points to *POMC* playing an important role in obesity (Pritchard *et al.* 2002). Leptin increases the expression of *POMC* in *POMC* neurons, and following processing cleaved *POMC* peptides, in particular α -melanocyte-stimulating hormone (α -MSH), are able to bind the melanocortin-4 receptor in the arcuate nucleus, signalling satiety. The factors governing expression of *POMC* in these neurons are not characterised, but it is possible to speculate that the 5' *POMC* promoter region is subjected to differential methylation. If this were the case, the density of methylation may determine the capacity for expression and ultimately the level of cleaved α -MSH released. One might hypothesise that some obese individuals may have a fixed higher level of methylation in this region compared with lean individuals, and hence a lower capacity for expression, and fixed diminished signalling of satiety. Such 'hard wiring' of appetite control fits well with clinical experience of obese patients.

In summary, the tissue-specific regulation and overall level of expression of *POMC* is strongly influenced by the methylation pattern of the 5' promoter. Although these are studies on rare endocrine conditions much beloved of clinical endocrinologists, they may have far wider implications – potentially for our current modern epidemic of obesity.

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