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Pituitary Pro-Opiomelanocortin Gene Expression Requires Synergistic Interactions of Several Regulatory Elements

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The pro-opiomelanocortin (POMC) gene is expressed very early during pituitary development, before expression of the other pituitary hormone genes, growth hormone and prolactin, and before expression of the Pit-1/GHF-1 transcription factor which activates those genes. Thus, analysis of the POMC promoter should provide markers of the early stages of pituitary development at the time when cells are being committed to expression of one or the other pituitary hormone. We have previously localized the rat POMC promoter to a 543-bp 5′-flanking DNA fragment of the gene using transfection and transgenic mice experiments. We have now used mutagenesis and in vitro protein-DNA binding studies to define three domains of the promoter which have distinct and complementary activities. Within these domains which require each other for full activity, at least nine regulatory elements were defined by in vitro footprinting and replacement mutagenesis. Each element appeared equally important for promoter activity, as mutagenesis of any element had similar effect on promoter activity. Most of the elements bound different AtT-20 nuclear proteins in gel mobility shift experiments. Whereas only two elements appeared to be binding sites for the known transcription factors AP-1 and chicken ovalbumin upstream promoter, the seven other elements appeared to bind nuclear proteins with novel properties. Thus, in contrast to the predominant role of Pit-1/GHF-1 in transcription of the growth hormone and prolactin genes, the control of an early pituitary gene, POMC, appears to depend on the synergistic interaction of several regulatory elements which bind different nuclear proteins.

Hormones, which represent the hallmark of the differentiated phenotype of pituitary secretory cells, appear at different times during fetal development of the pituitary gland. At day 14.5 or 15 of rat fetal development, adrenocorticotropin peptide is the first pituitary hormone to appear (7, 22, 37); this hormone derives from proteolytic processing of pro-opiomelanocortin (POMC), which is specifically expressed in corticotroph cells of the anterior pituitary (12). POMC mRNA first appears on day 14 during fetal pituitary development (22). The other pituitary hormones occur later in development; for example, growth hormone (GH) and prolactin (PRL) appear at embryonic days 19 and 21, respectively (7). It was recently shown that a common cell-specific transcription factor, Pit-1/GHF-1, activates transcription of these two genes (23). This factor was almost exclusively found in GH and PRL-expressing cells (3, 18). It was not detected in POMC-expressing corticotroph and melanotroph cells (31). Thus, another factor(s) must be responsible for cell-specific activation of POMC.

We have previously localized 5′-flanking promoter sequences required for pituitary-specific expression of the rat POMC (rPOMC) gene (19, 34). In transgenic mice (34), these 5′-flanking sequences confer expression in POMC-expressing cells of both the anterior (corticotrophs) and intermediate (melanotrophs) pituitary gland. Using AtT-20 cells as a model for anterior pituitary corticotrophs, we have localized minimal sequences necessary for expression to a 543-bp 5′-flanking fragment of the gene (19). This analysis suggested that three different domains of the promoter contribute to its activity. We have now shown that the distal and central domains of the promoter must be present together to activate POMC transcription and that at least nine specific regulatory elements are present throughout the promoter. The DNA-binding specificity of putative transcription factors which bind these elements has been defined and found to be diverse. These results contrast with the organization of GH and PRL promoters and suggest that multiple regulatory elements are required for POMC transcription and that their interaction is synergistic.

MATERIALS AND METHODS

Plasmids and oligonucleotides. The various 5′ deletion mutants (19) of the rPOMC promoter fused to the firefly luciferase reporter gene (8) were constructed by insertion of the appropriate DNA fragment into the pXP1 luciferase vector (26). Additional 5′ deletion mutants were generated by digestion with restriction enzyme *Cla*I of rPOMC promoter fragments containing replacement mutations which introduced a unique *Cla*I site in the promoter. Replacement mutations were produced by oligonucleotide mutagenesis (33) with the thiol derivative in vitro mutagenesis kit sold by Amersham Corp. These mutations replaced 6 to 14 bp within the promoter as indicated by stars in Fig. 3. In each case, the replaced nucleotides included a *Cla*I site. The DNA sequence of each mutation in the promoter was determined (29). Standard procedures were used for plasmid constructions (24).

Oligonucleotides were synthesized by the phosphoramidite procedure with an Applied Biosystems instrument. For replacement mutagenesis, the mutant oligonucleotides contained 10 complementary nucleotides on either side of the mutated sequence. For gel retardation probes, synthetic oligonucleotides were synthesized to correspond to the sequence protected in DNase I footprinting experiments plus

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a few nucleotides on either side. The rPOMC sequences present in each oligonucleotide were as follows: oligonucleotide DE-4 from position −480 to −453, DE-3 from position −442 to −417, DE-2 from position −410 to −369, DE-1 from position −364 to −339, CE-2 from position −266 to −237, CE-1 from position −216 to −188, PE-4 from position −166 to −135, PE-3 from position −78 to −46, PE-2 from position −24 to +2, and PE-1 from position +20 to +53. Other oligonucleotides used as competitors had the following sequences: AP-2, 5′-GAACTGACCGCCCGCGGCCCTG-3′ (17); Sp1, 5′-CGGGGCGGGGC-3′ (5); chicken ovalbumin upstream promoter (COUP), 5′-TATGGTGTCAAAGGTC AAACTTCT-3′ (36); and mouse albumin CCAAT (NF-Y) box, 5′-GGAACCAATGAAATGCGAGG-3′ (21).

Cells, nuclear extracts, and whole-cell microextraction. AtT-20 cells were grown as described previously (19). Nuclear extracts were prepared as follows. The cells were harvested in cold phosphate-buffered saline (PBS) containing 0.6 mM EDTA while still in exponential growth and centrifuged at 2,000 × *g* for 5 min. Cell pellets were suspended in 10 volumes of buffer A [10 mM Tris-HCl [pH 7.5], 50 mM NaCl, 250 mM sucrose, 1 mM Na · EDTA, 0.25 mM Na · ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid, 0.5 mM spermidine, 0.15 mM spermine, 10 mM β-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride] and pelleted at 2,000 × *g* for 10 min. The cells were then lysed by being suspended twice in buffer B (which is buffer A containing 50 mM sucrose and 0.5% Triton X-100), and the nuclei were centrifuged at 2,000 × *g* for 10 min. This nuclear pellet was washed twice in 1 volume of buffer A and suspended to a final DNA concentration of ≈2 mg/ml in buffer C (which is buffer A containing 100 mM NaCl and 10% glycerol instead of sucrose). After gentle stirring for 15 min and centrifugation at 2,000 × *g* for 10 min, the nuclei were suspended at the same DNA concentration in buffer D (buffer A containing 400 mM NaCl and 10% glycerol instead of sucrose), gently stirred for 30 min, and pelleted at 10,000 × *g* for 20 min. The supernatant was collected, and proteins were precipitated by slow addition of $(NH_4)_2SO_4$ to 45% saturation. After stirring for 30 min and centrifugation at 10,000 × *g* for 20 min, the protein pellet was suspended in dialysis buffer containing 10 mM Tris-HCl (pH 7.5), 50 mM NaCl, 5 mM $MgCl_2$, 0.1 mM Na · EDTA, 1 mM dithiothreitol, and 20% glycerol and dialyzed twice for 2 h against 500 volumes of the same buffer. After removal of a slight precipitate by centrifugation for 5 min at 12,000 × *g*, the extracts were stored at −90°C.

Microextraction of proteins from whole cells was carried out on 2×10^7 growing cells. After two washes in cold PBS, the cells were harvested and centrifuged for 30 s at 2,000 × *g*. Cell pellets were gently suspended in 100 μl of extraction buffer which contained 20 mM Tris-HCl (pH 7.9), 100 mM NaCl, 0.2 mM EDTA, 0.5 mM dithiothreitol, 20% glycerol, 0.5 mM phenylmethylsulfonyl fluoride, 0.7 μg of pepstatin per ml, and 1 μg of aprotinin per ml. This suspension was adjusted to 450 mM NaCl and gently homogenized with a loose-fitting pestle for Eppendorf tubes. After 15 min of incubation on ice, the cell extract was obtained by centrifugation for 10 min at 12,000 × *g*, dialyzed twice against 500 volumes of extraction buffer, and stored at −90°C.

DNase I footprinting assays. Most DNase I footprinting assays were performed in conditions A (20) except for those performed in conditions B (Fig. 2d and f), which were done as follows: 1 to 2 fmol of probe (3,000 to 5,000 cpm) was incubated with 1 to 10 μg of nuclear extracts in a total volume of 20 μl containing 10 mM Tris-HCl (pH 7.9), 50 mM NaCl, 4 mM $MgCl_2$, 0.05 mM EDTA, 1 mM dithiothreitol, 10% glycerol, and 0.5 μg of poly(dI-dC). Incubation was for 30 min on ice followed by 1 min at 20°C, and then 2 μl of a DNase I solution (10 ng/μg of extract) freshly diluted in 100 mM Tris-HCl (pH 7.9), 10 mM $MgCl_2$, 1.5 mM $CaCl_2$, and 0.2 mg of bovine serum albumin (BSA) per ml was added and incubated for 30 s at 20°C. Reactions were stopped, and the reaction mixtures were extracted with phenol and analyzed by electrophoresis on 6 or 8% polyacrylamide sequencing gels.

Gel retardation assays. Gel retardation assays were done as described previously (2) with crude nuclear extracts or whole-cell microextracts from AtT-20 cells. Five femtomoles (≈20 kcpm) of 5′-end-labeled oligonucleotides was incubated with 5 to 10 μg of proteins for 20 min at 22°C in a total volume of 20 μl which contained 10 mM Tris-HCl, pH 7.9, 60 mM KCl, 1 mM EDTA, 1 mM dithiothreitol, 4% Ficoll, 1 to 3 μg of poly(dI-dC), and 0.25 to 1 μg of single-stranded M13-mp19 DNA. For competition experiments, both probe and competitor DNA were mixed together before the addition of proteins. The samples were loaded onto 4% polyacrylamide gels (acrylamide/bisacrylamide ratio, 30:1) buffered with 0.5× TBE (24) and separated by electrophoresis for 2 h.

Electroporation and enzyme assays. For electroporation, exponentially growing AtT-20 cells were harvested in PBS buffer as described elsewhere (27). AtT-20 cells (5×10^6) resuspended in 0.5 ml of PBS buffer were electroporated with a Bio-Rad instrument with a 200-V discharge (960-μF capacitance). Luciferase reporter plasmids (50 μg) were tested together with 100 μg of pRSVGH (30), which was used as internal control. These amounts of DNA were optimized for efficient expression within a linear response range for both activities. After electroporation, cells were plated and incubated for 16 h before preparation of extracts used in the luciferase assay as described previously (8). Tissue culture media were collected at the same time for an assay of human GH secretion, which was taken as an internal control to correct for variations in electroporation efficiency. Luciferase activity was measured with a LKB model 1250 luminometer, and the protein concentration of cellular extracts was also measured (4). In each experiment, plasmids were tested in duplicate dishes and each plasmid was tested in three or four different electroporations.

RESULTS

We have previously shown that three domains of the rPOMC promoter conferred transcriptional activity to a heterologous promoter despite the fact that deletion of the central domain had no effect in 5′ deletions (11, 19). In order to understand the role of these three domains, we tested their activities individually and in combination with the other domains of the promoter. These analyses (Fig. 1) indicated that both proximal and central domains increased transcription 5- to 10-fold when tested upstream of a minimal POMC promoter (−34 bp to +63 bp) which included the TATA box and exon 1 sequences. In contrast, the distal element was devoid of activity. Juxtaposition of either distal or central domains with the proximal domain did not further enhance activity. However, juxtaposition of the distal and central domains resulted in marked synergy. Thus, it appears that the distal and central domains function as a unit in which both are essential for activity.

In vitro localization of putative regulatory elements. In order to localize regulatory elements responsible for the

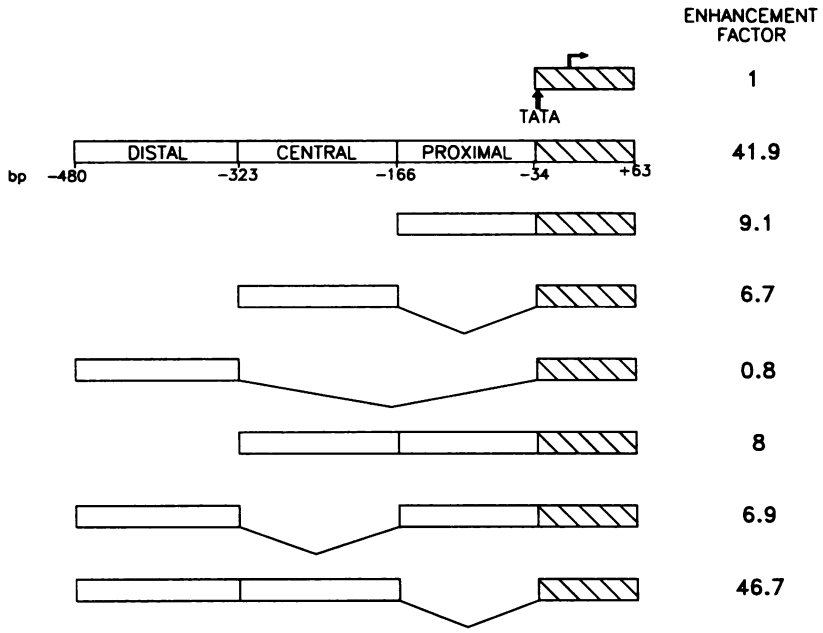

FIG. 1. Synergistic interaction between the distal and central domains of the rPOMC promoter. The activities of the three domains of the promoter were tested by insertion of these domains alone or in combination upstream of a minimal rPOMC promoter fragment extending from position −34 to position +63. The activity of each promoter construct is shown relative to the activity of this minimal promoter. The data represent the means of results of three to four experiments in which plasmids were assayed in duplicate.

activities of these three domains (19), we identified within this 543-bp promoter region binding sites for AtT-20 nuclear proteins by using the DNase I footprinting technique (14). The results of these analyses are shown in Fig. 2 and summarized in Fig. 3. In all cases, the footprints were localized on both strands in numerous experiments; Fig. 2 presents representative gels for functionally important elements. Four overlapping DNA fragments of the rPOMC promoter were end labeled at either their 5′ or 3′ end to serve as probes (Fig. 2a). Probe A, which contained the distal region of the promoter, revealed three footprints (Fig. 2b) labeled DE-4 (−481 to −458 bp), DE-3 (−440 to −426 bp), and DE-2 (−414 to −387 bp). The overlapping probe B also revealed (Fig. 2c) footprints DE-3 and DE-2 as well as footprints DE-1 (−361 to −341 bp), CE-2 (−283 to −248 bp), and CE-1 (−216 to −193 bp). Three footprints were revealed in the proximal region of the promoter by using probe D (Fig. 2e): they are PE-3 (−71 to −57 bp), which overlaps a previously described (13) glucocorticoid receptor-binding site, PE-2 (−18 to +6 bp), and PE-1 (+24 to +42 bp). We tested other DNA-binding conditions (conditions B, NaCl instead of KCl and higher protein concentration) to ensure that we had not failed to detect DNA-binding activities possessing different binding requirements. Indeed, with the same nuclear extracts, these different binding conditions (B) revealed one additional footprint, PE-4 (−144 to −109 bp), in the proximal region (Fig. 2d). They also resulted in displacement further downstream of footprint PE-1 from positions +24 and +42 to positions +33 and +53 (Fig. 2f). It thus appeared that these binding conditions revealed different AtT-20 proteins binding to different sequences within PE-1. However, it is noteworthy that rat anterior pituitary nuclear extracts produced footprints extending throughout both halves of PE-1 (+24 to +55 bp) in conditions A (Fig. 2e) but over the 3′ half in conditions B (Fig. 2f). These differences in footprinting activities could represent true differences in nuclear proteins present in AtT-20 extracts and in pituitary extracts. The precise position of each footprint is presented in Fig. 3 together with POMC promoter sequences required for cell-specific activity in AtT-20 cells (19). The footprints were labeled in accordance with previous analyses which defined proximal, central, and distal domains within the promoter (11, 19) (Fig. 2a). This figure also shows the alignment of sequences within footprints PE-3 and PE-1 with consensus binding sites for transcription factors COUP (16) and AP-1 (1).

Mutation analysis of putative regulatory elements. In order to establish the transcriptional role of the putative regulatory elements defined above by footprinting, we introduced replacement mutations (numbered and indicated by stars under the DNA sequence in Fig. 3) over each footprint in the promoter and tested the activity of each mutant promoter by electroporation into POMC-expressing AtT-20 cells (Fig. 4a). Most mutations decreased promoter activity by 30 to 70%, with the exception of mutations over elements DE-4 and PE-3 (mutants 1 and 15, respectively), which had no effect. In addition, one mutation located at the 5′ end of footprint PE-4 (mutant 10) and mutations upstream and downstream of PE-3 (mutants 14 and 16) did not affect activity. It is noteworthy that mutagenesis of so many elements decreased promoter activity to a similar extent for each element.

The effect of replacement mutations was compared with that of a series of 5′ deletions of the promoter (Fig. 4b); each sequential 5′ deletion removed one or two regulatory elements. Deletion of elements DE-3 and DE-2 led to progressive declines in promoter activity. However, further resection in the central domain did not result in a greater decrease of promoter activity, despite the deleterious effect of replacement mutations over elements DE-1, CE-2, and CE-1.

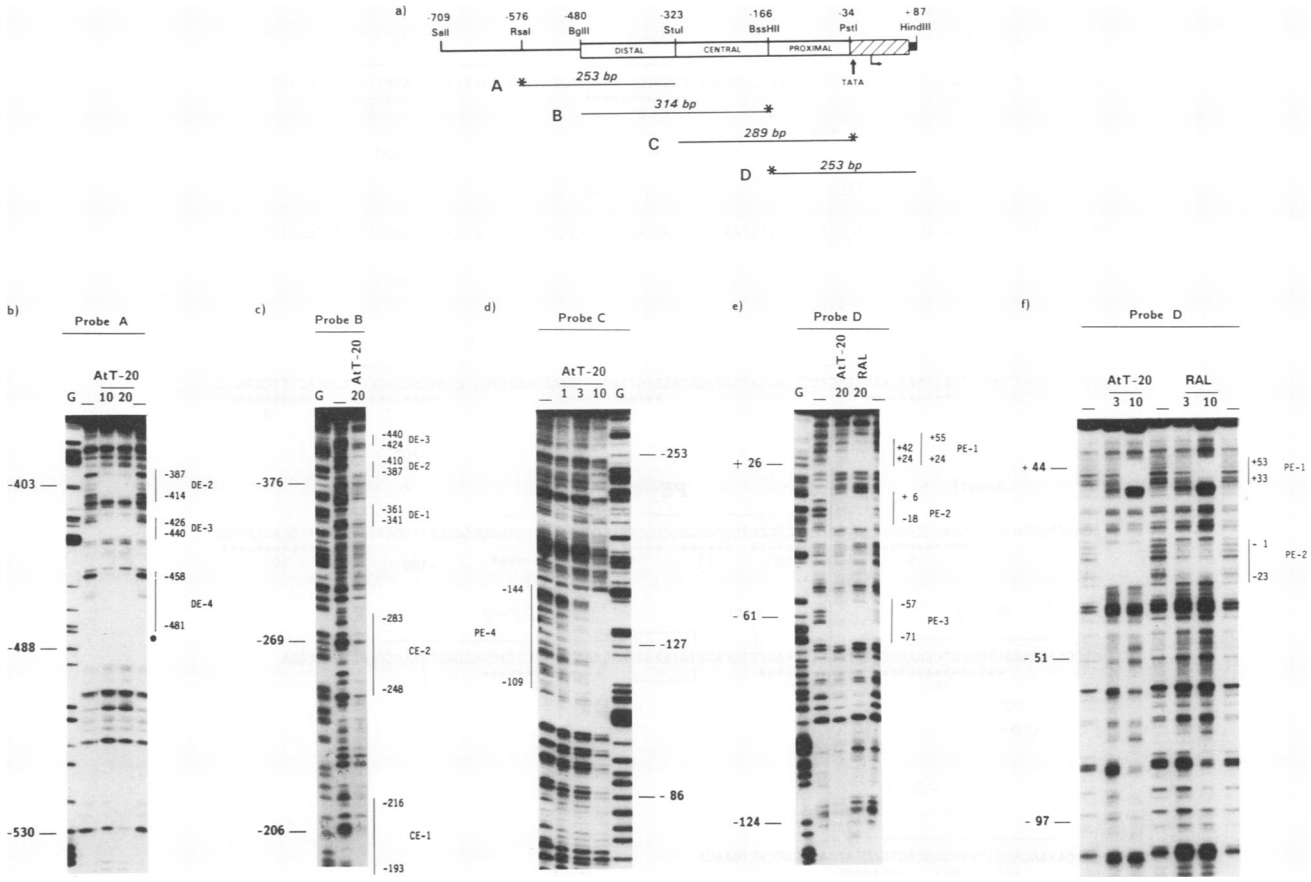

FIG. 2. Localization of binding sites for AtT-20 nuclear proteins within the POMC promoter by using DNase I footprinting. (a) Schematic representation of the rPOMC promoter showing restriction enzyme cutting sites used previously to define three domains within promoter sequences which are required for activity in AtT-20 cells (19). These sites were also used to generate four DNA probes used in DNase I footprinting experiments. Probes A to D were labeled as indicated by asterisks at either the 5′ or 3′ end. (b) Localization of binding sites for AtT-20 nuclear proteins in the distal region of the promoter by using probe A (5′-end labeling) in binding conditions A and 10 or 20 μg of AtT-20 cell nuclear extract as indicated. G refers to G sequencing track, whereas lanes labeled with a dash were controls to which BSA was added instead of nuclear extract. The positions of some guanine residues are indicated on the left for orientation, and the positions of the various footprints are indicated on the right by bars and labels. Footprints in the distal region of the promoter were labeled DE. (c) Localization of binding sites for AtT-20 nuclear proteins in the distal and central region of the promoter by using probe B (5′ end labeling). (d) Footprint PE-4 is revealed in binding conditions B (containing KCl and higher protein concentrations) by using probe C (3′-end labeling). (e) Localization of binding sites for AtT-20 nuclear proteins in the proximal region of the promoter by using probe D (5′-end labeling). The same conditions used in panels b and c were used for binding. In addition to AtT-20 nuclear extract (20 μg), a nuclear extract prepared from rat anterior pituitary lobe (RAL; 20 μg) was also assayed for comparison. (f) Localization of binding sites for AtT-20 and RAL nuclear proteins in the proximal region of the promoter by using probe D (3′-end labeling) and binding conditions B.

These results are consistent with the synergistic interactions between central and distal domains of the promoter (Fig. 1). Further deletions within the proximal domain led to progressive declines in promoter activity. Indeed, the decrease in promoter activity which resulted from deletion of element PE-4 is consistent with the effects of replacement mutations within PE-4. In contrast, subsequent deletions of the proximal domain resulted in lower promoter activity, whereas replacement mutations over the same DNA sequences (in and around PE-3) did not affect promoter activity (Fig. 4). In summary, the mutation analysis indicated that multiple regulatory elements are required for POMC transcription. None of these elements appeared to play a dominant role on its own. Rather, these elements appear to combine their activities to stimulate POMC transcription.

Different nuclear proteins bind POMC elements. In order to assess the nature of the *trans*-acting factors which interact with the POMC regulatory elements, we performed gel retardation using synthetic oligonucleotides corresponding to each *cis* element. Most oligonucleotides formed complexes of different mobility with AtT-20 cell nuclear proteins (Fig. 5a). However, some complexes comigrated in the gel retardation assay (DE-3 and DE-1, CE-1 and PE-4, and PE-3 and PE-1). In order to determine whether these comigrating complexes involved the same or different proteins, an AtT-20 cell nuclear extract was fractionated by heparin-agarose chromatography and the resulting fractions were assayed for binding to the different *cis* elements. The results of these analyses are summarized in Fig. 5b, which presents the elution profiles on heparin-agarose for the major DNA-binding proteins. The comigrating DE-3- and DE-1 (band C)-binding activities coeluted in this chromatography; similarly,

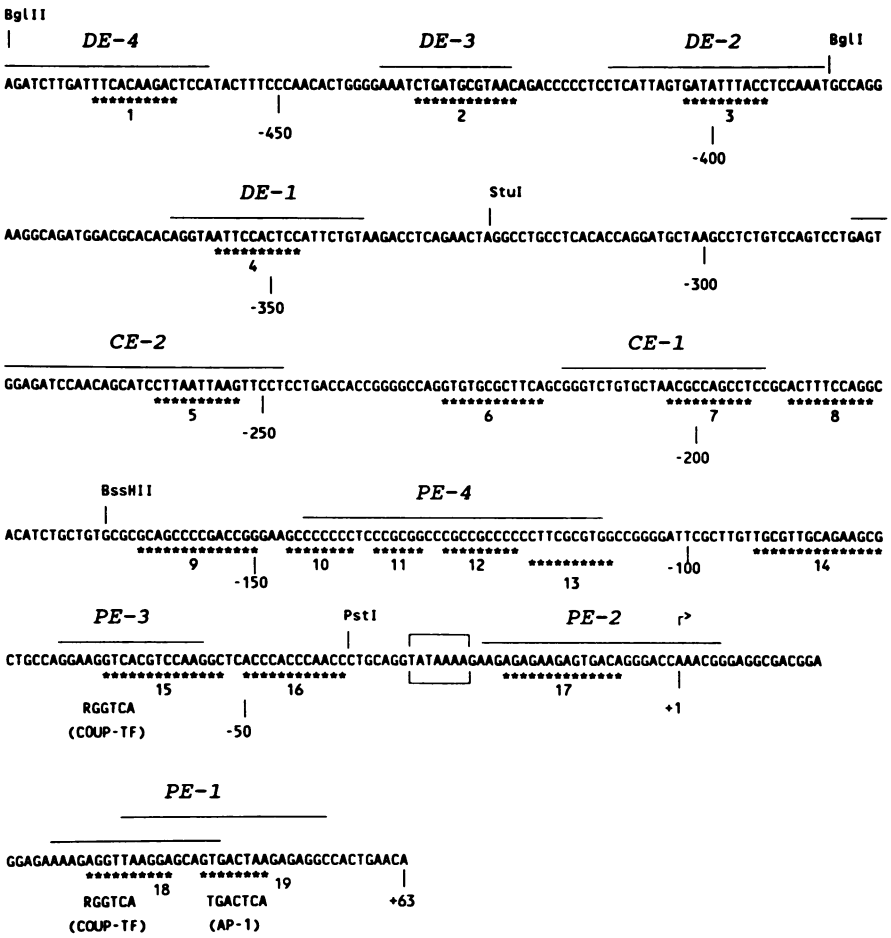

FIG. 3. Position of DNase I footprints on the DNA sequence of the rPOMC gene promoter. DNase I footprints identified in Fig. 2 are shown by lines over the DNA sequence (representing the outer borders of footprints performed on both coding and noncoding strands for each footprint) and labeled according to their positions within the proximal (PE), central (CE), or distal (DE) domain of the promoter. These domains were defined previously (11, 19), and the DNA sequence is taken from that of Drouin et al. (10). Stars indicate the nucleotides which were mutated in each replacement mutant (numbered from 1 to 19) described in the legend to Fig. 4. The TATA box and site of transcription initiation are indicated by a box and an arrow, respectively. The alignments of consensus binding sites for transcription factors AP-1 and COUP are shown below the DNA sequence.

PE-3- and PE-1-binding activities also coeluted. Binding activities for PE-4 and CE-1 were not recovered after heparin-agarose chromatography.

Since complexes formed with DE-1 and DE-3 oligonucleotides comigrated in the gel retardation assay (Fig. 5a) and coeluted on the heparin-agarose column (Fig. 5b), we tested their relatedness in competition binding assays. As shown in Fig. 6, DE-3 formed only one specific complex which comigrated with DE-1 complex C; in addition, DE-1 produced another slower-migrating complex which presented as a broad band in many gel retardation experiments (Fig. 6 and data not shown). This band was labeled A/B because two different DNA-binding activities were resolved by heparin-agarose chromatography (Fig. 5b); the elution profile of band A was similar to that of band C, whereas band B eluted at a higher salt concentration. In the competition experiment (Fig. 6), both DE-3- and DE-1-binding activities were inhibited by the cognate and the other oligonucleotide, whereas oligonucleotide DE-2 did not compete for binding. Thus, one set of protein(s) (band C) recognized elements DE-1 and DE-3. In addition, another protein(s) bound DE-1, but since band A/B was inhibited by excess DE-3, these bands may contain at least one DNA-binding protein in common with band C.

PE-4 is a 36-bp footprint encompassing a GC-rich region which contains sequences homologous to binding sites for transcription factors Sp1 and AP-2 (5, 17). We used competition binding assays to assess whether these transcription factors bind PE-4. We analyzed CE-1-binding activities in parallel because AtT-20 proteins binding CE-1 and PE-4 comigrated in gel retardation (Fig. 5a). Using oligonucleotide PE-4 as a probe (Fig. 7), we observed that both the homologous oligonucleotide and CE-1, albeit at higher concentrations, competed for binding. In addition, an Sp1 oligonucleotide (5) also competed very efficiently, whereas an AP-2 oligonucleotide (17) or CE-2 did not. Similar results were obtained with CE-1 as a probe (data not shown). Because binding of the same AtT-20 extracts to PE-4 and Sp1 probes resulted in complexes of different mobilities (the Sp1 probe formed a slower-migrating complex; data not shown), it may be that Sp1-related proteins bound elements PE-4 and CE-1.

Elements PE-1 and PE-3 produced similar complexes in gel retardation (Fig. 5a). Since elements PE-1 (5′ half) and PE-3 contained sequences related to the COUP transcription

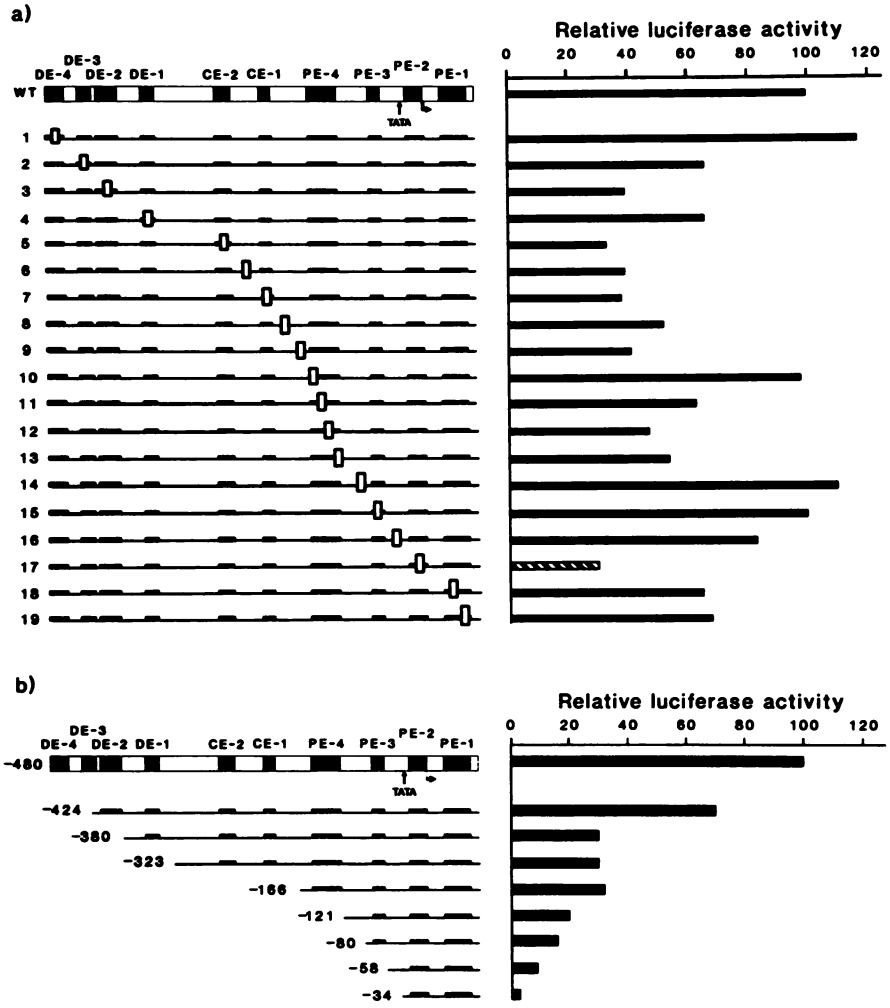

FIG. 4. Deletion and replacement mutagenesis indicate that most putative regulatory elements contribute to promoter activity. (a) The activity of the 19 replacement mutants numbered and shown by stars in Fig. 3 is presented relative to the activity of the intact rPOMC-luciferase reporter plasmid; in all cases, rPOMC sequences from position −480 to position +63 are present in plasmid constructs. The position of each replacement mutation is indicated by a box on a line drawing of the promoter. The data presented are the averages from three to four different experiments, each performed in duplicate, and in all cases, the data were corrected for protein concentration of the cell extract and for the activity of the internal Rous sarcoma virus-human GH control plasmid. The activity of a similar replacement mutation (mutant 17) constructed by Riegel et al. (28) over element PE-2 is also shown (hatched bar) for comparison. (b) Relative activity of 5′ deletions of the rPOMC promoter. The activity of each 5′ deletion of the promoter is shown relative to the activity of the intact rPOMC promoter fragment extending from position −480 to position +63. The data represent the means from three to four experiments, each performed in duplicate.

factor-binding site (Fig. 3), we tested the ability of oligonucleotides PE-3 and COUP to inhibit footprints PE-3 and PE-1 (5′ half). Indeed, both oligonucleotides inhibited footprints PE-1 and PE-3 but not footprint PE-2 (Fig. 8). An unrelated oligonucleotide corresponding to the mouse albumin gene CCAAT (NF-Y) box did not inhibit any footprints. Conversely, PE-1 oligonucleotides competed for binding to PE-3 (data not shown). Thus, both elements PE-1 (5′ half) and PE-3 interact at least in vitro with members of the COUP transcription factor family. No other COUP-binding sites were found elsewhere in the promoter; a detailed analysis of COUP interactions with PE-1 and PE-3 will be presented elsewhere (32a). Two different footprints were observed on element PE-1 depending on binding conditions (Fig. 2e and f). The downstream footprint contains an AP-1 transcription factor-binding consensus (Fig. 3); purified HeLa cell AP-1 (1) bound as efficiently to this sequence as to the collagenase gene TRE in gel retardation (32a). The POMC promoter contains another sequence (TGCCTCA, positions −321 to −315) related to the AP-1-binding site TGACTCA. This sequence did not exhibit footprints in conditions which revealed footprint PE-1 (3′-half), but we did not test for its binding with purified AP-1. Our inability to reveal simultaneous binding of COUP and AP-1 activities using AtT-20 extracts appears to depend on binding conditions.

DISCUSSION

The results of the present study have shown that transcription of the cell-specific rPOMC gene requires the synergistic interaction of different *trans*-acting factors which bind multiple regulatory elements (summarized in Fig. 9). This promoter organization contrasts with that of other pituitary-specific genes, the GH and PRL genes, which are predomi-

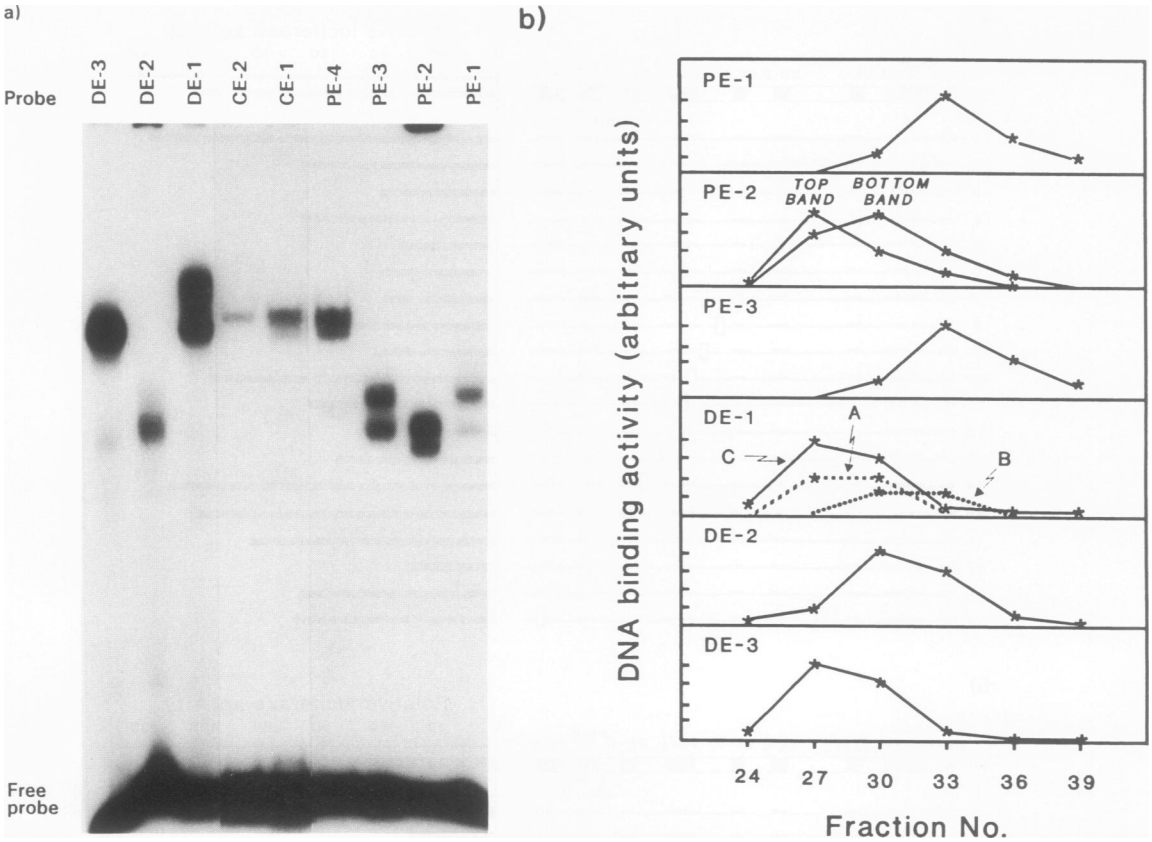

FIG. 5. Analysis of AtT-20 nuclear proteins binding the putative POMC regulatory elements by gel retardation. (a) Synthetic oligonucleotides containing the DNA sequence of each putative regulatory element were used as probes in the gel retardation assay to reveal AtT-20 nuclear proteins which specifically recognize them. (b) An AtT-20 nuclear extract was fractionated by heparin-agarose chromatography; after an initial wash with 0.1 M KCl, proteins were eluted with a linear gradient of KCl from 0.1 to 0.8 M. Forty-two individual fractions were collected and assayed for binding to each putative POMC regulatory element by the gel retardation assay. The elution profile of each binding activity is presented on a separate panel for each probe; profiles were derived by quantitation of the relative intensities of retarded bands. The different activities binding elements PE-1 and PE-3 showed similar elution profiles, so only one is presented. For element DE-1, profiles A, B, and C correspond to the bands indicated in Fig. 6. The elution profile was not determined for PE-4, CE-1, and CE-2, because these activities were lost after chromatography.

nantly activated by one cell-specific factor, Pit-1/GHF-1. The diverse putative POMC transcription factors appear to contribute equally to transcription, since mutation of each regulatory element only moderately reduced promoter activity (Fig. 4a). This interpretation is corroborated by the progressive decline in promoter activity observed after sequential removal of the elements in a 5′ deletion series (Fig. 4b). Although we cannot exclude the possibility that our analyses failed to identify other elements or factors required for POMC transcription, the correlation between the effects of single replacement mutations and the 5′ deletions suggests that this is not the case.

Despite the distinct properties of domains within the promoter (Fig. 1), it is noteworthy that mutagenesis of any upstream element of the promoter had the same effect as deletion of the distal and/or central domain (compare Fig. 4a with Fig. 1). These results suggest that all elements of these two domains must be present together for activity and that the distal and central domains function as one unit. The analysis of nuclear proteins which bind POMC regulatory elements has revealed that most elements are recognized by different proteins. Thus, POMC transcription appears to result from the compounded activity of multiple regulatory elements which, for the most part, interact with different nuclear factors (Fig. 9).

Within the proximal domain, the replacement mutations confirmed the importance of PE-1, PE-2, and PE-4 but not of PE-3 (Fig. 4a). This last result is somewhat at odds with the 5′-deletion results (Fig. 4b) which suggested that sequences located between PE-4 and the TATA box also contribute to promoter activity. It may be that otherwise redundant elements of the proximal domain become active in the absence of the central and distal domains. We have previously shown that a binding site for the glucocorticoid receptor overlaps element PE-3 and contains sequences homologous to the CCAAT box sequence and the cyclic AMP response element (12). Binding studies using AtT-20 nuclear extracts have provided evidence only for binding of COUP-related proteins to PE-3 (32a) (Fig. 8). We have previously suggested (12) that COUP transcription factor may represent a positive transcription factor whose activity is impeded by glucocorticoid receptor binding to an overlapping DNA sequence. This model of hormone-dependent repression does not appear to be possible, because replacement mutations which destroy the COUP-binding site (mutant 15) did not decrease basal promoter activity (Fig. 4a). Thus, models other than

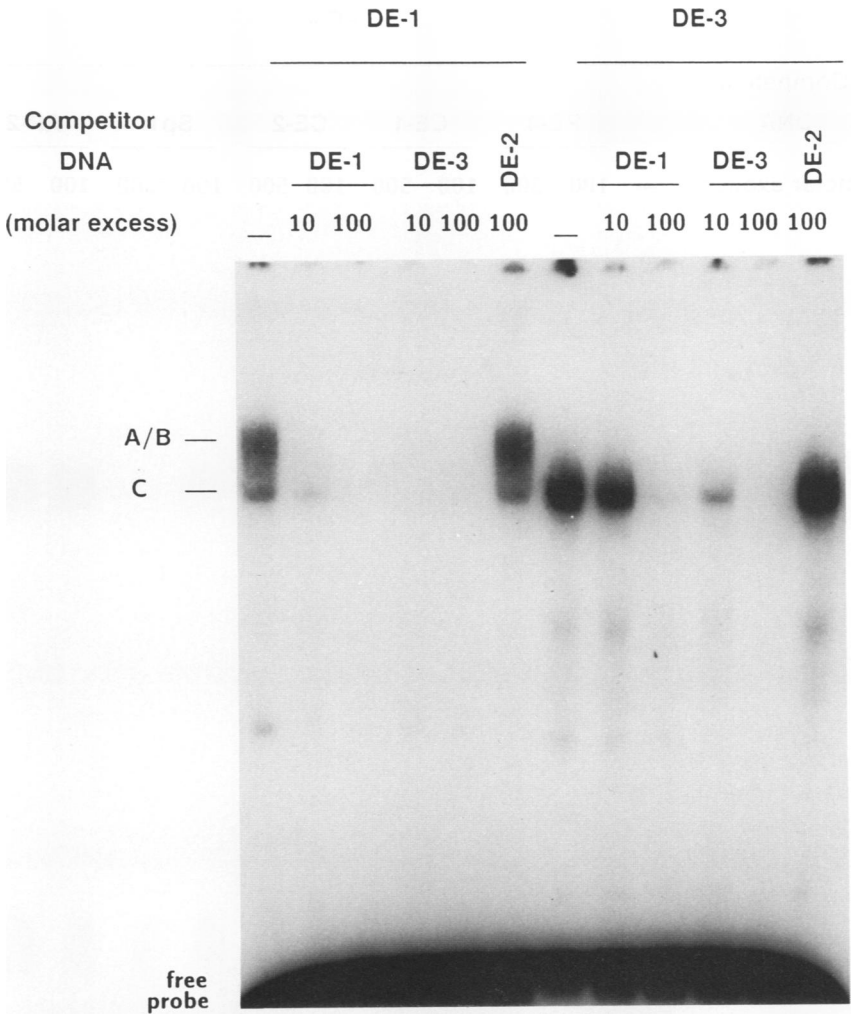

FIG. 6. DE-1 and DE-3 elements are recognized by related proteins. The competitive binding analysis by gel retardation assay used AtT-20 nuclear extracts (10 μg) and double-stranded DE-1 and DE-3 oligonucleotides (Fig. 5a) labeled at their 5′ extremities with ^{32}P. Three specific complexes (A, B, and C) were revealed by using DE-1 probe, and one specific complex (C) was revealed by using DE-3 probe. Nonlabeled double-stranded DE-1, DE-2, and DE-3 oligonucleotides (10 and 100 molar excesses) were used as competitor DNAs.

factor displacement by the glucocorticoid receptor must be envisaged to account for glucocorticoid repression of POMC.

We have shown that several different AtT-20 nuclear proteins bind specific DNA sequences within the rPOMC promoter (Fig. 9). Some of these are related to previously described transcription factors. Indeed, element PE-1 contains binding sites for both AP-1 and COUP transcription factors (Fig. 3). Purified HeLa AP-1 and COUP factors were shown to bind these sequences in vitro (32a). Whereas AtT-20 nuclear extracts contain proteins which bound these two elements under different conditions (Fig. 2e and f), extracts prepared from rat anterior pituitary tissues could bind both sequences at the same time. This difference between AtT-20 and pituitary extracts could be due to differences in the abundance of the proteins or the presence in these extracts of different members of the AP-1 and/or COUP family of transcription factors (9, 35, 36). Be that as it may, replacement mutants over either the AP-1 (mutant 19) or COUP (mutant 18) sequence decreased promoter activity (Fig. 4a), suggesting that both may be active in vivo.

Element PE-2 appeared important for POMC activity, since mutation 17 over this sequence decreased promoter activity (Fig. 4a) (28). Proteins binding PE-2 (Fig. 5) may be related to a protein characterized by Riegel et al. (28). Competition binding experiments indicated that PE-2-binding proteins do not bind the other elements of the promoter (data not shown). Despite its position between the TATA box and the initiation site, element PE-2 is not homologous to the DNA sequence of previously described initiator regulatory elements (32). Elements PE-4 and CE-1 are recognized in vitro by the same proteins which appeared related in binding specificity to transcription factor Sp1 (Fig. 7). However, these proteins differ in gel mobility compared with the slower-migrating complexes formed with the Sp1-binding site of simian virus 40 (data not shown). Thus, these proteins may be related to but distinct from Sp1. Whereas the affinity of these Sp1-related proteins appeared weaker for CE-1 than for PE-4 (Fig. 7), mutagenesis of either CE-1 or PE-4 had similar effects (Fig. 4a).

The central domain may contain regulatory elements in addition to CE-1 and CE-2 (which is bound by proteins that

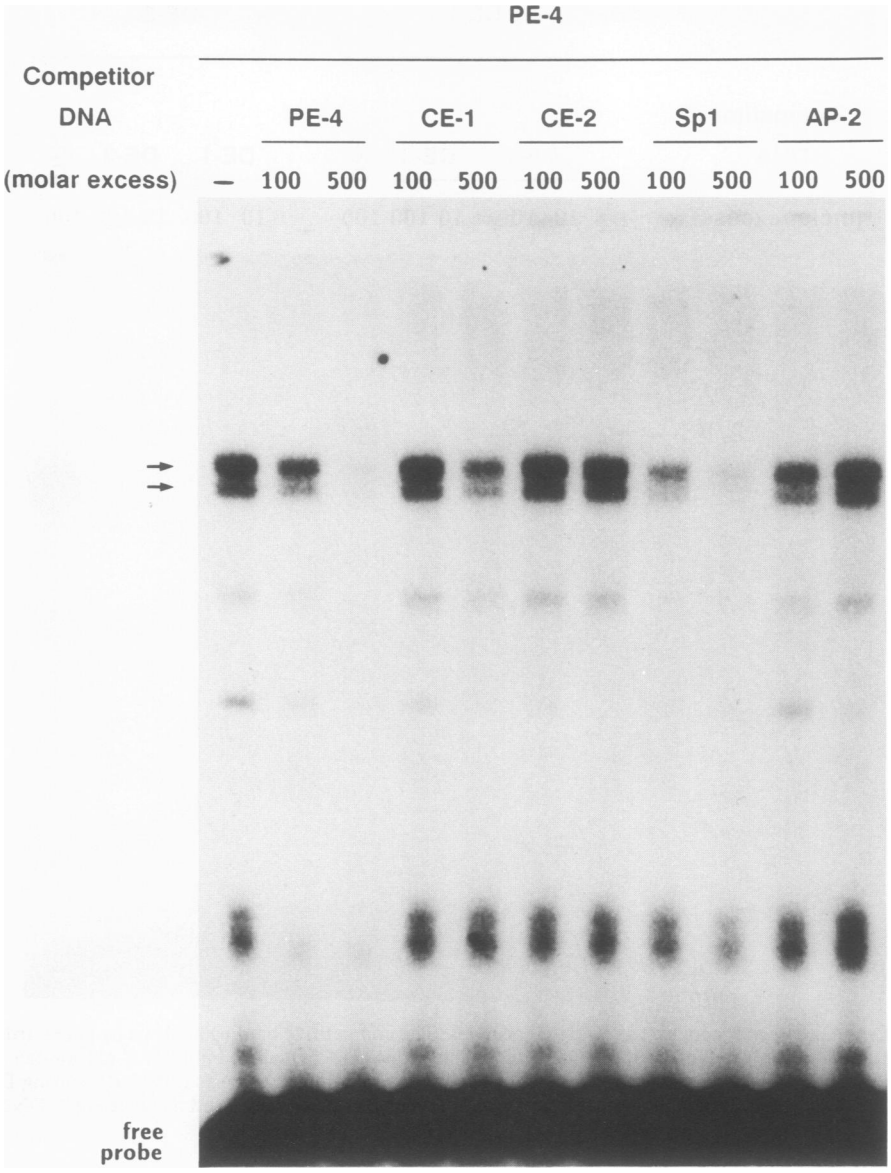

FIG. 7. Elements PE-4 and CE-1 are recognized by proteins related to Sp1 transcription factor. The competitive binding analysis used 5 μg of AtT-20 whole-cell extract and double-stranded PE-4 oligonucleotide probe. Excess unlabeled competitor oligonucleotides (PE-4, CE-1, CE-2, Sp1, and AP-2) were used at molar ratios of 100- and 500-fold. Arrows indicate the major species described in this study.

did not appear to recognize other elements of the promoter). Indeed, replacement mutations were introduced in three sequences of unclear function within the central domain. They include two sequences which exhibited footprints (data not shown) only in the absence of poly(dI-dC) competitor DNA (positions −230 to −214 [mutant 6] and −168 to −152 [mutant 9]) and one sequence (positions −188 to −182 [mutant 8]) which is similar to the simian virus 40 core enhancer consensus (38). Replacement mutations over these three sequences impeded promoter activity (Fig. 4a).

Within the distal domain, three elements appeared essential for activity, whereas DE-4 seemed dispensable (Fig. 4a). When tested as a whole on a minimal POMC promoter, the distal domain was inactive, suggesting that elements DE-1, DE-2, and DE-3 function only in association with an element(s) present in the central domain (Fig. 1). Although the same proteins bind DE-1 and DE-3 (Fig. 6), there is no obvious similarity in the DNA sequences of these two elements or with other transcription factor-binding sites (15). Since DE-2 is AT-rich and somewhat related to Pit-1 sites (20, 25), we tested whether Pit-1/GHF-1 might bind to DE-2. In competition binding experiments, it was clear that a Pit-1/GHF-1 oligonucleotide did not compete for DE-2 binding with AtT-20 nuclear extracts (data not shown). Further, the Pit-1/GHF-1 activity present in GH_3 extract did not bind DE-2 significantly, and its binding to the rat GHF-1 site 1 (6) was not inhibited by excess DE-2 (data not shown). In agreement with previous reports (31), we were unable to detect Pit-1/GHF-1 activity in AtT-20 cells. Furthermore, the role of Pit-1/GHF-1 appears restricted to activation of late pituitary genes like GH and PRL, as it is not present early in pituitary development when the POMC gene is

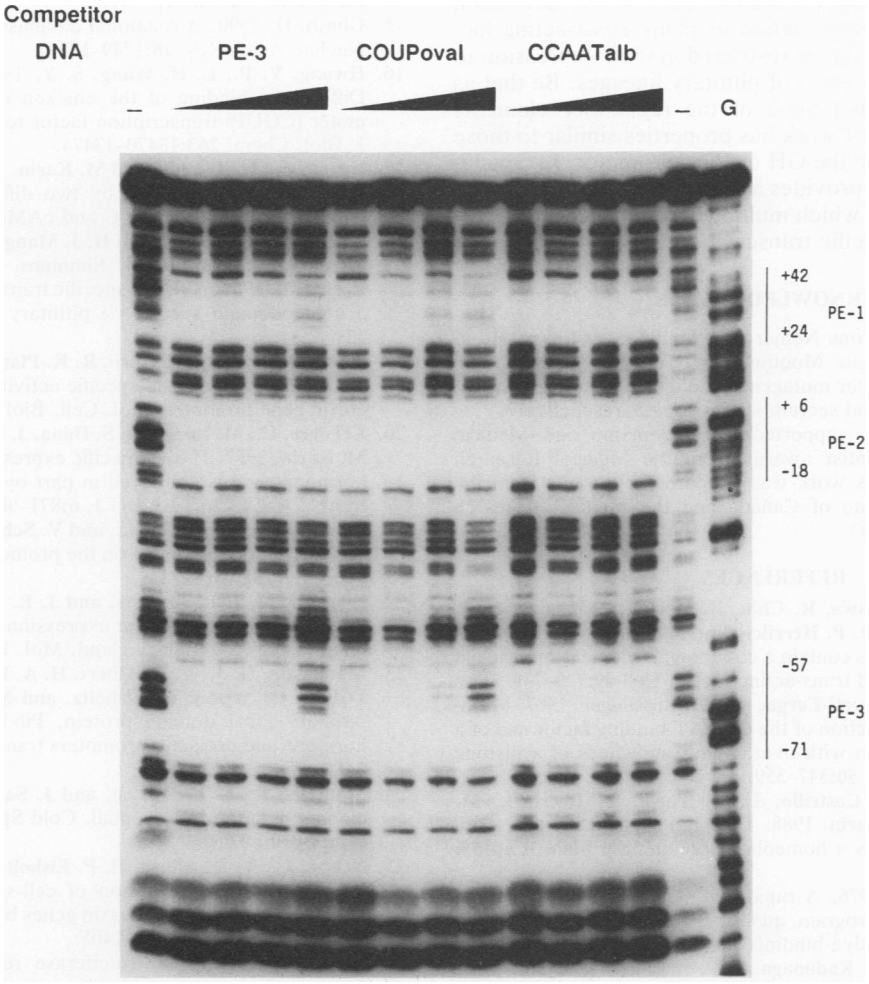

FIG. 8. Nuclear proteins related to COUP transcription factor bind elements PE-1 and PE-3 with similar affinities. The binding of AtT-20 nuclear extract (10 μg) was inhibited by specific double-stranded oligonucleotides, as indicated at the top in a DNase I footprinting assay. Lanes marked with a dash indicate control DNase I digestion in which the nuclear extract was replaced by BSA. Lane G indicates the DNA sequence ladder. The positions of footprints PE-1, PE-2, and PE-3 are shown alongside the gel. Increasing amounts of competitor DNA were added in the binding assay, as indicated by the wedges at the top of the gel.

activated. Thus, other pituitary factors must be responsible for gene activation and differentiation in the earlier stages of pituitary development. The POMC gene is likely to be an appropriate model to identify factors involved in early pituitary development and possibly involved in pituitary determination.

Comparison of our results with those obtained in other laboratories on the organization and activation of the GH and PRL genes suggests different mechanisms of cell-specific promoter recognition. Indeed, a single transcription factor, Pit-1/GHF-1, plays a predominant role in the activation of the GH and PRL genes late in pituitary development. In contrast, the POMC gene is expressed at an early stage of pituitary differentiation and its transcription requires multiple *trans*-acting factors, each contributing equally to promoter activity (Fig. 9). Thus, it may be that during differen-

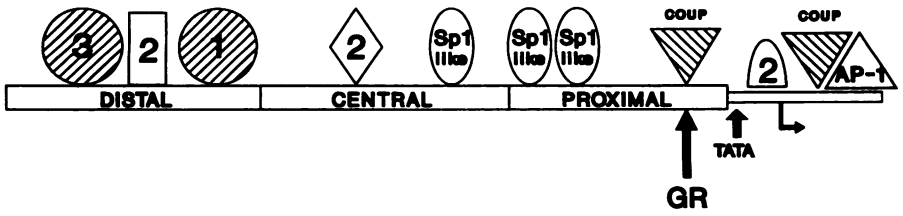

FIG. 9. Schematic representation of the various nuclear proteins documented in this work to bind regulatory elements of the rPOMC promoter. For elements which bind known transcription factors or protein related to them, the names of these factors are indicated on the symbols. GR indicates the position of the previously identified in vitro binding site for glucocorticoid receptor (13).

tiation of the corticotroph phenotype, POMC activation requires the simultaneous action of many *trans*-acting factors, some of which may be restricted in their expression to corticotrophs or to a subset of pituitary lineages. Be that as it may, it is clear that none of the regulatory elements identified in the present work has properties similar to those of Pit-1/GHF-1 sites in the GH or PRL promoter. In conclusion, the POMC gene provides a different model of pituitary-specific expression in which multiple factors must act simultaneously for cell-specific transcription.

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