Tissue-Specific Activity of the Pro-Opiomelanocortin Gene Promoter

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The pro-opiomelanocortin (POMC) gene is specifically expressed in corticotroph cells of the anterior pituitary. To define the POMC promoter sequences responsible for tissue-specific expression, we assessed POMC promoter activity by gene transfer into POMC-expressing pituitary tumor cells (AtT-20) and fibroblast L cells. The rat POMC promoter was only efficiently utilized and correctly transcribed in AtT-20 cells. 5'-End deletion analysis revealed two promoter regions required for activity in AtT-20 cells. When tested by fusion to a heterologous promoter, DNA fragments corresponding to both regions exhibited tissue-specific activity, suggesting the presence of at least two tissue-specific DNA sequence elements within the promoter. In summary, POMC promoter sequences from -480 to -34 base pairs appear sufficient to mimic the specificity of anterior pituitary expression.

Control of eucaryotic transcription results from the interaction of multiple DNA sequence elements that are presumably recognized by as many *trans*-acting factors (reviewed in references 21 and 33). In gene transfer experiments with tissue culture cells or transgenic animals, it was shown that various DNA sequence elements are responsible for tissue-specific expression of many cellular genes (2, 3, 9, 16, 24, 29, 31, 39, 45, 46). Tissue-specific sequence elements were identified in the 5'-flanking region of the genes coding for immunoglobulins (16, 24, 31), insulin (46), elastase (45), α_1 -antitrypsin (9), and many others. Some sequences conferring tissue specificity have enhancer properties and are located either upstream of the site of transcription initiation or elsewhere in the gene (1, 20, 37, 42). In many cases, multiple DNA sequence elements are required to confer tissue specificity (8, 13, 16, 24, 31, 48), including promoter-proximal and -distal sequences.

The pituitary gland represents an interesting system to investigate the role of tissue-specific DNA sequence elements in the expression of cell-specific genes. Pituitary hormone-secreting cells have a common embryological origin; however, each pituitary hormone gene is expressed in a distinct cell type (17). To identify DNA sequences required for tissue-specific expression in the pituitary, we studied the activity of promoter sequences present in the rat pro-opiomelanocortin (POMC) gene. The POMC gene is present in a single copy per haploid genome (12); it is expressed in two different pituitary cell types, the anterior pituitary corticotrophs, in which POMC is processed to adrenocorticotropin, and the pars intermedia melanotrophs, in which the same POMC protein precursor is processed to α -melanotropin (14). Regulation of POMC gene expression in these two tissues is also cell specific (18; J.-P. Gagner and J. Drouin, *Mol. Endocrinol.*, in press), highlighting the functional and developmental differences between the two secreting cell types.

We have assessed the specificity of POMC promoter utilization by gene transfer into cell lines of pituitary

(AtT-20) and fibroblastic (L) origin. The POMC promoter is only active and specifically recognized in the POMC-expressing tumor cell line AtT-20; in contrast, it is poorly and artifactually transcribed in L cells. POMC upstream DNA sequences localized in this work mimic the *in vivo* specificity of POMC expression.

MATERIALS AND METHODS

Plasmid constructions. Recombinant plasmids were constructed by standard procedures (30). Plasmid DNAs used for electroporation were purified by banding twice on cesium chloride gradients. The POMCneo plasmid constructs contain pBR327 plasmid sequences from the *SalI* site to the *EcoRI* site. Two rat POMC fragments (12) containing upstream sequences and part of exon 1 were fused to *neo* coding sequences. Both POMC fragments have the same 3' end produced by *XmnI* digestion after nucleotide 63 of exon 1; pPOMC₇₀₆neo contains 706 base pairs (bp) of upstream flanking sequences, while pPOMC₄₅₀₀neo includes flanking sequences up to an *EcoRI* site at position -4.5 kilobases. The POMCneo gene hybrids were constructed from similar recombinants containing the POMC promoter fragments linked to simian virus 40 (SV40) T antigen coding sequences (5). The exchange of coding sequences between the two sets of plasmids introduced a 24-bp segment of SV40 early 5' untranslated sequence (between *StuI* and *HindIII* sites) between POMC and *neo* sequences. This 24-bp segment was later used as a tag to differentiate in RNase mapping assays between endogenous and recombinant POMC transcripts present in electroporated cells. The viral enhancer sequence from Moloney mouse sarcoma virus (MSV) was inserted in both orientations upstream of the POMC promoter fragment at a single *XhoI* site (originally an *NruI* site) as illustrated in Fig. 1A. The MSV enhancer fragment is a 203-bp *XbaI*-*Sau3A* fragment (11) to which *SalI* linkers were added. A promoterless plasmid, pPLneo, devoid of any eucaryotic promoter sequences but otherwise similar to pPOMCneo plasmids, was used as a negative control. The 5' deletion mutants were constructed by deletion of POMC sequences from pPOMC₇₀₆neo by using convenient restriction sites.

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FIG. 1. (A) Plasmid constructs used to test the efficiency of POMC promoter utilization. (B) Southern blot analysis of plasmid DNA sequences present in genomic DNA isolated from pools of G418-resistant cells. The positions of linearized plasmids used as markers are shown by arrows.

Plasmid pRSV₁₃₀neo was constructed by exchange of an *Eco*RI fragment from pRSVneo (22) in place of equivalent RSVcat sequences present in pPr-LTR17 (27). Plasmid pRSV₄₈neo was constructed by internal deletion of Rous sarcoma virus (RSV) long terminal repeat (LTR) sequences between the *Xba*I and *Eco*RI sites of pRSV₁₃₀neo. Since both pRSV₁₃₀neo and pRSV₄₈neo plasmids contain a unique *Xba*I site at the 5' border of the LTR sequences, rat POMC promoter fragments destined for insertion in these vectors were flanked by synthetic *Xba*I linkers.

Tissue culture procedures and electroporation. AtT-20/D16-v cells (obtained from R. Kelly) were maintained in Dulbecco modified Eagle medium containing 10% fetal calf serum. L cells (TK⁻ APRT⁻, obtained from C. Stanners) were maintained in α -MEM medium containing 10% fetal calf serum. Cells for electroporation were maintained and harvested by trypsinization during log-phase growth. Electroporation was performed as described (41) for all cell lines. Cells (10^7 /ml) were electroporated with supercoiled DNA at a concentration of 50 μ g/ml. In preliminary electroporation experiments with plasmids pRSVneo and pPOMC₇₀₆neo, this DNA concentration fell within the linear portion of a dose-response curve of the number of G418-resistant foci as a function of DNA concentration. G418-resistant colonies were selected with concentrations of G418 (Geneticin; GIBCO Laboratories) producing 90% cell death in 7 days (44) in AtT-20 cells (750 μ g/ml) and L cells (600 μ g/ml). After 2 weeks of selection, foci were counted and pooled for DNA and RNA analyses.

Genomic DNA analysis. The copy number of electroporated plasmid DNAs was assessed by Southern blot (43) analysis of total genomic DNA isolated (23) from pools of G418-resistant foci. Genomic DNAs (10 μ g) were digested with *Bam*HI, which linearizes all the plasmids used. *neo* coding sequences were revealed by hybridization with a pSV0neo probe labeled by nick translation (30). Plasmid copy number was assessed by comparison of autoradio-

graphic signals produced by known amounts of plasmid DNA. The intensity of the *Bam*HI band should be proportional to the $n - 1$ copy number for clones containing more than one tandemly repeated copy of the plasmid DNA. This assessment of copy number may be slightly overestimated if a large number of clones contain only one integrated plasmid per cell.

Analysis of cytoplasmic RNA. Cytoplasmic RNAs were isolated (5) from pools of G418-resistant clones electroporated with various plasmids. RNase mapping of the 5' ends was performed as described with an RNA probe produced on an SP6 template (34). For this purpose, cytoplasmic RNA (10 μ g) was hybridized with about 400,000 cpm of ³²P-labeled RNA probe.

RESULTS

Specificity of POMC promoter utilization. The mouse pituitary tumor cell line AtT-20 provides a good host to study the tissue specificity of the POMC promoter, since this cell line greatly resembles anterior pituitary corticotrophs in terms of POMC expression, regulation, processing, and peptide release (14). We assessed the specificity of rat POMC promoter activity by using a focus formation assay dependent on expression of the bacterial neomycin resistance gene (*neo*) (44). Similar transformation assays were used to dissect thymidine kinase (TK) promoter sequences (32) and to localize regulatory elements present in the human metallothionein II gene (26). A group of related plasmids (Fig. 1A) were constructed and reintroduced by electroporation (41) into AtT-20 and mouse fibroblast L cells. Plasmids pRSVneo (22), pSV2neo (44), and pTKneo (13) were used as positive controls. However, the SV40 promoter present in pSV2neo appeared to be almost inactive in AtT-20 cells (Table 1) and in other pituitary cells (GH₃; not shown). In contrast, the pRSVneo and pTKneo plasmids appeared to be more active in AtT-20 than in L cells. The promoterless control plasmid

TABLE 1. Efficiency of various fusion genes

Plasmid	AtT-20 cells		L cells	
	No. of G418 ^r foci ^a ± SD	Enhancement factor	No. of G418 ^r foci ^a ± SD	Enhancement factor
pRSVneo	4,905 ± 1,512 (26)	1,226	886 ± 296 (10)	127
pSV2neo	22 ± 2 (4)	6	742 ± 215 (4)	106
pTK ₁₀₉ neo	87 ± 36 (13)	22	12 ± 7 (4)	2
pPLneo	4 ± 4 (14)	1	7 ± 5 (8)	1
pSV0neo	22 ± 14 (6)	6	84 ± 33 (6)	12
pPOMC ₄₅₀₀ neo	194 ± 45 (9)	49	3 ± 5 (8)	0.5
pPOMC ₇₀₆ neo	199 ± 89 (18)	50	8 ± 4 (11)	1
pPOMC ₇₀₆ neo + MSV (L→R) ^b	427 ± 176 (8)	107	65 ± 27 (6)	9
pPOMC ₇₀₆ neo + MSV (R→L) ^b	576 ± 248 (8)	144	70 ± 27 (5)	10

^a Average number of G418-resistant colonies per 5×10^6 electroporated cells. The number of determinations is shown in parentheses.

^b These plasmids contained MSV sequences in the indicated (right [R] to left [L] or vice versa) orientations.

pPLneo led to similar numbers of G418-resistant colonies in the two cell lines used in this study (Table 1). pPLneo as well as our other chimeric plasmids contains pBR327 plasmid vector sequences (up to the *SalI* site) instead of pBR322 sequences, as in pSV0neo (44), because the pBR322 sequences present upstream of the neomycin resistance gene in pSV0neo produce artifactual transcription and an important background of G418-resistant colonies (Table 1).

The pPOMCneo plasmids were 50 times more efficient in AtT-20 cells than pPLneo, whereas they were no more active than this negative control plasmid in L cells (Table 1). Similarly, the pPOMCneo plasmids were inactive in the mouse mammary tumor cell line C127I (J. Charron and L. Jeannotte, data not shown). POMC sequences upstream of position -706 bp were not required for promoter activity, since pPOMC₇₀₆neo and pPOMC₄₅₀₀neo were equally active. These data indicate that the POMC promoter is efficiently utilized in POMC-expressing corticotrophs such as AtT-20 cells, but not in fibroblasts.

The analysis of integrated plasmid DNA and transcripts was performed on pools of clones (obtained after G418 selection of 10^7 electroporated cells) to measure the most common event. Pool size varied according to the efficiency of electroporated DNAs (Table 1). The survival rate after electroporation was about 50% for all cell lines. The integrated DNA was characterized by digestion with the enzyme *Bam*HI, which cuts all the plasmids used at a single site (Fig. 1B). By comparison with the autoradiographic signal produced by known amounts of plasmid DNA (not shown), we estimated an average of about 5 plasmid copies per cell for most plasmids and pools of cells. Besides the unit-length plasmid DNA band, analysis of genomic DNA from pools of G418-resistant cells from four different experiments (only one shown in Fig. 1B) did not reveal any band present in more than one sample that could reflect preferential chromosomal integration. Southern analysis clearly showed that each plasmid was found at similar copy number in different pools of host cells and that there was no specific integration of plasmid DNA. Therefore, the number of G418-resistant colonies should reflect promoter utilization.

To measure the accuracy of transcription initiation, we characterized the 5' ends of the transcripts by RNase mapping with an RNA probe synthesized by the SP6 RNA polymerase (34). The probe (Fig. 2) allowed the simultaneous detection of recombinant rat POMCneo transcripts and endogenous mouse POMC mRNA present in AtT-20 cells. The endogenous AtT-20 mRNA protected the expected fragment of 63 nucleotides (nt) in control and

FIG. 2. Analysis of POMCneo transcripts and of endogenous POMC mRNA (AtT-20 cells) by RNase mapping (34). As illustrated at the bottom, the RNA probe contained the first 63 nt of the rat POMC mRNA and 24 nt of SV40 early 5' untranslated sequences. Hybridization of this probe to endogenous AtT-20 cell POMC mRNA protected a 63-nt fragment (arrow mPOMC), while hybridization of the same probe to POMCneo transcripts protected an 87-nt fragment (arrow rPOMCneo). The positions of single-stranded DNA markers are indicated.

electroporated cells. A ladder of smaller fragments was also present below the expected band of 63 nt. Although this ladder may be due to heterogeneous initiation, we suspect that it is caused by breathing at the ends of the hybrid: as expected in this case, about 5 bands were present in an S1 mapping assay with an equivalent end-labeled DNA probe (not shown), as opposed to the 10 or so bands observed in the RNase assay. Bands of about 70 and 115 nt were also present in the RNase mapping assay; they may represent minor initiation sites. Analysis of the RNA from AtT-20 cells electroporated with pPOMC₇₀₆neo or pPOMC₄₅₀₀neo revealed, in addition to the endogenous POMC bands, another band of 87 nt. This fragment corresponded exactly to the size expected of transcripts initiating at the correct site in the rat POMCneo gene.

Since L cells do not express endogenous POMC, the SP6 RNA probe did not protect any band in control cells, and all of the protected fragments in RNA samples isolated from electroporated cells must correspond to transcripts from the POMCneo gene. The RNase mapping analysis of POMCneo transcripts present in L cells suggested that these transcripts were very heterogeneous (Fig. 2). Multiple POMCneo transcripts were also detected in Northern blots (RNA blots) of RNA from the same pools of cells (not shown). This heterogeneity, which is consistent with the poor activity of the POMC promoter in L cells (Table 1), could result from multiple initiation sites or aberrant splicing of improperly initiated transcripts. The insertion of an MSV enhancer fragment in plasmid pPOMC₇₀₆neo increased promoter utilization in L cells about 10-fold (Table 1) and also increased the relative frequency of initiation from the correct site in the POMC promoter (Fig. 2). However, transcription initiation was still far from being as specific as for pPOMC₇₀₆neo in AtT-20 cells, suggesting that promoter strength is not the sole factor determining the specificity of transcription initiation. Thus, tissue-specific promoter recognition may determine promoter activity as well as correct initiation of transcription.

Deletion mutagenesis of POMC promoter. To delineate the sequences essential for activity, a series of 5' deletion mutants of the rat POMC promoter fragment were generated by using convenient restriction sites. The promoter efficiency of these mutants was tested by electroporation into AtT-20 cells, and their relative efficiency is shown in Fig. 3. Transcription of all promoter deletion mutants was initiated at the correct site as judged by RNase mapping analysis (not shown). No more than 480 bp of upstream sequences were required for full POMC promoter activity. Further resection of the POMC promoter fragment decreased its activity. From this analysis, two regions of the promoter (from position -480 bp to position -323 bp and from -166 bp to -34 bp) appear to contribute to promoter activity (Fig. 3). Deletion of POMC sequences beyond position -166 bp hampered promoter activity so much that cell growth in the presence of G418 was slower than for cells electroporated with pPOMC₇₀₆neo, in support of the interpretation that POMC sequences downstream of position -166 bp contribute significantly to promoter activity. When electroporated into L cells, the deletion mutants were no more active than the pPOMC₄₅₀₀neo or pPOMC₇₀₆neo plasmids (not shown), suggesting that promoter activity is tightly associated with tissue-specific recognition and that POMC upstream sequences do not have inhibitory activity in L cells.

Tissue-specific elements in POMC 5'-flanking sequences. We tested the activity of POMC 5'-flanking sequences to determine whether the two regions of the promoter revealed

by 5' deletion analysis conferred tissue specificity. For this purpose, POMC fragments were inserted upstream of RSV LTR sequences in plasmid pRSV₁₃₀neo or pRSV₄₈neo, which contain 130 or 48 bp of RSV 5'-flanking sequences, respectively. Plasmid pRSV₁₃₀neo contained one upstream promoter element, as defined by Laimins et al. (27), whereas pRSV₄₈neo only contained the TATA box region. While pRSV₄₈neo was as inactive as pPLneo or pPOMC₃₄neo, pRSV₁₃₀neo retained about 1 and 30% of pRSVneo activity in AtT-20 and L cells, respectively (Table 1 and Fig. 4).

To verify that POMC 5'-flanking sequences between positions -480 bp and -34 bp were sufficient to confer tissue-specific promoter activity, we fused these sequences to RSV LTR sequences present in plasmid pRSV₄₈neo. As shown in Fig. 4, these POMC sequences appeared to be sufficient to confer tissue-specific expression in AtT-20 cells.

Insertion of POMC fragments extending between positions -166 bp or -132 bp and -34 bp upstream of pRSV₁₃₀neo in either orientation specifically enhanced promoter efficiency (by a factor of 10- to 20-fold) in AtT-20 cells but not in L cells (Fig. 4). When assayed in the same plasmid, POMC fragments containing the most upstream promoter region defined by 5' deletions (between positions -480 bp and -166 bp or -132 bp) also specifically increased promoter efficiency in AtT-20 but not L cells. In support of the results of 5' deletion analyses, POMC sequences between positions -706 bp and -480 bp did not exhibit any transcriptional activity when tested in the same way (Fig. 4).

When equivalent POMC fragments were tested by insertion upstream of another heterologous promoter (herpes simplex virus TK promoter extending between positions

FIG. 3. Deletion analysis of POMC promoter sequences. The 5' deletion mutants were tested by electroporation in AtT-20 cells, and their efficiency is plotted relative to that of pPOMC₄₅₀₀neo. The relative efficiency of the promoterless control plasmid, pPLneo, is indicated (□). The values are the averages of 4 to 20 determinations, and the standard deviations are similar to those reported in Table 1.

FIG. 4. Tissue-specific elements in rat POMC 5'-flanking sequences. Plasmids pRSV₁₃₀neo and pRSV₄₈neo were used to test the transcriptional properties of different POMC 5'-flanking DNA fragments, represented by dark arrows aligned with a map of the POMC promoter. These fragments were inserted in either orientation upstream of the RSVneo gene as indicated. The various plasmids were electroporated (in duplicates) in AtT-20 and L cells. The average number of G418-resistant colonies per 5×10^6 electroporated cells $\pm$ standard deviation is presented.

–109 and 52 bp), the tissue-specific properties of the POMC fragments could not be observed (not shown).

DISCUSSION

The various pituitary hormone-secreting cells have a common embryological origin in Rathke's pouch (17), and each expresses a distinct hormone gene. Corticotroph cells of the anterior pituitary specifically express the POMC gene. By using gene transfer into nonpituitary cells, expression from the POMC promoter was previously shown to depend on the presence of viral sequences to increase promoter activity (5, 25, 35), as confirmed in this report with an MSV enhancer fragment (Table 1). However, none of these studies identified POMC sequences responsible for tissue-specific expression. We used gene transfer into pituitary corticotrophlike and fibroblast cell lines to show that rat POMC sequences between positions –480 bp and –34 bp confer tissue-specific promoter activity. The specificity of POMC promoter activity revealed in these gene transfer experiments into tissue culture cells was recently confirmed in transgenic mice. Indeed, a transgene containing POMC promoter sequences up to position –706 bp was almost exclusively expressed in the pituitary (Y. Tremblay and J. Drouin, data not shown).

Progressive resection of POMC upstream sequences suggested the presence of at least two regions involved in promoter activity (Fig. 3). The most upstream element would be located between positions –480 bp and –323 bp, while promoter-proximal sequences between positions –166 bp and –34 bp also appear to be important for promoter activity. However, the deletion analysis performed in this work does not completely rule out a role in promoter activity for sequences between positions –323 bp and –166 bp, since sequences in this region could act in synergy or be redundant with other promoter sequence elements.

The activity of the two putative POMC elements was assessed independently by fusion to a heterologous promoter (Fig. 4). POMC fragments extending from position –480 bp to position –166 bp or –132 bp exhibited tissue-specific transcriptional activity when tested by fusion to a 5'-deleted RSV promoter. POMC promoter-proximal sequences (between position –166 bp or –132 bp and position –34 bp) have similar activity; these fragments have enhancerlike properties, since they were active in both orientations. These results suggest that DNA sequences between positions –166 bp and –132 bp do not contribute to promoter activity; however, the 5' deletion analysis (Fig. 3) suggests otherwise, since deletion of these sequences significantly reduced promoter activity. The role of these sequences may not be detected by fusion to the RSV heterologous promoter because they may only exert their effect in their natural context; alternatively, they may be redundant with RSV sequences. The localization of the 5' border of POMC sequences required for promoter activity at position –480 bp is consistent with both 5' deletion and heterologous promoter analyses (Fig. 3 and 4). The lack of any activity of POMC sequences from positions –706 bp to –480 bp in the heterologous promoter assay contrasts sharply with the tissue-specific activity of the two promoter elements defined above. Replacement of POMC promoter sequences downstream of position –34 bp by equivalent sequences from the RSV LTR (plasmid pRSV₄₈neo) did not alter promoter specificity, suggesting that POMC sequences responsible for tissue-specific expression are localized between positions –480 bp and –34 bp.

It is now realized that enhancers and promoters are constituted of multiple elements which interact to produce maximal promoter activity (38, 50). We have demonstrated tissue-specific enhancer activity in POMC promoter fragments by fusion to an RSV promoter fragment, indicating

that transcriptional regulatory elements present in these sequences can complement each other. In contrast, we were unable to detect this activity by fusion to the TK promoter. Thus, assessment of enhancer activity with a heterologous promoter appears to be dependent on the organization of the test promoter (40).

Many cellular genes contain DNA sequences which confer tissue specificity of expression. More than one tissue-specific regulatory element was identified in the genes encoding immunoglobulins (16, 24, 31), insulin (13), complement protein factor B (48), and α A-crystallin (8). Similarly, the POMC promoter contains two regulatory elements which both confer tissue specificity independently of each other (Fig. 4). In contrast, other pituitary hormone genes, those encoding growth hormone and prolactin, apparently contain only one DNA region which confers tissue-specific expression (15, 36, 47). However, in both the human (28) and rat (4, 47, 49) growth hormone genes, this region was shown to contain binding sites for different DNA-binding proteins, one of which appears to be specific to growth hormone-expressing cells.

In addition to its expression in anterior pituitary corticotrophs, the POMC gene is expressed in melanotrophs of the intermediate pituitary (10, 19), specific neurons of the brain (10, 19), testes (7), ovaries, and placenta (6). In brain, testes, ovaries, and placenta, shorter POMC transcripts are found compared with pituitary POMC mRNA; these transcripts appear to arise by intragenic initiation of transcription (L. Jeannotte, J. P. H. Burbach, and J. Drouin, *Mol. Endocrinol.*, in press). Since POMC is a single-copy gene (12), POMC promoter activity may be determined by different *cis*- and *trans*-acting factors in these different tissues. In this report, we have identified two POMC promoter elements which confer tissue-specific expression in anterior pituitary corticotrophs. We are currently investigating the role of these elements in the control of POMC expression in other tissues.

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