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Cell-Specific Helix-Loop-Helix Factor Required for Pituitary Expression of the Pro-Opiomelanocortin Gene

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Pro-opiomelanocortin (POMC)-expressing cells appear to be the first pituitary cells committed to hormone production. In this work, we have identified an element of the POMC promoter which confers cell-specific activity. This element did not exhibit any activity on its own and required at least one other element of the promoter to manifest its cell-specific activity. Fine mutagenesis of this element indicated that a CANNTG motif is responsible for activity. This E-box motif is typical of binding sites for helix-loop-helix (HLH) transcription factors; however, the POMC cell-specific E box cannot be replaced by other E boxes like the κE2 site of the immunoglobulin gene or a muscle-specific E box. Similar E boxes which are present in the insulin gene promoter were shown to contribute to the pancreatic specificity of the insulin promoter. However, E-box-binding proteins found in nuclear extracts from POMC-expressing AtT-20 cells and from insulin-expressing cells have different electrophoretic mobilities. The AtT-20 proteins were named CUTE (for corticotroph upstream transcription element-binding) proteins, and they were not found in any other cells. CUTE proteins have DNA-binding properties characteristic of HLH transcription factors. Overexpression of the dominant negative HLH protein Id or of the ubiquitous positive HLH factor rat Pan-2 decreased or augmented POMC promoter activity, respectively. These observations are consistent with the hypothesis that CUTE factors might be heterodimers. This hypothesis was further supported by antibody shift experiments and by abrogation of DNA binding in the presence of bacterially expressed Id protein. Thus, the cell-specific CUTE proteins and their binding site in the POMC promoter appear to be important determinants for cell specificity of this promoter. The requirement for HLH factors in POMC transcription also presents the possibility that these factors are involved in differentiation of pituitary cells, in analogy with the role of HLH factors in muscle development.

During development of complex organisms, differentiation of specific cell types is thought to be mediated by several converging cues or signals. Extracellular signals, including growth factors and matrix components, exert their effects through specific receptors coupled to intracellular signal transduction pathways (13, 49, 55). Ultimately, extracellular signals and their intracellular mediators alter cell phenotype by their effect on transcription factors and gene expression (40). Different classes of transcription factors have been identified through genetic and biochemical means and shown to be key players during differentiation events (28, 36). Accordingly, these transcription factors often exhibit spatially and temporally restricted distribution which in several cases corresponds to a specific pattern of target gene expression (19, 57, 73). In particular, one class of transcription factors which has been associated with cell differentiation and cell-specific expression is the helix-loop-helix (HLH) family of factors (52, 78). Various members of this family were shown to be important in specifying cell type; examples are the MyoD gene family, which plays a pivotal role in mammalian muscle differentiation (78), and the *daughterless* and *achaete-scute* gene products, which regulate *Drosophila* neurogenesis and sex determination (8, 75). The myogenic factors, including MyoD, are unique in that their forced expression in various cells leads to activation of the myogenic phenotype (17, 79). Thus, these factors may function as determination factors in addition to their role as transcription factors.

The HLH family of transcription factors now includes nearly 40 independent genes which are characterized by a conserved DNA binding domain (52). This domain is constituted of a stretch of basic amino acids followed by a conserved sequence of 60 amino acids (HLH domain) presumed to form two α helices interrupted by a loop. Recent studies indicated that the HLH domain functions as a dimerization interface while the basic region is required for DNA recognition (15, 76). HLH factors were shown to bind DNA as homodimers or heterodimers (53). The sequence motif corresponding to the DNA binding site consensus for HLH factors (CANNTG), the so-called E box, was initially identified in the immunoglobulin heavy-chain enhancer (11, 21). Since then, several other functional E boxes have been identified within enhancer regions of many cell-specific genes, e.g., in control regions of other immunoglobulin genes (27, 42), in skeletal muscle-specific genes such as the muscle creatine kinase (MCK) gene (6) and the acetyl choline receptor α-subunit gene (58), in pancreatic-specific genes such as the insulin I and II genes (14, 38, 80), and in the chymotrypsin B gene (51).

HLH factors have not been yet implicated in specific expression of pituitary genes. So far, only one pituitary-specific transcription factor, Pit-1/GHF-1, has been identified, and it is a prototypic member of the POU family of transcription factors (5, 32). We now present evidence for the requirement of HLH factors in the cell-specific expres-

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sion of the pro-opiomelanocortin (POMC) gene in the pituitary. This gland offers an excellent model with which to investigate molecular mechanisms responsible for cell-specific expression and differentiation. It originates from an invagination of the somatic ectoderm called Rathke's pouch, which in turn gives rise sequentially to five distinct hormone-secreting cell types. The POMC-expressing corticotroph cells are the first hormone-producing cells to appear during differentiation of Rathke's pouch (9, 46, 77). The other cell types, i.e., thyrotrophs, gonadotrophs, somatotrophs, and lactotrophs, which each produce a distinct hormone, appear later during pituitary development. Analysis of the Pit-1/GHF-1 factor indicated that it is required for growth hormone (GH) and prolactin (PRL) gene expression in somatotroph and lactotroph cells, respectively (47). In addition to this role in cell-specific transcription, it has been suggested that Pit-1/GHF-1 is responsible for either the commitment or maintenance of three pituitary cell lineages. Indeed, a natural mutation occurring in the Pit-1/GHF-1 gene that resulted in a protein that no longer binds DNA produced dwarf mice that lack thyrotroph, somatotroph, and lactotroph cells (7, 43). Since corticotroph and gonadotroph cells are not affected by this mutation, it is likely that other factors are responsible for their determination. Thus, analysis of the POMC promoter and the characterization of factors responsible for its cell-specific expression might identify other pituitary factors that act during the early events of pituitary ontogenesis.

We have previously localized the rat POMC (rPOMC) promoter to a 543-bp 5′ flanking DNA fragment of the gene which is sufficient to confer cell-specific expression in transfection experiments (33) and in transgenic mice (74). We recently showed that at least nine regulatory elements are present in the rPOMC promoter and that they each contribute to a similar extent to promoter activity (72), although it was not clear which is (are) cell specific. Interestingly, the distal and central domains of this promoter (about 150 bp each) appeared to function in a unique manner, in that they absolutely required each other for activity. We now present evidence that the synergistic activity of central and distal domains is cell specific. This activity requires a CAGATG motif present in element DE-2 of the distal domain. Furthermore, transfection and in vitro protein-DNA binding experiments have indicated that corticotroph-specific HLH factors bind this motif, and they appear to be responsible for the corticotroph-specific activity of the distal domain. Thus, in addition to their role in muscle, pancreas, and lymphocytes, this study presents evidence for a role of HLH factors in pituitary-specific transcription.

MATERIALS AND METHODS

Plasmids and oligonucleotides. The various rPOMC promoter constructs were made in vector pXP1-luciferase (56) and were described previously (72). Oligonucleotides were cloned into *Bam*HI sites located either at the 5′ end of the minimal rPOMC promoter (−34 to +63 bp) or at the 5′ end of the central domain (−323 to −166 bp) luciferase construct as shown in the figures.

Oligonucleotides were synthesized by the phosphoramidite procedure, using an Applied Biosystems instrument. PE-2, DE-1, and DE-3 oligonucleotides were described previously (72). The 47-mer DE-2 oligonucleotide included rPOMC sequences between −410 and −369 bp; shorter DE-2 oligonucleotides contained a subset of this sequence as indicated in the figures. Sequences corresponding to insulin gene E-box (IEB), κE2, and MCK oligonucleotides were taken from the rat insulin I gene (38), the mouse κ-light-chain immunoglobulin gene (20), and the mouse MCK gene (6) and were as follows: IEB, 5′-AGGTAGGCAGATGGCGAGAG GGGC-3′; κE2, 5′-TCCCAGGCAGGTGGCCCAGATTAC 3′; and MCK, 5′-CAGGCAGCAGGTGTTGGGGG-3′. To facilitate their directional cloning, the oligonucleotides contained *Bam*HI and *Bgl*II extremities.

Cell culture and nuclear microextraction. AtT-20 (murine corticotroph tumor) and GH_3 (rat sommatotroph tumor) cells were grown in Dulbecco modified Eagle medium (DMEM) containing 10% fetal calf serum (33). HIT (hamster insulinoma) cells were maintained in DMEM containing 15% horse serum, 2.5% fetal calf serum, 10 μg of glutathione per ml, and 0.1 μM selenous acid. L (murine fibroblast) cells were grown in α-MEM containing 10% fetal calf serum. Raji (human Burkitt lymphoma) cells were grown in RPMI 1640 containing 10% fetal calf serum and 10 μM 2-mercaptoethanol. L6 (rat myoblast) cells were grown in DMEM containing 10% horse serum.

Nuclear microextraction from various cell lines was performed essentially as previously described (62), with slight modifications. Cells (2×10^7) were harvested in 2 ml of cold phosphate-buffered saline (PBS) containing 0.6 mM EDTA and pelleted at 5,000 × *g* for 30 s. Cell pellets were gently resuspended in 800 μl of cold buffer A (10 mM Tris-HCl [pH 7.9], 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol [DTT], 0.5 mM phenylmethylsulfonyl fluoride, 1 μg of aprotinin per ml, 1 μg of pepstatin per ml) and allowed to swell on ice for 15 min; then 25 μl of a 10% Nonidet P-40 solution was added to the cell suspensions, and the cells were vortexed for 5 s. After centrifugation at 12,000 × *g* for 5 s, the nuclear pellets were resuspended in 150 μl of cold buffer B (20 mM Tris-HCl [pH 7.9], 400 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride, 1 μg of aprotinin per ml, 1 μg of pepstatin per ml) and rocked for 1 h at 4°C. After removal of nuclear debris by centrifugation at 12,000 × *g* for 5 min at 4°C, the microextracts were stored at −90°C.

Electroporation and luciferase assays. For electroporation, exponentially growing AtT-20, GH_3 and L cells were harvested, washed, and resuspended in cold PBS buffer. Then 350 μl of cells (10^7/ml) was electroporated as described previously (72), with 25 μg of reporter plasmids and 25 μg of pRSVGH (65) used as the internal control to correct for variations in transfection efficiency. Coelectroporation was performed essentially as described above with 1.25 to 5 μg of reporter plasmids, 2.5 to 10 μg of effector plasmids, and 15 μg of pRSVGH. After electroporation, cells were plated and cultivated for 16 h before preparation of extracts used in the luciferase assay as described previously (16).

Gel retardation assays. Gel retardation assays were done as described previously (1), using crude nuclear microextracts from various cell lines. Briefly, 5 fmol of 5′-end-labeled oligonucleotides was incubated with 2 to 4 μg of proteins for 20 min on ice in a total volume of 15 μl containing 25 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES; pH 7.2), 150 mM KCl, 5 mM DTT, 10% glycerol, and 1 to 2 μg of a 1:1 mixture of poly(dI-dC) and poly(dA-dT) used as nonspecific competitor DNA. For competition experiments, probe and competitor DNA were mixed together before addition of proteins. The samples were resolved on 6% polyacrylamide gels (acrylamide/bisacrylamide ratio of 44:0.8) buffered and run for about 2.5 h in 40 mM Tris-HCl–195 mM glycine (pH 8.5) at 4°C.

For antibody supershift experiments, the antibody (53)

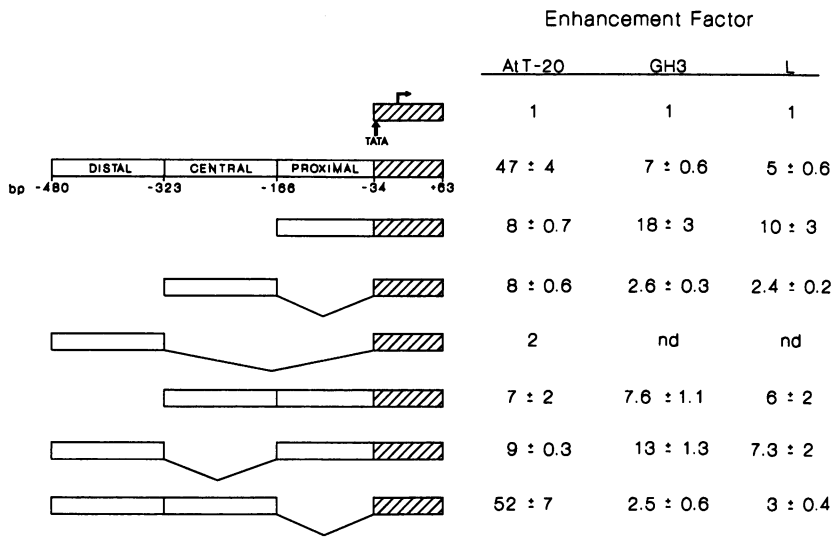

FIG. 1. Corticotroph specificity of the synergistic activity of the central and distal domains. The three rPOMC domains were inserted either individually or in combination upstream of a minimal rPOMC promoter (hatched box) fused to the firefly luciferase reporter gene and tested by electroporation into AtT-20, GH_3, and L cells. The luciferase activity of each construct is presented relative to the activity of the minimal rPOMC promoter construct. The data represent the means ± standard errors of the means of 2 to 16 experiments, each performed in duplicate. nd, not determined.

was added to the nuclear extracts and incubated for 10 min at room temperature before addition of the probe. The binding reaction mixture was further incubated for 15 min at room temperature and applied to the gel as described above.

The glutathione *S*-transferase (GST) and GST-Id proteins were expressed in bacteria and purified as described previously (67). Purified GST or GST-Id protein (1 μg) was added to AtT-20 nuclear extracts (2.5 μg) and denatured by dialysis twice for 1 h at 4°C against 6 M guanidine HCl in 20 mM Tris-HCl (pH 7.9)–50 mM KCl–0.5 mM EDTA–1 mM DTT–10% glycerol. The extracts were similarly renatured by dialysis against the same buffer without guanidine. The extracts were cleared of a slight precipitate by centrifugation and use in electrophoretic mobility shift assays (EMSA) as described above.

Methylation interference assays. DE-2 oligonucleotides were selectively labeled at either 5′ end and subjected to partial methylation at guanine residues as detailed by Maxam and Gilbert (48). Binding reactions were in the same conditions as above. After electrophoresis, the gel was wrapped with Saran Wrap and exposed for 1 h at 4°C. Bound and free probes were excised and electroeluted to recover the DNA. After ethanol precipitation, the DNA was redissolved in 50 μl of 10% piperidine solution, and base cleavage reactions were carried out for 20 min at 90°C. The samples were resolved on 15% polyacrylamide sequencing gels.

RESULTS

Localization of a corticotroph-specific element. To localize rPOMC sequences required for corticotroph-specific activity, we used various cell lines as hosts to test the activity of rPOMC promoter constructs. We used two cell lines of pituitary origin, POMC-expressing AtT-20 cells and GH-expressing GH_3 cells, and a cell line of fibroblastic origin (L cells) that does not express any pituitary hormone gene. We have previously shown that the rPOMC promoter contains three functional domains, the proximal, central, and distal domains (72). When inserted independently upstream of a minimal rPOMC promoter (−34 to +63 bp), the proximal and central domains activated transcription in AtT-20 cells about eightfold, but the distal domain was almost inactive (Fig. 1). When tested in pairs, the proximal and central domains appeared redundant, whereas the central and distal domains exhibited a marked synergy in AtT-20 cells. We tested the transcriptional specificity of these three domains by transfecting the same constructs into non-POMC-expressing cells. As shown in Fig. 1, the proximal and central domains also activated transcription in GH_3 and L cells. Most significantly, the synergistic activity of the central and distal domains observed in AtT-20 cells was completely absent in GH_3 and L cells. Thus, the corticotroph-specific activity of the rPOMC promoter appears to reside within the central/distal domain. Consequently, we looked for cell-specific elements within this functional unit.

Three regulatory elements, named DE-1, DE-2, and DE-3, were previously localized within the distal domain of the rPOMC promoter. To determine which of these elements is responsible for corticotroph specificity and/or for the synergistic interaction with the central domain, we constructed chimeric promoters in which oligonucleotides corresponding to each distal element were inserted upstream of the central domain. The constructs were transfected into AtT-20, GH_3, and L cells (Fig. 2). Elements DE-1 and DE-3 were weakly active in this context. In sharp contrast, one copy of a 47-bp oligonucleotide containing element DE-2 was as active as the entire distal region in AtT-20 cells, and two copies of this element enhanced activity of the central domain 65-fold. This activity was exquisitely cell specific, as element DE-2 was almost inactive in GH_3 and L cells. The strong AtT-20-specific activity of DE-2 was entirely dependent on the presence of the central domain, as two or four copies of DE-2 were devoid of any activity when inserted upstream of the minimal promoter (Fig. 2 and data not shown). The synergistic activity of DE-2 and central domain is independent on specific positioning of these two entities. Indeed, DE-2 oligonucleotides were also active in the reverse orientation, and the central and distal domains could be inverted

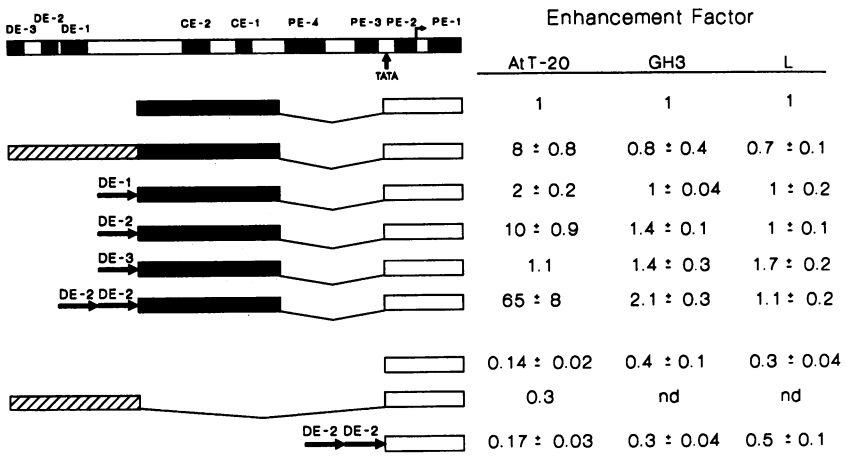

FIG. 2. Evidence that corticotroph-specific activity is conferred by DE-2 element. The activity of the distal domain (hatched box) or its elements (DE-1, DE-2, and DE-3) was tested by insertion upstream of the minimal POMC promoter (open box) in either the presence or absence of the central domain (dark box). The luciferase activity for each construct is presented relative to the activity of the construct containing the central domain fused to the minimal rPOMC promoter. A schematic drawing of regulatory elements previously identified within the promoter is shown at the top (72). The PE, CE, and DE elements are located within the proximal, central, and distal domains, respectively. The data represent the means ± standard errors of the means of 2 to 10 experiments, each performed in duplicate. nd, not determined.

without loss of activity (data not shown). Furthermore, DE-2 oligonucleotides could be inserted at various distances relative to the central domain and retained activity; the insertion of other sequences in place of the central domain did not confer DE-2-dependent activity (for example, juxtaposition of distal and proximal domains [Fig. 1 and data not shown]). Taken together, these data suggest that DE-2 is a major determinant for corticotroph-specific activity of the rPOMC promoter.

DE-2 activity depends on E box. Detailed examination of the DE-2 DNA sequence (Fig. 3) revealed three motifs of interest. First, an A/T-rich stretch (labeled DE-2A) is present within the DE-2 footprint that we previously characterized (72). Also, two CANNTG motifs (labeled DE-2B and DE-2C) are present downstream of the A/T-rich stretch. These E boxes might represent binding sites for transcription factors of the HLH family (52). To determine which of these sequence motifs contribute to DE-2 activity, we tested the activity of various DE-2 mutant oligonucleotides in AtT-20 cells (Fig. 3). Truncation of DE-2A resulted in a two- to threefold drop in activity, in agreement with a previous mutation of this motif within the native rPOMC promoter (72). A DE-2A oligonucleotide was found to be inactive on its own in this context (data not shown). Nevertheless, the DE-2 element deleted of DE-2A retained significant activity. Three block mutations, over and between the E boxes, indicated that motif DE-2C is the most active. Indeed, mutation over DE-2C and DE-2B E boxes resulted in 7- and 2.5-fold reduction of DE-2 activity, respectively, while a mutation between the E boxes did not significantly affect activity (Fig. 3). The importance of the CANNTG motif for activity was further highlighted by the 18-fold activation produced by a short oligonucleotide containing only 11 bp of DE-2C in AtT-20 cells (Fig. 3). None of these oligonucleotides were active in GH_3 cells (data not shown). Thus, the cell-specific activity of DE-2 appeared to reside mostly within an E box present in DE-2C.

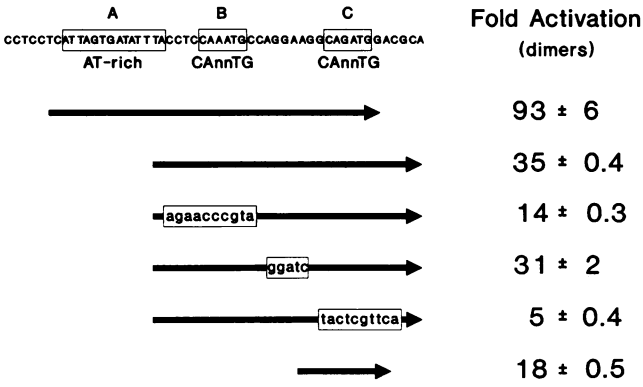

FIG. 3. Evidence that DE-2 is a composite element. The relative role of the three motifs identified within DE-2 was assessed by electroporation into AtT-20 cells of various oligonucleotides inserted in two copies upstream of the central domain as in Fig. 2. Motif A represents sequences previously identified by DNase I footprinting (72). Motifs B and C correspond to the conserved CANNTG consensus binding site for HLH transcription factors. The DNA sequence of each oligonucleotide (47, 37, or 16 bp) is indicated by arrows, and boxes indicate the positions of replacement mutations introduced in some oligonucleotides. Data from a representative experiment performed in duplicate are shown (means ± standard deviations).

To determine whether this E box or CANNTG motif is responsible for corticotroph-specific activity, we generated a series of DE-2C mutations. Mutations M2 to M5, which destroyed the HLH or E box, also abolished the activity of DE-2C (Fig. 4). Mutations M1 and M7, which change sequences flanking the DE-2C motif, had no effect, whereas mutation M6, which changes two nucleotides immediately 3′ of the E box, abrogated DE-2C activity. Thus, it appears that the corticotroph-specific activity of DE-2C can be ascribed to a NCANNTGN motif, in agreement with previous studies on E boxes (4, 31, 70).

HLH proteins bind element DE-2C. We performed EMSA using DE-2C as a probe in order to analyze AtT-20 nuclear proteins which bind DE-2C. AtT-20 nuclear extracts pro-

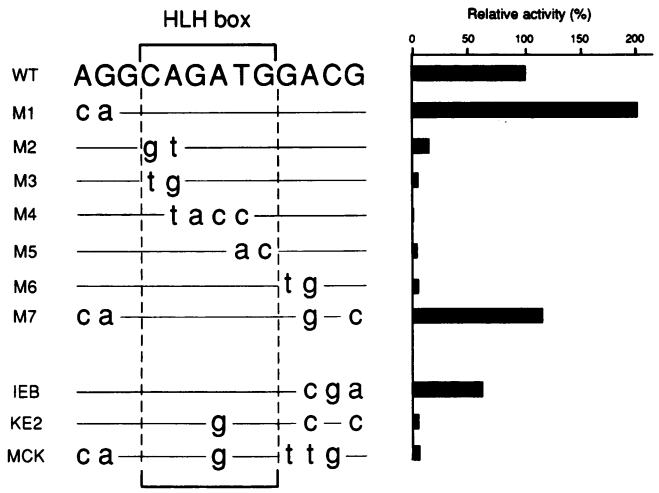

FIG. 4. Evidence that the DE-2C CANNTG motif is essential for cell-specific activity. The WT DE-2C sequence from −379 to −367 bp in the promoter is presented at the top and used as reference. Seven DE-2C mutant oligonucleotides (M1 to M7) are shown, with the mutated nucleotides indicated in lowercase. These and other naturally occurring binding sites for HLH factors (HLH box) were tested as dimers by electroporation in AtT-20 cells as described for Fig. 3. HLH binding sites tested include an IEB from the rat insulin I gene (39), the κE2 site from the mouse κ-light-chain immunoglobulin gene (20), and the MCK E box from the mouse MCK gene (6). The data represent the averages of two to five experiments, each performed in duplicate. The standard error of the mean represents a margin of error ranging from 5 to 25% of the value as in preceding figures.

duced four specific complexes with the DE-2C probe: two slow-migrating complexes named CUTE (for corticotroph upstream transcription element-binding proteins) and two faster-migrating complexes labeled PE-2 (Fig. 5). This latter doublet was so labeled because competitive binding experiments indicated that an oligonucleotide containing the PE-2

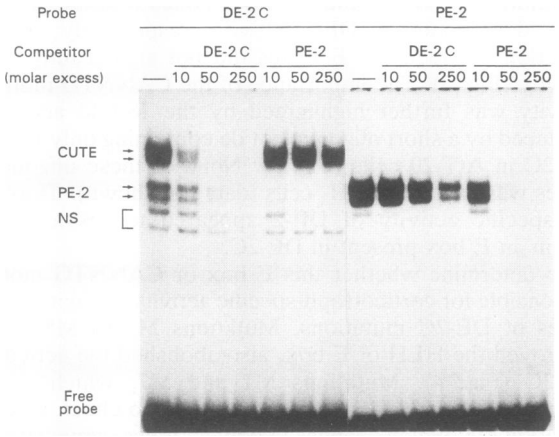

FIG. 5. Recognition of elements DE-2C and PE-2 by related proteins. A gel mobility shift assay was used to characterize AtT-20 nuclear proteins (4 μg of nuclear extract) which bind DE-2C (−383 to −359 bp) and PE-2 (−24 to +2 bp) oligonucleotides. A doublet of retarded bands, labeled CUTE, was competed for only by excess DE-2C, while another doublet, labeled PE-2, was competed for by excess DE-2C or PE-2. Bands labeled NS were either nonspecific or not observed reproducibly.

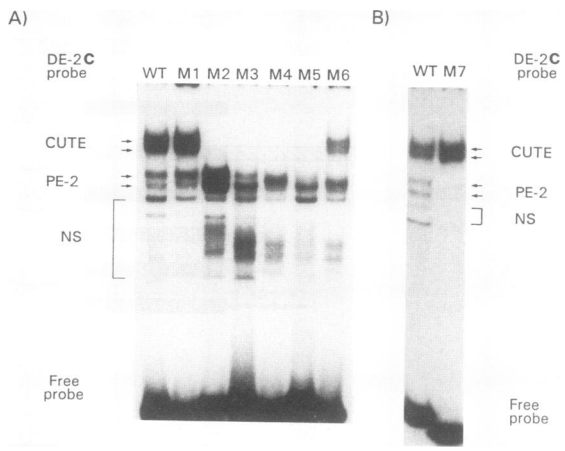

FIG. 6. Evidence that binding of CUTE proteins to DE-2C is prevented by mutations in the CANNTG motif. Binding of DE-2C oligonucleotides described in the legend to Fig. 4 to AtT-20 nuclear extract (4 μg) is shown. Binding conditions and the labeling of bands are as for Fig. 5. The various probes were all labeled to similar specific activity.

element (located at −18 to +6 bp [72]) was able to compete for the faster-migrating DE-2C complexes (Fig. 5). Similarly, DE-2C displaced binding to PE-2, although with at least fivefold-lower affinity. The PE-2 sequence did not compete for binding of the CUTE proteins, suggesting that there is no relationship between the CUTE and PE-2 complexes. This conjecture was further confirmed by the analysis of dimethyl sulfate (DMS) interference patterns of CUTE and PE-2 complexes, which indicated that PE-2 proteins bound downstream of CUTE proteins (data not shown and see below). Although the DNA sequences of DE-2C and PE-2 show only limited homology, greater similarity was observed for DMS interference patterns of the two probes; this pattern was similar to that reported for the PO-B factor, which was characterized in C127 mammary tumor cells and found to bind PE-2 (60).

To correlate binding of AtT-20 proteins with the ability of DE-2C to activate cell-specific transcription, we analyzed the binding properties of DE-2C mutations M1 to M7 (Fig. 4), using them as probes in EMSA. Mutations M1 and M7 were fully active (Fig. 4). Whereas mutation M1 had a binding pattern identical to that of wild-type (WT) DE-2C, mutation M7 bound CUTE as well as did WT DE-2C but did not bind PE-2 proteins at all (Fig. 6B). Thus, PE-2 binding is dispensable for corticotroph-specific activity. This finding is consistent with the complete lack of activity of plasmid constructs which contained one to three copies of the PE-2 oligonucleotide in place of DE-2 (data not shown). Mutations M2 to M5, which destroyed the CANNTG motif and were all transcriptionally inactive (Fig. 4), showed a complete loss of CUTE binding. Mutation M6, which was inactive in transfections, still bound CUTE, although this binding appeared to be of lower affinity than that of WT DE-2C (Fig. 6A). Competitive binding assays confirmed that CUTE proteins have at least three- to fivefold-lower affinity for mutation M6 than for WT DE-2C (Fig. 7). The analysis of the DMS methylation interference patterns of CUTE complexes (Fig. 8) revealed patterns similar to those previously reported for other E boxes and HLH factors (Table 1). The two CUTE complexes appeared to be closely related because they produced identical interference patterns. Taken together,

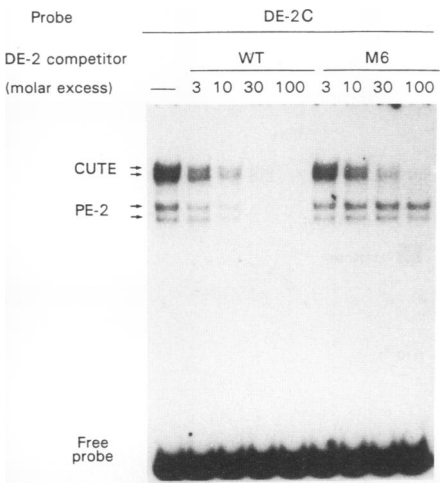

FIG. 7. Evidence that CUTE proteins have lower affinity for mutant M6 than for WT DE-2C. The binding of AtT-20 nuclear extract (2.7 μg) to DE-2C was competed for by excess unlabeled WT DE-2C or mutant M6 oligonucleotide.

these results indicate that the corticotroph-specific activity of DE-2C correlates with the binding of CUTE proteins to the HLH motif, NCANNTGN. In addition, the binding of CUTE proteins to DE-2C was competed for by excess κE2

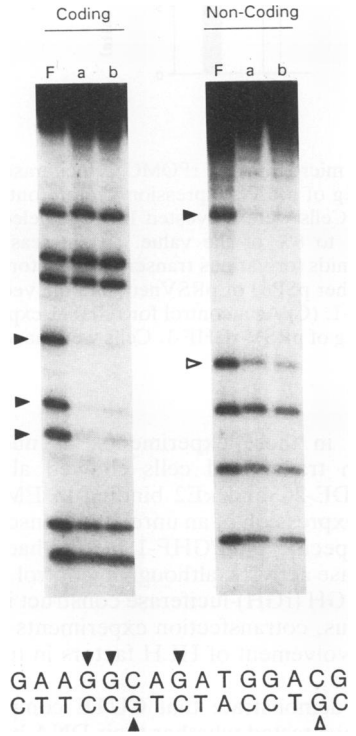

FIG. 8. Methylation interference analysis of CUTE complexes. Double-stranded DE-2C oligonucleotide was labeled with ^{32}P at the 5′ end of either the coding or noncoding strand, partially methylated with DMS, and used for gel mobility shift. Free (F) and bound probes (a [upper CUTE complex] and b [lower CUTE complex]) were excised from the gel, cleaved with piperidine, and separated on a 15% sequencing gel. The sequence of DE-2C from −381 to −367 bp is presented at the bottom. Closed and open arrowheads indicate methylated guanines which interfered completely and partially with binding, respectively.

TABLE 1. Comparison of methylation interference patterns for different HLH proteins[a]

Target gene (binding site)	Factor	Sequence
rPOMC (DE-2C)	CUTE	GGCAGATGGAC CCGTCTACCTG
Rat insulin I (Nir)	shPan-1	GGCAGATGGCG CCGTCTACCGC
Rat insulin II (Ripe 3a)	Ripe 3a1	AGCAGATGGCC TCGTCTACCGG
Immunoglobulin κ (κE2)	E47S/AS-C T3	GGCAGGTGGCC CCGTCCACCGG
Cardiac α-actin (E box)	E12/myogenin	GGCAGGTGCGG CCGTCCACGCC

[a] The methylation interference pattern of CUTE complexes with DE-2C (Fig. 8) is compared with patterns produced by other HLH factors. Data for binding of the Nir box of the rat insulin I gene are from German (25); data for binding of the Ripe 3a site of the rat insulin II gene are from Shieh and Tsai (1991); data for binding of the κE2 site of the κ-light-chain immunoglobulin gene are from Murre et al. (53); data for binding of an E box in the cardiac α-actin gene are from French (22). Residues that interfere completely or partially with protein binding are indicated with closed or open circles, respectively.

(Fig. 9), the well-characterized HLH factors binding site of the κ-light-chain immunoglobulin gene (52). Thus, CUTE proteins displayed a DNA binding specificity similar to that of HLH factors. This specificity can be contrasted with that

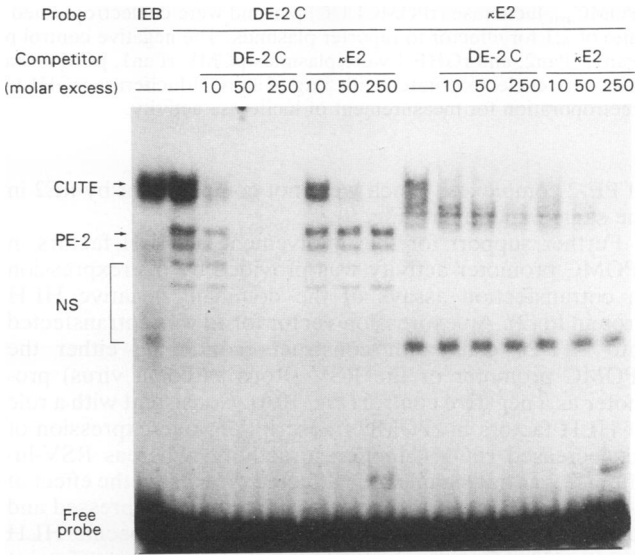

FIG. 9. Evidence that binding of DE-2C CUTE complexes is competed for by the κE2 E box. A gel mobility shift assay was performed as for previous figures, using AtT-20 nuclear extracts and an IEB, DE-2C, or κE2 probe. Unlabeled oligonucleotides were also used at the indicated molar excesses as competitors. Bands are labeled as in previous figures.

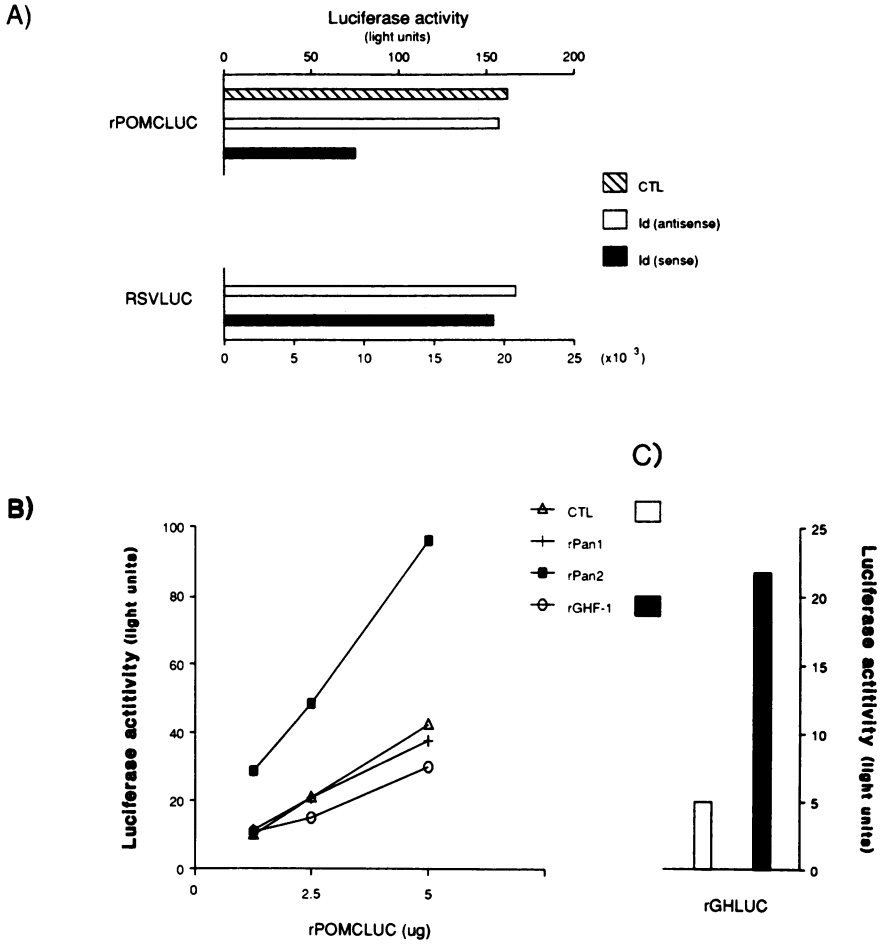

FIG. 10. Modulation of rPOMC promoter activity by HLH proteins. (A) Twenty-five micrograms of $rPOMC_{480}$-luciferase (rPOMCLUC) or RSV-luciferase (RSVLUC) plasmid was coelectroporated into AtT-20 cells with 25 μg of pMSV expression vector containing no insert (CTL) or Id cDNA inserted in either coding (sense) or inverted (antisense) orientation. Cells were harvested 10 h after electroporation for measurement of luciferase activity. The standard deviation represents an error of 1 to 8% of the value. (B) Increasing amounts of $rPOMC_{480}$-luciferase (rPOMCLUC) plasmid were coelectroporated with expression plasmids for various transcription factors, using a molar ratio of 2:1 for effector to reporter plasmids. The negative control plasmid (CTL) was either pSP64 or pRSVneo, and the vectors for factors rPan1, rPan2, and rGHF-1 were plasmids pCMV-rPan1, pCMV-rPan2, and pRSV-rGHF-1. (C) As a control for rGHF-1 expression, AtT-20 cells were coelectroporated with 5 μg of an rGH-luciferase (rGHLUC) construct and 10 μg of pRSV-rGHF-1. Cells were harvested 16 h after electroporation for measurement of luciferase activity.

of PE-2 complexes, which were not competed for by κE2 in the same experiment (Fig. 9).

Further support for the involvement of HLH factors in rPOMC promoter activity was provided by overexpression in cotransfection assays of the dominant negative HLH protein Id (2). An expression vector for Id was cotransfected into AtT-20 cells with constructs containing either the rPOMC promoter or the RSV (Rous sarcoma virus) promoter as a negative control (Fig. 10A). Consistent with a role of HLH factors in rPOMC transcription, overexpression of Id decreased rPOMC-luciferase activity, whereas RSV-luciferase was not significantly affected. We tested the effect of positive HLH factors which are ubiquitously expressed and known to form heterodimers with some cell-specific HLH factors (53). For this purpose, expression vectors for rat Pan-1 (rPan-1) and rPan-2 (54), which are homologous to the human E12 and E47 factors (52), were cotransfected with the rPOMC promoter construct (Fig. 10B). Interestingly, rPan-2 activated by threefold the rPOMC promoter, whereas rPan-1 had no effect. Both rPan-1 and rPan-2 appeared to be overexpressed in these experiments, as nuclear extracts prepared from transfected cells showed altered and increased total DE-2C and κE2 binding in EMSA (data not shown). Overexpression of an unrelated transcription factor, the pituitary-specific Pit-1/GHF-1 factor, had no effect on POMC-luciferase activity, although in control experiments it activated a rat GH (rGH)-luciferase construct in AtT-20 cells (Fig. 10C). Thus, cotransfection experiments provided support for the involvement of HLH factors in transcription of the POMC gene.

To directly demonstrate that CUTE complexes contain HLH factors, we tested whether their DNA binding activity can be blocked by bacterially expressed Id (34). A GST-Id fusion protein and GST itself were added to AtT-20 nuclear extracts and tested for binding to different DNA probes from the POMC promoter. GST-Id blocked CUTE binding to DE-2C or mutant M7 probe but not binding of the PE-2 proteins to the DE-2C or PE-2 probe; similarly, the binding of AtT-20 proteins to a POMC DE-3 probe was not affected by GST-Id (Fig. 11A). The hypothesis that CUTE complexes

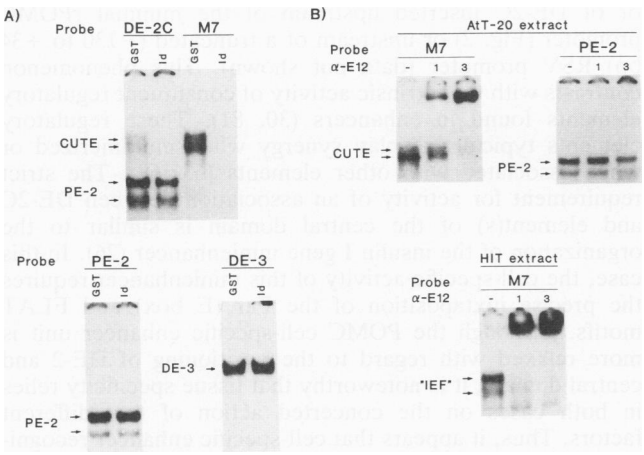

FIG. 11. Identification of CUTE proteins as HLH factors. (A) Bacterially expressed GST or GST-Id (Id) fusion protein was renatured with AtT-20 nuclear extracts. The extracts were then used in gel mobility shift assays with different oligonucleotide DNA probes from the POMC promoter as indicated. Probes DE-2C and M7 bind CUTE proteins, and probes DE-2C and PE-2 bind PE-2 proteins, as indicated. (B) An antiserum raised against the human E12 HLH factor (α-E12; 53) was added as indicated (in microliters) to gel mobility shift assays using the M7 or PE-2 probe and AtT-20 or HIT cell nuclear extracts. The E-box binding activity detected in HIT cell extracts is indicated (IEF) by analogy to previous work (1).

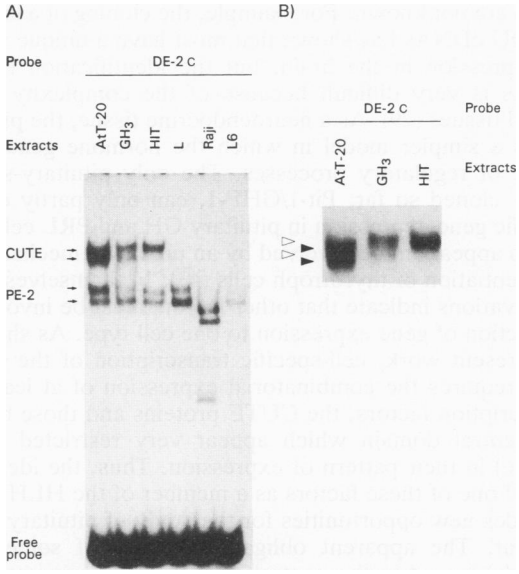

FIG. 12. Restriction of CUTE proteins to AtT-20 cells. (A) Gel mobility shift assay using 4 μg of nuclear extracts from various cell lines in binding reactions with the DE-2C oligonucleotide as a probe. CUTE and PE-2 complexes are indicated by arrows. (B) Gel mobility shift assay performed in high-resolution gel conditions to distinguish AtT-20 cell CUTE complexes (closed arrowheads) from complexes observed with GH_3 or HIT nuclear extracts (open arrowheads). The gels were 4% polyacrylamide and were run for 5 h.

may share epitopes with other HLH factors was tested directly by using an antiserum against the human E12 and E47 proteins (53) in EMSA. The CUTE complexes formed with the M7 probe were further retarded by the antiserum, whereas complexes formed with PE-2 probe were not (Fig. 11B). In agreement with previous work (66), the antiserum retarded HIT cell protein complexes with the E-box probe. Thus, the CUTE complexes contain proteins which share epitopes with the E12/E47 ubiquitous HLH factors, and their DNA binding activity is specifically blocked by an Id fusion protein, presumably through heterodimerization. These data suggest that CUTE proteins are either heterodimers of ubiquitous and cell-specific HLH factors or dimers of a cell-specific HLH factor(s).

CUTE proteins are corticotroph-specific HLH proteins. In view of the competition of CUTE complexes by κE2 (Fig. 9), we tested whether the κE2 E box could substitute for DE-2C. This E box did not exhibit any corticotroph-specific activity, and similarly, the E box from the MCK gene was devoid of activity (Fig. 4). This lack of activity could be due to the lower affinity of CUTE proteins for these E boxes, as shown for κE2 (Fig. 9). The comparison of various E boxes also indicated that the IEB1 site of the rat insulin I promoter (39) and the ICE site of the rat insulin II promoter (12) are identical to DE-2C. Accordingly, it was found that IEB could substitute for DE-2C in transfection experiments (Fig. 4) and that CUTE proteins bound efficiently to IEB (Fig. 9). It is thus possible that the same factors bind DE-2C and IEB and that they are present in POMC- and insulin-expressing cells.

To define the cellular distribution of CUTE proteins, we assessed their presence in various cell lines by EMSA and found them to be unique to AtT-20 cells (Fig. 12A). In contrast, the PE-2 complexes, which also form with DE-2C but do not correlate with activity, appeared more generally distributed. GH_3 nuclear extracts produced two complexes which migrated close to CUTE complexes, but they were clearly different when more resolving conditions were used (Fig. 12B). Similarly, insulin-expressing HIT cells nuclear extracts produced one complex which did not comigrate with CUTE complexes but which comigrated with the upper GH_3 complex (Fig. 12B). It was previously shown that HIT and GH_3 cell nuclear extracts produce complexes of similar mobility with IEB (1), in agreement with our observation. The L, Raji (human B-cell line), and L6 myoblast (rat skeletal muscle cell line) nuclear extracts did not produce any detectable CUTE complexes. Thus, AtT-20 nuclear extracts appeared to contain unique HLH proteins which form CUTE complexes with the DE-2C E box, and their binding correlated with corticotroph-specific activity of this element.

DISCUSSION

The analysis of gene expression in the pituitary gland has provided novel insight into the mechanism of cell-specific transcription. For instance, a new class of transcription factors, the POU proteins, was defined partly by the characterization of the pituitary-specific factor Pit-1/GHF-1 (5, 32). The identification of this factor on the basis of its specific action on the GH and PRL promoters has led to the demonstration of its role in the development of three pituitary cell types (7, 43). Outside of the pituitary, the POU and the related homeodomain proteins have also been most often implicated in the control of development, starting with the *Drosophila* homeotic genes in which the homeodomain was first identified (24, 64). In mammals, HOX genes have been shown to be essential for correct development (10, 45), and they were shown to have a specific spatial and temporal pattern of expression (41, 50). They are thought to act as transcription factors, but in most cases, their specific target

genes are not known. For example, the cloning of a large set of POU cDNAs has shown that most have a unique pattern of expression in the brain, but the identification of their targets is very difficult because of the complexity of the neural tissues (29). As a neuroendocrine tissue, the pituitary offers a simpler model in which the hormone gene is the target of regulatory processes. The only pituitary-specific factor cloned so far, Pit-1/GHF-1, can only partly explain specific gene expression in pituitary GH and PRL cells, and it also appears to be involved by an unknown mechanism in differentiation of thyrotroph cells (43). In themselves, these observations indicate that other factors must be involved in restriction of gene expression to one cell type. As shown in the present work, cell-specific transcription of the POMC gene requires the combinatorial expression of at least two transcription factors, the CUTE proteins and those binding the central domain which appear very restricted (if not unique) in their pattern of expression. Thus, the identification of one of these factors as a member of the HLH family provides new opportunities for the study of pituitary development. The apparent obligate presence of some other factor(s) bound to the central domain of the promoter also suggests that the HLH CUTE proteins must interact with some other factor(s) for activity. The finding that the binding site of the other factor is not an E box (unpublished observations) suggests interactions between members of different family of factors, although this point will be conclusively settled only once the factors are cloned.

Model for cell specificity of POMC promoter. In the pituitary, POMC expression is restricted to corticotroph and melanotroph cells of the anterior and intermediate lobes, respectively. We have previously mimicked this specific pattern of expression in transgenic mice (74) and in transfection studies (33) using a short POMC promoter fragment of only 543 bp. The activity of this promoter fragment in AtT-20 cells is dependent on the presence of at least nine regulatory elements which are binding sites for nuclear proteins present in AtT-20 cells (72) and other cells (unpublished observations). However, as most DNA-binding proteins identified in previous work were found to be widely distributed, they could not provide a working model to explain the cell specificity of POMC transcription.

We have now used deletion analysis and transfection in various cell lines to define a corticotroph-specific element of the POMC promoter. Interestingly, this element, DE-2, is devoid of activity on its own and absolutely requires the central domain of the promoter for activity (Fig. 1). The central domain also did not confer cell-specific activity on its own, although it exhibited transcriptional activity in many cell lines (Fig. 1). Thus, the synergistic activity of DE-2 with an element(s) of the central domain might represent the mechanism for cell-specific activation of POMC transcription. In agreement with this model, we have recently found that one of the many regulatory elements of the central domain exhibits marked synergy in association with DE-2 (data not shown). This synergistic activity is observed only in AtT-20 cells (Fig. 2 and 3 and data not shown). The strict specificity of DE-2C activity and the cell-specific distribution of CUTE proteins (Fig. 12) indicate that the DE-2C E box is an important determinant of POMC specificity. In addition, the central domain element which acts in synergy with DE-2C also appears to contribute to the cell specificity of the promoter.

The complete lack of activity of DE-2 on its own (even as multimers) is striking. Indeed, we have been unable to detect any significant activity of dimers of the 47-mer DE-2 element or of DE-2C inserted upstream of the minimal rPOMC promoter (Fig. 2) or upstream of a truncated (−130 to +34 bp) RSV promoter (data not shown). This phenomenon contrasts with the intrinsic activity of constituent regulatory elements found in enhancers (30, 81). These regulatory elements typically display synergy when multimerized or when associated with other elements (63, 68). The strict requirement for activity of an association between DE-2C and element(s) of the central domain is similar to the organization of the insulin I gene minienhancer (26). In this case, the cell-specific activity of this minienhancer requires the precise juxtaposition of the Far (E box) and FLAT motifs. Although the POMC cell-specific enhancer unit is more relaxed with regard to the positioning of DE-2 and central domain, it is noteworthy that tissue specificity relies in both cases on the concerted action of two different factors. Thus, it appears that cell-specific enhancer recognition by multiple factors is a mechanism shared by many genes to achieve specificity, using a pool of transcription factors with restricted distribution. This mechanism contrasts with the simple one gene/one factor model that has been proposed for the GH and Pit-1/GHF-1 system (37). The concerted action of multiple factors with limited but overlapping tissue distribution is more compatible with a combinatorial model of cell-specific control of gene expression. The requirement for two different elements and their cognate factors suggests models of transactivation in which both factors are required concurrently to activate a common target. This common target could be one component of the basic transcription machinery such as TFIIB or TFIID (44, 69) or a coactivator which creates a bridge between the factors and the basic transcription machinery (3, 59).

HLH factors in POMC transcription. Transcription factors of the HLH family have not yet been implicated in transcription of pituitary genes, including POMC. Indeed, previous analysis of the POMC promoter by DNase I footprinting failed to reveal DE-2C (72). Thus, the DE-2C CANNTG motif characterized in the present work represents a novel POMC regulatory element. The search of the POMC promoter for a similar motif identified two other E boxes, both localized in the central domain of the promoter. It is noteworthy that both differ in sequence from DE-2C at either internal or flanking nucleotides which are important for binding specificity (4, 31, 70) and that neither can substitute for DE-2C in transfection experiments (data not shown). Thus, the DE-2C E box plays a unique role in the POMC promoter.

The corticotroph-specific activity of DE-2C was correlated by fine-structure mutagenesis with the GCAGATGG motif (Fig. 3 and 4) and with the binding of CUTE proteins (Fig. 5, 6, 9, and 12). All DE-2C mutants which failed to form CUTE complexes also failed to activate. Mutant M6, κE2, and MCK oligonucleotides were also inactive; however, the affinity of their interaction with CUTE proteins appeared to be three- to fivefold lower than that of WT DE-2C (Fig. 4, 6, and 9 and data not shown). Since the κE2 and MCK E boxes are good binding sites for ubiquitous HLH factors E12 and E47 (or rPan-1 and rPan-2), their inactivity in the POMC system suggests that other HLH factors are implicated, even though they may be involved in heterodimer formation, as suggested by the antibody supershift experiment (Fig. 11). The DMS methylation interference pattern of CUTE complexes is consistent with interference pattern of other HLH factors (Table 1). All known HLH factors bind the CANNTG motif as dimers, either homo- or heterodimers (53). Some HLH proteins, such as Id, act as dominant

negative factors through heterodimerization with HLH transactivators (2, 71). Thus, the decrease of rPOMC promoter activity resulting from Id overexpression (Fig. 10A) and the blockade of CUTE binding to DE-2C by GST-Id (Fig. 11A) further support the conclusion that HLH factors are involved. Also, the enhancement of rPOMC promoter activity by rPan-2 overexpression (Fig. 10B) and the antibody supershift experiment (Fig. 11B) are consistent with the idea that CUTE complexes may be heterodimers which contain a ubiquitous HLH protein such as Pan-2 and a cell-specific HLH factor. Interestingly, rPan1 did not enhance rPOMC promoter activity, suggesting that only rPan-2 can act on POMC either as a homodimer or by heterodimerization with another HLH factor present in AtT-20 cells. Since both Id and Pan-2 have been shown to interact with class A or B HLH factors, such E12/E47 or MyoD, respectively, but not with Myc-related factors (class C), it is likely that the CUTE HLH proteins which act on POMC transcription belong to class A or B (53, 71).

Cell-specific HLH factors. HLH transcription factors recognize DNA sequences related to the consensus NCANN TGN. However, specific members of the family interact preferentially with some E boxes which contain specific variant nucleotides (N) within their binding site (4, 31, 70). It is thus significant that DE-2C differs from previously characterized E boxes like the κE2 and MCK E boxes at one and two variant nucleotides, respectively (Fig. 4). For example, the one-nucleotide difference between the DE-2C and κE2 E boxes is sufficient to confer corticotroph-specific activity to DE-2C. The other genes containing E boxes identical to DE-2C are the insulin I and II genes (12, 38, 66). It is thus possible that the same or a closely related factor is present in both POMC-expressing and insulin-expressing cells and that it is involved in cell-specific transcription in each case. However, E-box-binding proteins of the HLH family (IEF-1) characterized in three insulin-producing cell lines (hamster HIT, rat RIN-m, and mouse βTC-1 cells) appeared similar to each other in EMSA (1) but distinct from mouse CUTE complexes (Fig. 12). In addition, IEF-1 showed a similar affinity for IEB and κE2 (1), whereas AtT-20 CUTE proteins appeared to have lower affinity for κE2 than for DE-2C (Fig. 9). Also, it was shown that mutagenesis of IEB (ICE) into a μE2-type E box did not affect its pancreatic-specific activity in HIT and βTC-1 cells (12), while a similar type of mutation abolished the corticotroph-specific activity of DE-2C in AtT-20 cells (Fig. 4). Furthermore, IEB was shown to be active on its own in HIT cells (39), whereas DE-2C required the POMC central domain for activity in AtT-20 cells (Fig. 2). Collectively, these differences strongly suggest that despite the identity of target sequences, the CUTE proteins of AtT-20 cells differ from IEF-1 found in pancreatic cells. Nonetheless, these two activities might have similarities in the sense that both may be HLH heterodimers which include a ubiquitous component like Pan-2 and a cell-specific component. At this point, we do not know how the cell-specific components of these two activities may differ to account for their difference in electrophoretic mobility; they may be the products of different genes, differentially spliced products of the same gene, or even the result of cell-specific posttranscriptional modifications.

The effect of GST-Id on CUTE binding to DNA (Fig. 11A) and the antibody supershift experiment (Fig. 11B) support the idea that CUTE proteins are HLH and that they may be heterodimers which contain a ubiquitous component. Also, the enhancement of POMC promoter activity produced by overexpression of the ubiquitous Pan-2 HLH factor might involve the formation of heterodimers (Fig. 10B). The Pan2 gene is expressed in AtT-20 cells because we have cloned AtT-20 cDNAs (unpublished results) for the ubiquitous HLH factors Pan-2, Pan-1, and HTF-4/HEB (31, 82). However, the Pan-2 factor cannot account for DE-2C activity on its own since extracts from AtT-20 cells overexpressing rPan-2 showed greater increase of binding activity for κE2 than for DE-2C (data not shown). Yet despite being a preferential target for rPan-2 binding, the κE2 site was inactive in this system (Fig. 4). In summary, the presence of corticotroph-specific CUTE complexes in AtT-20 cells and the corticotroph specificity of their CANNTG target motif strongly support the conclusion that a cell-specific HLH factor is involved in transcription of the POMC gene.

Concluding remarks. HLH factors are unique in that a subfamily, the myogenic factors, were originally described by their property to change the phenotype of various differentiated cells into muscle (79). Indeed, no other mammalian transcription factors, including the pituitary factor Pit-1/GHF-1, was shown to reprogram the phenotype of cells. Genetic studies in *Drosophila* cells have also established a role for HLH gene products such as the *achaete-scute* complex (75), *daughterless* (8), *hairy* (61), and *extramacrochaetae* (18, 23) in neuronal determination. The involvement of HLH factors in mammalian neurogenesis was also suggested by Johnson et al. (35), who cloned two *achaete-scute*-related cDNAs, *MASH-1* and *MASH-2*, which are transiently expressed during central and peripheral nervous system development. The presence of specific HLH factors in the neuroendocrine corticotroph cells of the pituitary allows one to speculate that these factors may be involved in pituitary development in addition to acting as POMC-specific transcription factors. This hypothesis is appealing when one considers that the corticotroph cells appear to be the first pituitary cells committed to terminal differentiation, as assessed by expression of POMC.

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