DNA elements with AT-rich core sequences direct pituitary cell-specific expression of the pro-opiomelanocortin gene in transgenic mice

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Corticotrophs are the first fully differentiated cells to appear in the anterior pituitary during organogenesis and are distinguished by pro-opiomelanocortin (POMC) gene expression. Earlier studies in our laboratory defined three DNA regions (sites 1, 2 and 3) within promoter sequences at the 5'-end of the rat POMC gene (–323/–34) that cooperatively targeted cell-specific gene expression to corticotrophs and melanotrophs in transgenic mice. In this study we analysed the DNA–nuclear protein interactions underlying this functional activity. We demonstrated that the transcriptional activator SP1 interacts with GC-rich regions in sites 1 (–146/–136) and 2 (–201/–192) and an unidentified protein, which we call PP1 (putative pituitary POMC1), interacts with AT-rich regions in sites 2 (–202/–193) and 3 (–262/–253). The PP1-binding activity appears to be

specific to cells that express the POMC gene because it was detected in nuclear extracts prepared from AtT20 corticotroph cells and mouse melanotroph tumours but not from GH₄ pituitary tumour cells, HeLa cells or liver. Site-directed mutagenesis of core binding sequences demonstrated that PP1 is required for the correct cell-specific expression of the POMC gene in the pituitary gland of transgenic mice and SP1 appears to support such an expression. The best core binding sequence for PP1 is TAAT, a possible transcription factor homeodomain contact site. However, PP1 is distinct from Brn 3.0, a POU protein that also binds to site 3. We conclude that PP1 is a transcriptional activator for pituitary-specific POMC gene expression.

INTRODUCTION

Eukaryotic cells differentiate by activating or repressing particular genes. Therefore, the unique phenotype of each cell is controlled primarily at the transcriptional level. In the anterior pituitary gland, there are five major cell phenotypes that differentiate from progenitor cells of Rathke's pouch [1]. Each type is characterized by the expression of distinct hormone genes in a unique pattern of spatial and temporal compartmentalization [2]. Studies on cell-specific expression of the growth hormone (GH) and prolactin (PRL) genes led to the cloning of transcriptional activator Pit-1/GHF-1 [3,4], which in addition defines the precise developmental appearance of three pituitary cell types, somatotrophs, lactotrophs and thyrotrophs [5,6]. Corticotrophs expressing the pro-opiomelanocortin (POMC) gene are the first cell type to fully differentiate and their differentiation is independent of extra-pituitary influences [7]. Defining the mechanism controlling POMC gene expression will therefore be important for the understanding of early events in pituitary organogenesis.

We defined previously a 290 bp 5'-end DNA sequence from –323 to –34 of the rat POMC (rPOMC) gene that targeted pituitary-cell-specific and early developmental expression of transgenes to anterior lobe corticotrophs and intermediate lobe melanotrophs [8]. Within this DNA fragment three identified regions [site 1 (–148/–114), site 2 (–218/–190) and site 3 (–265/–249)] interacted with nuclear proteins from AtT20 cells, a mouse corticotroph cell line which expresses the POMC gene. Transgenes containing either sites 2 or 3 in combination with site 1 showed correct cell-specific expression in transgenic

pituitaries, demonstrating that multiple DNA elements were required for detectable expression [8]. In this study, we characterized further these DNA elements and their cognate nuclear protein factors, and also examined their functional relevance to the cell-specific expression of the POMC gene in transgenic mice. Here we report that transcriptional factor SP1 binds to GC-rich regions in sites 1 and 2 and a chemically unidentified protein factor which we term PP1 (putative pituitary POMC1) binds to AT-rich regions in sites 2 and 3. Transgenic studies demonstrated that binding of PP1 to the AT-rich regions was required for cell-specific POMC gene expression in the pituitary gland while SP1 played a less important role. PP1 protein was detected only in POMC gene-expressing cells or tissue in gel-shift assays, and DNA mutagenesis experiments showed that PP1 had the best core binding site of TAAT, a transcriptional factor homeodomain contact sequence [9,10]. We demonstrated that PP1 is not Brn-3.0, a POU protein present in AtT20 cells that also binds to POMC site 3 [11,12]. We therefore concluded that the sequences with an AT-rich core located in site 2 and site 3 had cell-specific enhancer activities, and their cognate binding protein, PP1, is required for cell-specific POMC gene expression in pituitary gland.

EXPERIMENTAL

Protein–DNA interaction assays

Nuclear extracts were prepared from cells or tissues as described previously [8]. Footprint assays and the making of probes were

Abbreviations used: ANOVA, analysis of variance; CRH, corticotropin releasing hormone; GH, growth hormone; hSP1, human SP1; PRL, prolactin; POMC, pro-opiomelanocortin; PP1, putative pituitary POMC 1; rPOMC, rat POMC; SV40, simian virus 40.

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described earlier [8]. Gel-shift assays were performed as before [8] but all the oligonucleotides corresponding to rPOMC promoter sequences used in these assays were first cloned into the *EcoRV* site of plasmid Bluescript (BS/SK; Strategene, La Jolla, CA, U.S.A.) and released with *EcoRI*/*HindIII*, then end-labelled with ^{32}P by T4 DNA kinase.

Methylation interference assays were performed as follows: oligonucleotide site 1 (−160/−124) and site 2 (−222/−190) were cloned into the vector BS/SK at the *EcoRV* site. Probes were made by fill-in at one end (*EcoRI* for down strand and *HindIII* for up strand) and partially methylated by DMSO (Sigma, St. Louis, MO, U.S.A.). Approx. 10^5 c.p.m. of probe was incubated with 10 μg of AtT20 nuclear extracts and 10 μg of poly[d(I-C)] in 100 μl of gel-shift assay buffer. Bound and free probe fractions were separated on 4% PAGE gels and electro-eluted. Clean, dried DNA pellets were resuspended in 20 μl of freshly diluted 1 M piperidine (Sigma) and heated at 95 °C for 30 min. Reaction mixtures were dried in a speed-vac and resuspended in 100 μl of distilled water. Equal amounts of DNA (c.p.m.) from both bound and free fractions were separated on a 10% polyacrylamide/urea sequencing gel (19:1) and autoradiographed. A cloned rPOMC promoter DNA (−234/−133) was also used in this assay and identical DNA bases were found to interact with the PP1 protein (results not shown).

DNA mutagenesis and sequencing

SP1 binding sites were mutated by PCR with wild-type rPOMC promoter DNA. Two pairs of oligonucleotides with mutated SP1 sites were used: pair 1 to amplify promoter DNA fragment (−480/−200): 5′-GAAACAGAGATCTTG-3′, and 5′-TACGAGCTCTATTAGCACAGACCCGCTG-3′; and pair 2 to amplify fragment (−191/−129): 5′-TAGAGCTCGTAGCAC-TTTCAGGCACAT-3′, and 5′-GGCCGCGGTGATCAATT-GTCCCGGTCGGGGCT-3′. After restriction enzyme digestion by *BglII*/*SacI* and *SacI*/*SacII* to produce compatible ends, they were subcloned into plasmid 770rPOMC-PGEM7Z [8]. Then a fragment between *StuI*/*PstI* was excised and recloned into *EcoRV*/*PstI* of BS/SK to generate plasmid rPOMC290mSP1-BS. To mutate the PP1 binding site in site 2, a restriction fragment *SacI*/*BamHI* from rPOMC290mSP1-BS and an oligonucleotide DNA with rPOMC promoter sequences from −234 to −199, but carrying the PP1 mutation, were ligated into BS/SK (plasmid rPOMC200mSP1mPP1-BS). These mutations were confirmed by sequencing with a DNA sequencing kit (USB, Cleveland, OH, U.S.A.).

Transgene construction and transgenic mice production

Procedures to construct transgenes were as described previously [8]. The luciferase cDNA was obtained from plasmid pSV232AL-A5′ [13] and promoter DNAs were from plasmid 770rPOMC-7Z [8], rPOMC290mSP1-BS and rPOMC200mSP1mPP1-BS. Transgenic mice were produced with each transgene as described previously [8] using either B6D2F₁ or FVB inbred mice as oocyte donors. All experiments with mice were performed in accordance with the Guide for the Use and Care of Laboratory Animals under approved institutional protocols.

Luciferase assays and immunohistochemistry

Total pituitary glands or separated anterior and intermediate lobes were homogenized by sonication in 100 mM K₃PO₄ buffer, pH 7.4 (containing 0.1% Nonidet P-40) at 4 °C. Enzymic activity in transgenic pituitary extracts was measured according to the

manufacturer's protocol (Promega, Madison, WI, U.S.A.) with a luminometer (United Technologies Packard, Downers Grove, IL, U.S.A.). Cell specificity of transgene expression was examined by simultaneous dual immunofluorescence histochemistry with a luciferase antibody (Cortex Biochem, San Leandro, CA, U.S.A.) and an anti-(adrenocorticotrophic hormone) antibody on pituitary sections as described earlier [8].

Statistical analysis

The data for luciferase expression in transgenic mouse pituitary glands were analysed by a non-parametric analysis of variance (ANOVA) due to the violation of assumptions of normality and equal variance. The Kruskal–Wallis one-way ANOVA was chosen because the underlying populations are continuous and have the same shape. *Post-hoc* comparisons between groups for significance were performed by the Mann–Whitney U test.

RESULTS

In a previous report we demonstrated that three regions within a 290 bp rPOMC promoter DNA footprinted by AtT20 nuclear extracts were involved in the cell-specific expression of the POMC gene in transgenic mouse pituitaries [8]. The identity of the AtT20 transcriptional factors interacting with the POMC gene remained unknown, although oligonucleotide competition for DNA–protein interaction in gel-shift and Southwestern assays suggested the involvement of an SP1-like protein [8]. Further analysis demonstrated that the transcriptional activator SP1 binds to site 1, because in gel-shift assays the major shifted band (band a) showed an identical mobility to purified human SP1 (hSP1) (Figure 1a), and in methylation interference assays band a showed an identical protein–DNA interaction pattern to that of hSP1 (Figure 1b). In addition, band a was supershifted by anti-hSP1 antibody, gradually abolished by increased concentrations of EDTA in the reaction buffer, and recovered by addition of Zn²⁺ in gel-shift assays (results not shown). The chemical nature of the other two minor shifted bands (b and c) remains uncertain, but the available data indicate that they are SP1-related proteins, because band b showed the same protein–DNA interaction pattern as band a. Band c may represent a degraded SP1 which we consistently detected as a 37 kDa SP1 immunoreactive protein by Western blot assays of AtT20 nuclear extracts (results not shown). We also found that SP1 bound to footprint site 2, as shown in gel-shift assays (Figure 1c) and in methylation interference assays (Figure 1d). The consensus sequence for SP1 sites in the rPOMC promoter is CCN(G/C)CCTCC. Under the same *in vitro* conditions, SP1 seems to bind to site 1 with higher affinity.

The major factor interacting with site 2 was detected in AtT20 cell nuclear extracts as a pair of bands in gel-shift assays and we term it PP1 (Figure 1c). DNA bases directly contacting PP1 protein were determined by methylation interference assay (Figure 1d). Each band in the doublet produced an identical interference pattern (results not shown). The PP1 binding site is immediately adjacent to, but does not overlap, the SP1 site. Two minor complexes migrating ahead of the lower PP1 were not detected consistently and therefore may represent some non-specific binding of the nuclear extract to the probe (Figure 1c).

Therefore, the major proteins causing footprint sites 1 and 2 are SP1 and PP1. This conclusion was confirmed by an additional experiment shown in Figure 2. We used a cloned DNA fragment (−234/−133) encompassing both sites 1 and 2 as a probe to perform gel-shift assays. Major shifted bands representing the binding of SP1 and PP1 are indicated. Competitive binding by

Figure 4 Interactions of purified Brn 3.0 protein and AtT20 nuclear extracts with the oligonucleotides CRHwt, POMC site 2 and site 3 in a gel-shift assay

CRHwt is a natural Brn 3.0 protein binding site in the CRH gene promoter [11]. 1 μ l of Brn 3.0 and 2 μ g of AtT20 nuclear extracts were used in this assay. Free probes (F) and bound fractions are marked (both the CRHwt oligonucleotide and purified Brn 3.0 were gifts from Dr. M. G. Rosenfeld). From left to right in this gel-shift assay: lane 1, CRH wild-type probe and purified Brn 3.0 protein; lane 2, POMC site 2 oligonucleotide probe and purified Brn 3.0; lane 3, POMC site 3 oligonucleotide and purified Brn 3.0 protein; lane 4, CRH wild-type probe and AtT20 cell nuclear extract; lane 5, POMC site 2 oligonucleotide probe and AtT20 cell nuclear extract; lane 6, POMC site 3 oligonucleotide and AtT20 cell nuclear extract.

performed a series of gel-shift assays using several specifically mutated oligonucleotides as competitors of PP1 binding to the mSP1 probe. Mutation of the trinucleotide sequence TAA to GCA in the PP1 binding sequences of site 2 (mPP1-2) resulted in a marked loss of competition compared with mutations in the adjacent SP1-binding nucleotides (Figure 3a). Similarly, mutation of the dinucleotide TA to GC in the context of the site 3 oligonucleotide (AT-M3) largely blocked competition for binding to the probe (Figure 3b). Taken together these data indicate that the final four nucleotides TAA(C/G) are essential for PP1 binding.

Next we examined whether PP1 is the cloned POU protein Brn 3.0 which has been reported to bind to POMC site 3 and transactivate a reporter gene containing multimers of the Brn 3.0 binding site in heterologous cells [11,14]. We found that purified Brn 3.0 protein bound to a corticotropin releasing hormone (CRH) oligonucleotide, which contains a well-defined Brn 3.0 binding site [11], and to a POMC site 3 oligonucleotide, but not to a POMC site 2 oligonucleotide probe. Both the POMC site 3 and site 2 oligonucleotides contain a PP1 binding site in gel-shift assays (Figure 4). In addition, we detected presumptive Brn 3.0 protein in our AtT20 nuclear extracts with similar mobility to the purified one complexed to the CRH probe and the POMC site 3 probe (Figure 4). In this experiment PP1 protein clearly bound to sites 2 and 3 as double bands with similar affinities, migrating slower than the Brn 3.0 complex. Therefore, we concluded that PP1 is not Brn 3.0.

The chemical nature of PP1 remains unknown but it is possibly another homeodomain protein since the TAAT sequences generated in the mutation for the mSP1 probe showed the highest binding affinity to PP1 (compare Figure 4, lanes 6–8 with lanes 1–3 in Figure 2). This tetranucleotide actually matches the native sequence present in the human POMC gene promoter at the homologous position (Figure 5). TAAT is generally believed

Figure 5 Alignment of POMC promoter sequences from rat [24], mouse [25], human [26] and bovine [27]

Hyphens indicate identical nucleotides to rat and dots represent gaps introduced to achieve the best alignment. PP1, SP1 and Brn 3.0 binding sites are boxed.

Figure 6 Gel-shift assay with mSP1 probe and nuclear protein extracts (2 and 4 μ g) from different sources

The tumour extract was obtained from a POMC-expressing melanotroph tumour induced in a transgenic mouse [28]. 2621-2 is a SV40 Tag-transformed pituitary cell line that ceased to express the POMC gene (M. J. Low, unpublished work). Nuclear extracts from GH₄, HeLa cells and mouse liver tissue were tested in footprinting and gel-shift assays as a control for integrity of nuclear proteins and contained SP1 binding activity (results not shown).

to be the sequence directly contacted by homeodomain proteins [9,10]. Notably, PP1 binding sites at these two locations are among the best-conserved POMC promoter regions in different mammalian species (Figure 5).

Gel-shift data showed that the PP1 protein was present only in POMC-expressing cells or tissues (Figure 6). Interestingly, PP1 binding activity was present at lower levels in a melanotroph tumour with depressed POMC gene expression, but was totally absent from a melanotroph tumour-derived cell line that had silenced POMC gene expression. In contrast, SP1 is an ubiquitously present protein and Brn 3.0 was also detected in different tissues and cell lines in addition to AtT20 cells [11].

Therefore, the functional relevance of SP1, PP1 and Brn 3.0 to pituitary-cell-specific expression of the POMC gene was studied further in transgenic mice. The degree of transgene expression was calculated in two ways. Penetration refers to the percentage of independent founder mice with $> 10^4$ light units per pituitary. This level was clearly above background counts of about 10^3 . Quantitative comparisons were made among the values of light

Figure 7 Functional relevance of SP1 and PP1 to transgene expression

(a) Level of transgene expression is represented as total light units per pituitary measured in 30 s in a luminometer. Machine background level was very consistent at about 800 light units. No luciferase activity was detected in pituitary samples from normal mice. Pituitaries were extracted fresh and luciferase activity measured in duplicate. One line of transgenic mice (BC-Luc no.9) was bred for several generations and was used as an internal control in each assay to eliminate the inter-assay variations. Each asterisk in (a) represents an independent adult transgenic founder mouse. Luciferase activity was detected in both anterior and intermediate lobes. Brn 3.0 binding location was determined by Dr. M. G. Rosenfeld [2]. [†] $P = 0.013$ or 0.017 compared with transgenes nos. 2 and 3 respectively, Mann-Whitney U test. ^{††} $P < 0.001$ compared with transgene no. 1, Kruskal-Wallis one-way ANOVA. (b) rPOMC DNA probes (–234/–133) and the probe (mSP1) carrying SP1 mutations at both sites 1 and 2 equivalent to transgene no. 3 were incubated with 1 unit of purified hSP1 protein and fractionated in a gel-shift assay. The shifted band was competed for by unlabelled oligonucleotide sites 1, 2 and SP1 (results not shown). (c) mSP1 probe and a probe (mSP1 + mPP1) carrying the SP1/PP1 double mutations equivalent to transgene no. 4 were incubated with 2 μ g of AtT20 nuclear extract in a gel-shift assay. The shifted band (PP1) was competed for by site 2 oligonucleotide (results not shown). SP1 sites are underlined, the PP1 site is boxed, and mutated nucleotides are shown in bold type.

units/pituitary expressed as $\log_{10}$ of the raw data. A Kruskal-Wallis one-way ANOVA showed highly significant inter-group differences with $P < 0.001$. As we observed previously with simian virus 40 (SV40) K1Tag reporter transgenes [8], luciferase was most highly expressed in transgenic pituitaries with intact sites 1, 2 and 3, and remained highly expressed after a truncated deletion of site 3 (Figure 7a, compare transgenes nos. 1 and 2). Site-directed mutagenesis of the SP1 core binding sites dramatically reduced SP1 binding to the promoter *in vitro* (Figure 7b), but showed no statistically significant reductions of the promoter activity *in vivo* (compare transgene no. 3 to no. 2). Additional mutation of the remaining PP1 binding site abolished *in vitro* PP1 binding to the promoter DNA, which already included the SP1 mutations (Figure 7c). The transgene with SP1/PP1 double mutations (Figure 7a, transgene no. 4) had a significantly lower expression of luciferase and a marked drop in penetrance compared with transgene no. 3 or no. 2. Transgene no. 5, containing a truncated deletion of sites 2 and 3, showed a similar expression pattern to transgene no. 4, even though it still contained a strong SP1 binding site. Expression specificities were examined in several selected transgenic lines by immuno-histochemical staining with an anti-luciferase antibody (Cortex Biochem, San Leandro, CA, U.S.A.) and as described in our earlier report [8] with a rPOMC-SV40 K1Tag transgene, the luciferase protein was co-localized specifically to POMC-expressing cells in both lobes of the pituitary gland (results not shown).

DISCUSSION

Within the minimal pituitary-cell-specific rPOMC promoter DNA (–323/–34) we identified *cis*-DNA elements that interact

with three different types of protein factors. GC-rich regions in sites 1 and 2 interact with the ubiquitous transcriptional factor SP1 and sequences with an AT-rich core in sites 2 and 3 interact with a protein (PP1) that was detected only in POMC-expressing cells or tissue by gel-shift assays. Additionally, POU protein Brn 3.0 binds to site 3 adjacent to the distal PP1 binding site and is present in AtT20 cells.

The functional relevance of these protein factors to pituitary-cell-specific expression of the POMC gene was evaluated in transgenic mice. We were able to co-localize the luciferase protein to the POMC-expressing cells with a luciferase-specific antibody in F₁ mice having the highest levels of enzymic activities in their pituitaries, although the SV40 Tag gene appeared to be more suitable for histochemical studies [8]. However, luciferase appears to be a better choice as a reporter for this study because enzymic activity of this protein in tissues from transgenic mice was measured quantitatively with great sensitivity in a luminometer. Three previously analysed rPOMC promoter DNA sequences directed luciferase gene expression to the pituitary in a similar pattern to the three transgenes using SV40 Tag as a reporter [8], indicating that the reporter sequences themselves do not contribute to the specificity of transgene expression. With the 323 bp rPOMC promoter we detected luciferase transgene expression above basal levels ($> 10^4$ light units) in 80 % of independently derived transgenic mice pituitaries (transgene no. 1) but in only 50 % using the SV40 Tag gene as a reporter [8]. Truncated deletion of site 3 (transgene no. 2) minimally reduced the promoter activity (penetrance: 64 % 10^4 light units); however, further deletion of site 2 (transgene no. 5) virtually abolished pituitary expression (penetrance: 7 % 10^4 light units). The significance of two newly analysed transgenes (transgenes nos. 3 and 4) is to link directly the identified protein factors to pituitary-

cell-specific expression of the POMC gene in transgenic mice. Mutagenesis of the core binding sequences of SP1 in sites 1 and 2 (CCTCC) dramatically reduced SP1, but increased PP1 binding to the promoter *in vitro*; however, this caused no significant reduction in the promoter activity *in vivo* (transgene no. 3, penetrance: 44% 10^4 light units). It is not known if increased PP1 binding to the promoter could compensate for the loss of SP1. However, an additional mutation that abolished PP1 binding to the promoter *in vitro* clearly reduced the promoter activity by two logarithmic orders of magnitude from transgene no. 1 (transgene no. 4, penetrance: 11% 10^4 light units). This result demonstrated that PP1 plays a critical role for the correct pituitary-specific gene expression. The two rare high-level expressors of transgene no. 4 and no. 5 are probably due to the transgenic position effect [15], since values from both mice were hundreds to thousands of times higher than the median in these groups. Therefore, abolishing PP1 binding to the promoter by combined deletion and mutation dramatically reduced the promoter activity as measured both by transgene penetrance (80% to 11%) and median luciferase expression (100-fold reduction). SP1 binding alone clearly was insufficient to activate transgene expression (transgene no. 5). Considering its tissue-specific distribution by gel-shift assay we concluded that PP1 is the cell-specific transcriptional activator required for POMC gene expression in the pituitary, and SP1 and Brn 3.0 are likely to support such expression.

The conservation of PP1 binding sites in promoter regions of the POMC gene in different species suggest that this protein may be a common pituitary activator for the POMC gene in all species. It has been demonstrated repeatedly that a transcriptional factor (e.g. Pit-1/GHF-1) can bind multiple times to its cognate promoter (e.g. GH and PRL genes) [3,4]. PP1 protein binds twice to the 323 bp rPOMC DNA promoter that activates transgene expression indistinguishably from transgenes with much longer promoter sequences [8,16,17]. These observations suggest that PP1 is probably the essential activator protein required for cell-specific expression of the POMC gene in the pituitary gland. However, these data do not exclude the involvement of other protein factors. The complexity of cell-specific gene expression has been demonstrated for the well-studied GH gene, which requires more protein factors in addition to the cell-specific transactivator Pit-1 [18–20].

By cell transfection assays other laboratories have demonstrated that additional proteins were involved in POMC gene expression in AtT20 cells. Within the context of the 323 bp rPOMC promoter there are binding sites for another DNA-binding protein PO-B [21,22]. PO-B binds to sequences close to the TATA-box and activates POMC gene expression in transfected AtT20 cells [22]. However, this protein is present ubiquitously and deletion of its binding sequences by substitution of a minimal viral *tk* promoter for the POMC promoter did not influence transgene expression in transgenic mice [8,22]. Brn 3.0 binds to POMC site 3 adjacent to the distal PP1 site and overexpression of this protein in transfected cells activated expression of transgenes containing the POMC promoter [11]. Our truncated deletion of site 3 removed both the Brn 3 binding site and the distal PP1 binding site and apparently reduced the POMC promoter activity, but the remaining proximal PP1 binding site in site 2 was still sufficient to target high-level transgene expression to the mouse pituitary. Other proteins have been detected that bind to sequences beyond the promoter region used here. A protein named CUTE that bound to E-box-like sequences in the rPOMC promoter more distal to our 323 bp

promoter DNA and which activated transgene expression in transfected cells has been proposed to be a helix–loop–helix protein [23]. However, our earlier study [8] and current data demonstrated that CUTE is not required for the correct cell-specific expression of transgenes in mice. Therefore, those proteins may play a role like SP1 in supporting the action of PP1 on the POMC gene. A test of this hypothesis requires the cloning of PP1 and the direct examination of interactions between PP1, other proposed transcriptional activators and components of the basal transcriptional machinery.

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