Binding of Upstream Stimulatory Factor and a Cell-specific Activator to the Calcitonin/Calcitonin Gene-related Peptide Enhancer*

(Received for publication, February 21, 1997, and in revised form, May 7, 1997)

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The calcitonin/calcitonin gene-related peptide (CT/CGRP) gene is selectively transcribed in thyroid C cells and neurons. We have previously shown that the rat CT/CGRP cell-specific enhancer is synergistically regulated by a helix-loop-helix (HLH) protein and the OB2 octamer-binding protein. In this report, we show that the HLH-OB2 enhancer is required for full promoter activity, even in the context of other HLH elements. Since this enhancer appears to be a major controlling element, we have characterized the HLH and OB2 DNA binding proteins. We have identified the major HLH complex as a heterodimer of the ubiquitous upstream stimulatory factor (USF)-1 and USF-2 proteins. USF bound the enhancer with a reasonably high affinity ($K_a = 1.6$ nM), comparable to other genes. Characterization of a series of mutations revealed that a portion of the HLH motif is also recognized by OB2 and confirmed that HLH activity requires OB2. We have shown that OB2 is a single DNA binding protein based on UV cross-linking studies. The 68-kDa protein-DNA complex was detected only in C cell lines, including a human C cell line that has robust HLH-OB2 enhancer activity. These results suggest that the calcitonin/CGRP gene is controlled by the combinatorial activity of a ubiquitous USF HLH heterodimer and an associated cell-specific activator.

The calcitonin/calcitonin gene-related peptide (CT/CGRP) gene encodes the hormone CT and the neuropeptide CGRP (1). CT lowers serum calcium levels during calcium homeostasis and is used as a therapeutic agent to maintain bone calcium in certain types of osteoporosis and Paget's disease (2, 3). CGRP has pleiotrophic effects but has been best characterized as a potent vasodilatory neuropeptide (4, 5). Elevated CGRP levels has pleiotrophic effects but has been best characterized as a potent vasodilatory neuropeptide (4, 5). Elevated CGRP levels have been found in a number of cardiovascular disorders (5) and in vascular headaches (6). The CT/CGRP gene is transcribed in a large number of neurons of the peripheral and central nervous systems and in the neuroendocrine thyroid C cells. Interestingly, thyroid C cell lines and cultured C cells have a more neuronal phenotype that includes neurofilament expression, serotonergic properties, and CGRP production (7–9). This acquisition of neuronal properties is consistent with the common origin of C cells with serotonergic enteric neurons from a vagal sympatheticadrenal progenitor in the neural crest (10). Consequently, thyroid C cell lines can be used as a model to study the transcriptional regulation of CT/CGRP in both C cells and neurons.

Cell-specific transcription of the CT/CGRP gene is controlled by a distal cell-specific enhancer. Transgenic mice containing 1.3 or 1.7 kilobase pairs of flanking DNA express reporter genes in thyroid C cells and peripheral neurons (11, 12). Both the rat and human CT/CGRP distal enhancers contain several helix-loop-helix (HLH) binding sites that contribute to enhancer activity (11, 13–16). Additional sequences near the promoter are responsive to signal transduction pathways induced by cAMP (17, 18), nerve growth factor (19), and activated Ras (20). We have found that the rat CT/CGRP enhancer requires not only the HLH factor but also a cell-specific protein that binds an adjacent octamer motif (16). Synergism between the HLH protein and the octamer-binding protein, referred to as OB2, is required for activity of the enhancer (designated as HLH-OB2). This type of combinatorial control is becoming an increasingly common theme in gene transcription (21).

Since the CT/CGRP HLH complex had been detected from both CT/CGRP expressing and non-expressing cell lines (16), this suggested that ubiquitous HLH proteins might recognize the HLH-OB2 enhancer. The ubiquitous E12/E47, ITF-2, and USF proteins have been implicated in combinatorial control with other proteins, including cell-specific factors. The E12/E47 and ITF-2 proteins have been shown to functionally interact with homeodomain proteins to help direct cell-specific gene expression (22–24). The USF proteins were initially identified as upstream stimulatory factors that control the adenovirus major late promoter (25–27). Since then, USF binding sites have been found in a number of cellular genes (28–33). USF often consists of a heterodimer of the ubiquitously expressed 43-kDa USF-1 and the 44-kDa USF-2 gene products, although homodimers can also bind DNA (34, 35). Furthermore, USF has been shown to work in combination with other factors (32, 33, 36–39). We have found that the CT/CGRP enhancer (20). We have found that the rat CT/CGRP enhancer requires not only the HLH factor but also a cell-specific protein that binds an adjacent octamer motif (16). Synergism between the HLH protein and the octamer-binding protein, referred to as OB2, is required for activity of the enhancer (designated as HLH-OB2). This type of combinatorial control is becoming an increasingly common theme in gene transcription (21).

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In this report, we have demonstrated the functional impor-
tance of the CT/CGRP HLH-OB2 enhancer in the context of full promoter sequences containing other regulatory elements, including other HLH motifs, and have characterized the DNA binding proteins. We found that a heterodimer of USF-1 and USF-2 proteins comprises the major HLH binding complex. The USF binding site was shown to overlap the site recognized by the OB2 protein. OB2 consists of a single 68-kDa cell-specific polypeptide that was identified in rat and human C cell lines. These results demonstrate that the CT/CGRP HLH-OB2 enhancer, a key regulatory element of the CT/CGRP gene, is bound by the ubiquitously expressed USF HLH proteins and a cell-specific protein.

EXPERIMENTAL PROCEDURES

Cell Culture—The CA77 thyroid C cell line was maintained in Dulbecco’s modified Eagle’s medium (DMEM) (low glucose)/Ham’s F12 (1:1), 10% fetal bovine serum (FBS) (HyClone). For the other cell lines, the media were as follows: 44-2C, DMEM (high glucose), 10% equine serum; B103, DMEM (low glucose), 10% FBS; HeLa, Ham’s F12, 10% FBS; GH3, DMEM (low glucose), 2.5% FBS, 15% equine serum; Rat-1, DMEM (high glucose), 10% bovine calf serum; Penicillin (100 units/ml) and streptomycin (100 mg/ml) were added to all media. Cells were incubated at 37 °C in 5% CO2.

Cell Transfections and Luciferase Assays—The 1250-bp CT/CGRP plasmids contain a fragment from the Kpn I site at −1250 bp to an artificial HindIII site +21 bp (16). The fragment was cloned into both the pSDR-lacZ vector provided by Dr. J. Rossant and the pGL3 luciferase vector (Promega). The herpes simplex thymidine kinase (Tk) promoter (105 bp) and the 205-bp Bgl II CT/CGRP enhancer (−1125 to −920 bp) fragment upstream of the TK promoter plasmids have been described (42). The 1125−290 + BamHI-TK-luc plasmid was constructed by inserting an 8-bp BamHI linker (5’gggatccg3’) (New England Biolabs) into the unique PvuII site of 1125−920-TK-luc. This yielded the enhancer sequence 5’CAaggatccgCCTGAAT3’, which contains a disrupted HLH motif with a reconstructed OB2 site. The 1250 CT/CGRP + BamHI-lacZ plasmid was constructed by replacing the BglII fragment (−1125 to −920 bp) with the BglII fragment containing the BamHI linker at the PvuII site. The CT/CGRP HLH-OB2 enhancer (−1043 to −1025 bp) plasmids (HO-TK-luc) contained two or four tandem inserts in a (+) orientation or a (−) orientation, respectively. Similar activities were seen with 2 to 4 elements in either orientation. The HO + A-TK-luc reporter contains four copies of the HLHm1 element in the minus orientation and the HLHm1-TK-luc reporter contains 2 copies of the HLHm1 element in the plus orientation. These plasmids were constructed from annealed oligonucleotides containing BamHI ends ligated into the TK-luciferase plasmid, as described (16). CA77 and COS-7 cells were transfected by electroporation as described (42), except that COS-7 cells were electroporated at 260 V. The transfected cells from a single cuvette were grown on a 60-mm dish as described (42), except that COS-7 cells were electroporated at 260 V. The transfected cells from a single cuvette were grown on a 60-mm dish as described (42), except that COS-7 cells were electroporated at 260 V.

RESULTS

Activity of the CT/CGRP Enhancer in the Context of Flanking Sequences—We have previously shown that the CT/CGRP enhancer contains adjacent HLH and octamer binding sites (referred to as HLH-OB2, or HO, enhancer) (−1043 to −1025 bp) and that this DNA is sufficient for cell-specific enhancer activity (16). In this study, we have addressed whether the HLH-OB2 enhancer is required for enhancer activity in the context of flanking DNA. We made an insertion mutation in the HLH motif of the enhancer and measured activity in a C cell line and a non-C cell line. The mutation was tested in the context of two different 5′-flanking fragments of the CT/CGRP gene, a 1250-bp fragment that is sufficient to direct expression to thyroid C cells in transgenic mice (11) and a 205-bp fragment that contains other HLH sites implicated in control of the human CT/CGRP gene (13, 14), as well as consensus sites for SP1 and AP-2 factors. The 1250-bp region also contains elements responsive to cAMP and Ras-activated signal transduction pathways (17, 18, 20). The HLH site within the CT/CGRP HLH-OB2 enhancer was disrupted by inserting an 8-bp BamHI linker into the HLH site. Reporter genes containing either the 1250-bp CT/CGRP promoter (CT/CGRP-lacZ) linked to the β-galactosidase gene or the 205-bp distal enhancer (1125−920-TK-luc) linked to the thymidine kinase promoter and luciferase gene were transfected into the CA77 C cell line. Mutation of the HLH site reduced the promoter activity of the 1250-bp CT/CGRP reporter gene to about 40% of the wild type promoter (Fig. 1A). Similarly, mutation of the HLH site reduced the distal enhancer activity to 20–25% of the wild type enhancer (Fig. 1A). When these constructs were transfected into the heterologous COS-7 cells, there was little to no significant change in promoter activity upon mutation to nullify the HLH motif (Fig. 1B). Similar results were obtained when 1125−920-TK-luc was transfected into the CT/CGRP promoter, 44-2C cells or the non-C/CT/CGRP producing HeLa cells (data not shown).

In the heterologous COS cells, there was a relatively small increase in TK promoter activity mediated by the 205-bp distal enhancer. We had previously observed this increase in other heterologous cells and suggested that the distal enhancer contains both cell-specific and non-cell-specific elements (16).
Since the mutant and wild type distal enhancers have essentially the same activity in COS cells, this demonstrates that the enhancement in heterologous cells is not due to a low level of HLH-OB2 enhancer activity but rather to the non-cell-specific sites. Taken together, these results indicate that the HLH-OB2 enhancer plays a major role in cell-specific CT/CGRP expression.

The CT/CGRP HB1 Complex Is a Heterodimer of USF-1 and USF-2 HLH Proteins—We then set out to identify the HLH protein that binds the HLH-OB2 enhancer. We have previously used electrophoretic mobility shift assays to characterize several factors that bind this enhancer (16). These factors include an HLH protein (HB1) and two octamer-binding proteins, the ubiquitous Oct-1 and the cell-specific OB2 protein. Weaker complexes, including some that may contain HLH proteins (16), have been detected with some nuclear extracts; however, characterization of these complexes has been hampered by inconsistent detection and variable competition results. It should be noted that a faint complex beneath Oct-1 that initially fits the criteria of a complex containing OB2 and an HLH (16) does not appear to contain OB2 upon examination of additional mutations discussed below (e.g., HOM4, HO + A). Consequently, we have focused on the major HLH complex, HB1.

Identification of HB1 in the mobility shift assay is shown by competition with an excess of unlabeled DNA containing an AP4 HLH protein binding site and lack of competition by mutated HLH-OB2 DNA containing two point mutations in the HLH motif (HLHmut1) (Fig. 2A).

To identify the HLH protein within the HB1 complex, we tested several antisera against candidate proteins. Addition of a USF antibody to the mobility shift assay disrupted the HB1 complex, suggesting that HB1 is comprised of USF (Fig. 2A). Since this antibody (USF-2 C-20) recognizes both USF-1 and USF-2, we then determined which USF protein, or both, was present in HB1. To do this, we took advantage of antibodies that are specific for epitopes on USF-1 and USF-2. Addition of either antibody completely removed the HB1 complex (Fig. 2A). The USF-1 (C-20) antibody supershifted the HB1 complex, whereas addition of USF-2 (N-18) disrupted the HB1 complex. To more clearly resolve the supershifted complex from the Oct-1 complex, a small excess of consensus octamer competitor DNA was included in the binding assay (Fig. 2A, lanes 8–11). As a control, an excess of a DNA competitor containing the consensus USF binding site was also included and shown to compete the supershifted complex (Fig. 2A, lane 10).
results were seen in the absence of the octamer DNA competitor (data not shown). As additional controls, the effect of the USF-2 (C-20) antibody was blocked by preincubation with 0.5 μg of the C-20 peptide, and addition of preimmune sera from a different rabbit did not affect any of the complexes (data not shown). Consequently, three different polyclonal antisera raised against USF-1 and USF-2 specifically recognize HB1. Since antibodies to either protein were able to displace the entire complex, this demonstrates that the enhancer is bound by a heterodimer of USF-1 and USF-2.

We then tested antibodies against other proteins, including HLH proteins. Addition of antisera directed against the human E12 HLH protein or CREB-binding protein (CBP) did not affect the HB1 complex (Fig. 2B), even though these antisera cross-react with their rat homologs. Similarly, HB1 was not affected by addition of antisera against the ubiquitous HLH proteins Pan (rat homolog of E12/E47) and ITF-2 or the cell-specific MASH-1 HLH protein found in C cell lines (data not shown). These results demonstrate that the CT/CGRP HLH-OB2 enhancer selectively binds the USF HLH proteins. Furthermore, similar tests done on the HB1 complex from a variety of cell lines, including the human TT C cell line, showed similar results (data not shown). Consequently, we will refer to HB1 as USF.

Binding Affinity of USF for the HLH-OB2 Enhancer—The absolute and relative binding affinities of USF for the HLH-OB2 enhancer were measured by mobility shift assays. This was a pertinent question since the central dinucleotide of the HLH-OB2 HLH site (CAGCTG) differs from the USF consensus (CACGTG), and this dinucleotide has been shown by others to play a role in USF binding (34, 40, 41, 44). Scatchard plot analysis of DNA binding to USF yielded a dissociation constant of 1.6 nM (Fig. 3). This is similar to measurements of recombinant and crude USF binding to the consensus element (0.75–1.24 nM) reported by others (35, 45). To directly compare the relative binding affinities of USF for the HLH-OB2 enhancer and the consensus USF motifs, competition assays were done using oligonucleotides containing the HLH-OB2 sequence, the AP4 HLH site, which also contains the GC central dinucleotide, and the USF consensus site (Fig. 4A). There was about 3-fold greater binding to the USF consensus site than either the AP4 or HLH-OB2 motifs based on the amount of DNA competitor required for 50% competition of binding to the HLH-OB2 probe (Fig. 4B).
**TABLE I**

**HLH-OB2 enhancer oligonucleotide sequences and relative binding activities**

Oligonucleotides containing wild type (WT) and mutant CT/CGRP HLH-OB2 enhancer sequences, as well as AP4, USF, and octamer sequences, are shown. The boxed sequences represent the HLH and octamer motifs. Underlined nucleotides indicate differences from the HLH-OB2 enhancer. All oligonucleotides except AP4, USF, and Oct contain Bam HI ends (not shown). Relative binding activities of USF and OB2 for each element are indicated.

|  |  |  |  |
|---|---|---|---|
| WT HO | GCCAGCTG | TGCAGTCCT | Yes | Yes |
| HLM1 | GCCAGCTG | TGCAGTCCT | Yes | No |
| HOm1 | GCCAGCTG | TGCAGTCCT | No | No |
| HOm2 | GCCAGCTG | TGCAGTCCT | Low | Yes |
| HOm3 | GCCAGCTG | TGCAGTCCT | No | No |
| HOm4 | GCCAGCTG | TGCAGTCCT | Yes | No |
| HO + A | GCCAGCTG | TGCAGTCCT | No | Yes |
| HO + 5 | GCCAGCTG | TGCAGTCCT | Yes | Yes |
| HO + 10 | GCCAGCTG | TGCAGTCCT | Low | Yes |
| HOhum | GCCAGCTG | TGCAGTCCT | Yes | Yes |
| Octm1 | GCCAGCTG | TGCAGTCCT | Low | Yes |
| Octm2 | GCCAGCTG | TGCAGTCCT | Low | Yes |
| AP4 | GCCAGCTG | TGCAGTCCT | No | Yes |
| USFcon | GCCAGCTG | TGCAGTCCT | No | Yes |
| Oct con | GCCAGCTG | TGCAGTCCT | Low | No |

**USF and OB2 Binding Sites Overlap**—We then performed a detailed analysis of the USF binding site on the CT/CGRP HLH-OB2 enhancer in a competition binding assay using a series of mutations in the enhancer (Table I). Binding of both USF and OB2, as well as Oct-1, were monitored in this assay. Mutation of the two 5'-nucleotides of the HLH motif (HLHm1) (ACGCTG) reduced binding of USF but not OB2 or Oct-1 (Fig. 5, lane 3). Interestingly, mutation of other nucleotides within the HLH motif (HOm1, HOM2, HOM3, and HOM4) reduced both USF and OB2 binding. In contrast, Oct-1 binding was not changed by these mutations. Conversion of the central GC dinucleotide of the HLH site to create a consensus USF site (HOM2) partially reduced the binding of OB2 (Fig. 5, lane 5). The reduction was comparable to that seen with the Octm2 mutation (Fig. 5, lane 13), which we have previously shown to have greatly reduced activity (16). This result argues that the GC dinucleotide contributes to OB2 binding. In support of this conclusion, both USF and OB2 bound a DNA containing the 3'-GCTG nucleotides of the HLH motif inserted between the HLH and octamer motifs (HO + 5) (Fig. 5, lane 9); insertion of 10 bp (HO + 10) that restores only the last two 3'-nucleotides (TG) of the HLH site has reduced OB2 binding similar to the HOM2 DNA (Fig. 5, lane 10). These data demonstrate that the OB2 site overlaps the 3' four bases of the USF site. The functional consequence of both the HO + 5 and HO + 10 insertions is greatly reduced enhancer activity (16).

Finer mutations of the USF and OB2 sites were then performed. Point mutations of the 3'-TG of the HLH site (HOM3 and HOM4) reduced USF binding, without affecting Oct-1 binding (Fig. 5, lanes 6 and 7). Mutation of the thymidine of the 3'-TG to a guanosine (HOM3) also prevented OB2 binding. However, mutation of the terminal guanosine to an adenosine (HOM4) did not affect OB2 binding. The HOM4 mutation created a consensus octamer motif. To conclusively test whether a consensus octamer motif would yield greater enhancer activity, we then inserted an adenosine residue, so as to not disrupt the HLH motif (HO + A). However, the HO + A DNA did not bind the OB2 complex (Fig. 5, lane 8). As expected, both the HOM4 and HO + A mutations bound Oct-1 better than the HLH-OB2 DNA. This confirms that OB2 does not prefer a consensus octamer motif and that the OB2 binding site extends from the octamer motif into the HLH motif.

Finally, the 3’ boundary of OB2 binding was established. We had previously shown that mutation of the AT dinucleotide in the octamer motif (Octm2) reduced OB2 binding and activity.

**Fig. 5. USF and OB2 recognition sites overlap.** A, mobility shift assay using the CT/CGRP enhancer probe with 44-2C nuclear extract and a series of competitor DNAs. The extracts were incubated without competitor DNA (lanes 1 and 16), or preincubated with 50-fold excess wild type HLH-OB2 DNA (lane 2), mutated HLH-OB2 DNA (lanes 3–10, 12 and 13), human HLH-OB2 DNA (lane 11), AP4 HLH DNA (lane 14), or consensus octamer DNA (lane 15). Similar results were seen with 25-fold molar excess competitors and using CA77 nuclear extract (not shown). B, schematic of the rat CT/CGRP HLH-OB2 enhancer showing the USF and OB2 binding sites determined from A. The positions of the HLH-OB2 mutations are indicated by asterisks, and the point of insertion of the +A, +5 bp, and +10-bp nucleotides are indicated by the arrow.
Combinatorial Control by USF and a Cell-specific Activator

The human CT/CGRP HLH-OB2 enhancer differs from the rat sequence in the four most 3’ nucleotides, including the thymidine mutated in Octm2. We have now shown that the human enhancer is capable of binding OB2 (Fig. 5, lane 11). It does not bind Oct-1, which underscores our previous argument that Oct-1 does not play a functional role at the HLH-OB2 enhancer (16). For comparison, additional competitors previously used to characterize the HLH-OB2 element are also shown (Fig. 5) (16). Mutation of a single residue in the 5’ region of the octamer motif (Octm1) disrupts OB2 binding, the AP4 element selectively removes the USF complex, and the consensus octamer element completely removes the Oct-1 complex, and partially removes the OB2 complex. Taken together, these results suggest that the OB2 binding site overlaps with the HLH motif and that OB2 binding is fundamentally different from Oct-1.

To confirm the competition assay results we also directly tested binding and activity of the HO + A mutation. This mutation was chosen since it had the most deleterious effect on OB2 binding without affecting USF binding. Using HO + A as a probe in the mobility shift assay, the Oct-1 binding was easily observed, but OB2 binding could not be detected (Fig. 6A). This agrees with the competition assay results. The assignment of Oct-1 was confirmed by specific competition with consensus octamer DNA. In agreement with the competition studies, there was no detectable OB2 complex on the HO + A DNA. For comparison, the HLH-OB2 enhancer was used as a probe to mark the relative position and intensity of the OB2 complex.

We then tested the functional consequence of the HO + A mutation. Tandem repeats of the HO + A enhancer were fused to the thymidine kinase promoter-luciferase reporter gene and transfected into CA77 C cells. The wild type HO-TK-luc reporter gave about an 8-fold increase in activity as compared with TK-luc alone, where as HO + A-TK-luc had little or no increase in activity over the parental TK-luc reporter (Fig. 6B). This indicates that the HO + A mutant enhancer has greatly reduced activity in CA77 C cells. These results are in agreement with our previous studies showing loss of activity with other mutations that affect binding (16). These results demonstrate that both the USF and OB2 binding sites are required for activity and confirm that the OB2 site functionally overlaps with the USF site.

Cross-linking of a Cell-specific Protein to the OB2 Binding Site—To characterize the factors that are contained within the OB2 complex, UV cross-linking reactions were performed using radiolabeled HLH-OB2 oligonucleotides with CA77 nuclear extract. The cross-linked products were resolved on a SDS-polyacrylamide gel and detected by autoradiography. Several bands were detected that were dependent on addition of nuclear extract and exposure to UV light (Fig. 7A). As a control, a 50-fold molar excess of unlabeled HLH-OB2 DNA was added as a competitor to determine the specificity of the cross-linking reaction (Fig. 7A, lane 4). Only the 68-kDa protein-DNA complex specifically bound the CT/CGRP HLH-OB2 enhancer. While we do not know the exact contribution of the cross-linked DNA to the 68-kDa complex size, we estimate that the minimal protein size would be about 60 kDa. This is too large to be USF (43 and 44 kDa) and too small to be Oct-1 (90 kDa). Hence it seemed likely that it could be OB2. Faint bands at the approximate sizes of USF and Oct-1 were detected using extracts from other cell lines and after competition with the HLHm1 DNA (see below), although further experiments will be needed to identify those complexes.

To determine whether the apparent 68-kDa protein was OB2, oligonucleotides containing mutations in the HLH-OB2 enhancer or consensus HLH and octamer motifs were added as competitors in the cross-linking reactions (Fig. 7B). Both OB2 and the 68-kDa protein did not bind the HO + A mutant sequence or the AP4 HLH motif yet did bind the HLH-OB2 HLH mutant (HLHmut1) DNA and partially bound the octamer consensus sequence. These results indicate that the 68-kDa protein has the same binding properties as the OB2 complex defined in the mobility shift assay (compare Figs. 5 and 7).

Detection of HLH-OB2 Enhancer Activity and OB2 Protein in a Human C Cell Line—To test the significance of the 68-kDa OB2 protein in CT/CGRP gene expression, we asked whether the human TT C cell line has HLH-OB2 enhancer activity. As described above, other labs had reported that HLH motifs are important for human CT/CGRP enhancer activity; however, those studies did not directly test the HLH-OB2 motif. To do this, a reporter gene containing the enhancer (HO-TK-luciferase) was transfected into the TT C cell line. The HO-TK-luciferase reporter had 10-fold greater promoter activity over the parental TK-luciferase reporter (Fig. 8A). To test whether both the HLH and OB2 sites are required for activation, the HO + A-TK-luciferase and HLHm1-TK-luciferase reporters were transfected into the TT cells. These mutations greatly reduced promoter activity, demonstrating that the enhancer requires both the HLH and OB2 motifs (Fig. 8A). These data demonstrate that the rat CT/CGRP HLH-OB2 enhancer is ac-
We then asked whether the OB2 protein was present in the human C cell line nuclear extract. A 68-kDa protein-DNA complex was detected by cross-linking reactions, similar to that seen with the rat CA77 cells (Fig. 8B). Specific competitions confirmed that this protein had the same binding properties as the rat OB2 protein. The HLH-OB2 (self) and HLH-OB2 HL-Hmut1 competitors removed the human 68-kDa protein-DNA complex, whereas HO1A mutant DNA did not affect the binding of this protein (Fig. 8B). This agrees with our detection of OB2 binding in mobility shift assays using the human HLH-OB2 element (Fig. 5) and using TT nuclear extracts (data not shown). Hence, both rat and human C cell lines contain HLH-OB2 enhancer activity and the 68-kDa OB2 protein.

To further characterize the cell specificity of the 68-kDa protein-DNA complex, several CT/CGRP expressing and non-expressing cell lines were surveyed. The CT/CGRP producing CA77 and 442C nuclear extracts contains the 68-kDa protein, whereas HeLa, GH3, and Rat-1 cells, which do not express CT/CGRP, did not yield a 68-kDa cross-linking product (Fig. 8C). Since GH3 cells are a pituitary neuroendocrine cell line, this suggests that OB2 is apparently not expressed in all neuroendocrine cell types. However, we cannot rule out the possibility that OB2 is expressed at a low concentration or that it is not activated in these cells. Interestingly, the neuronal-like B103 cells do express CT and CGRP mRNAs, yet do not appear to have OB2 binding activity. This is consistent with our findings that the HLH-OB2 enhancer is not active in B103 cells in transfection studies and has little or no detectable OB2 complex in mobility shift assays (data not shown). These results...
suggest that OB2 is a cell-specific factor found in a subset of neuroendocrine cells.

DISCUSSION

We have found that the CT/CGRP HLH-OB2 enhancer contains overlapping motifs bound by USF HLH proteins and the cell-specific OB2 protein. The combination of OB2 with the HLH protein is required for activation of the enhancer. The relative importance of this enhancer was demonstrated by the reduced activity seen upon mutation of the USF site even in the context of flanking DNA containing other enhancer elements, including HLH sites that lack an adjacent OB2 motif. The significance of the HLH-OB2 enhancer was further underscored by its activity in a human C cell line and the presence of both USF and OB2 protein in these cells.

USF bound the CT/CGRP enhancer exclusively as a heterodimer of USF-1 and USF-2, which is consistent with reports that these proteins often dimerize with each other (34, 35). The finding that USF bound the HLH-OB2 enhancer was somewhat unexpected since USF has a fairly well-established consensus binding site of CACGTG (25–27), which differs in the central dinucleotide from the CT/CGRP HLH-OB2 HLH motif of CACCGT. This latter sequence is preferably recognized by the E12 and myoD class of HLH proteins, not the USF proteins (46). Hence, it was important to establish that USF was binding the HLH-OB2 enhancer with reasonable affinity. Our calculated dissociation constant of 1.6 nM and the finding that USF prefers the consensus site is in agreement with published observations for USF (34, 35, 40, 41, 45). It should be noted that USF binding to the CACCGT element in vitro has been reported to be strongly influenced by magnesium concentration (44); however, we did not detect any effect of 0.1–2.5 mM MgCl2 on USF binding (data not shown). Irrespective of the in vitro data, the CACCGT motif from the amylod β-protein promoter gene promoter has been shown to be bound and transactivated in vitro by USF (40), and USF has been shown to transactivate nonconsensus elements in other promoters (28, 29). These studies support the possibility that USF can recognize a nonconsensus site such as found in the CT/CGRP enhancer. The question then is why might the CT/CGRP enhancer have retained a less than optimal USF site? Based on the HOm2 mutation, we suggest that the nonconsensus USF site has been maintained to allow optimal binding of OB2.

OB2 was shown to be a single ~68-kDa DNA binding protein whose binding site extended from the octamer motif into the HLH motif. Fine mapping of the OB2 binding site strongly argues that OB2 differs from Oct-1 and that Oct-1 binding to the CT/CGRP HLH-OB2 enhancer is nonfunctional, as previously suggested (16). This is best exemplified by the HO + A mutation, which created a consensus octamer site, yet virtually eliminated OB2 binding and enhancer activity. Likewise, the human CT/CGRP HLH-OB2 enhancer binds OB2 but does not bind Oct-1. Another interesting feature of OB2 that came from these studies is its cell specificity. The B103 cerebellum cell line expresses the CT/CGRP gene, yet lacks CT/CGRP HLH-OB2 enhancer activity and OB2 protein. Consistent with this observation, CT/CGRP promoter fragments containing the HLH-OB2 enhancer can direct expression to peripheral neurons and C cells in transgenic mice but apparently not to the central nervous system (11, 12). These results suggest that different enhancer factors may control CT/CGRP gene expression in the central nervous system.

The synergistic CT/CGRP HLH-OB2 enhancer activity is an ideal target for regulation. We have previously demonstrated this point by showing that retinoic acid (42), dexamethasone (15), and a serotoninergic agonist3 all can repress CT/CGRP gene expression through the CT/CGRP HLH-OB2 enhancer. The possibility that USF activity can be regulated has been suggested by Riccio et al. (32), who have shown that transforming growth factor-β can regulate gene expression through overlapping USF-CTF/NF-1 sites in the type 1 plasminogen activator inhibitor gene (32). Mutations in either the USF or CTF/NF-1 sites reduced transcriptional activation upon exposure to transforming growth factor-β. These results suggest that synergistic interactions between USF and other factors may be a common target for transcriptional regulation.

Based on this study, we propose a model in which the CT/CGRP gene is controlled by the combinatorial action of a ubiquitous USF HLH heterodimer and the cell-specific OB2 activator. The mechanism by which USF-1 and/or USF-2 interact with OB2 to activate gene expression remains to be determined but may involve a direct protein-protein interaction between these proteins or interactions through unidentified cofactors. Using the mobility shift assay, we have been unable to unambiguously identify a complex containing both USF and OB2. Whether USF and OB2 can co-occupy the enhancer or bind in a mutually exclusive or sequential manner remains to be determined. In either case, USF and OB2 are apparently not required for each other’s DNA binding activity since both proteins could bind to DNA with relatively high affinities, at least in vitro.4 While we cannot exclude the possibility that cell-specific or other HLH proteins can fulfill the HLH role in vivo, we can rule out the MASH-1 protein, since we and others have now shown that the CT/CGRP gene is expressed in mice lacking MASH-1 (47).2 There is precedence for ubiquitous HLH proteins allowing cell-specific gene expression via functional interactions with other transactivators. For example, the insulin gene has been proposed to be controlled by the combinatorial actions of E47 HLH proteins and cell-specific homeodomain proteins (22, 23). The USF HLH proteins have also been shown to functionally interact with other DNA binding proteins to activate transcription, including in a cell-specific manner (32, 33, 38). In the case of the CT/CGRP enhancer, we propose that cell specificity is provided by the OB2 protein.

Acknowledgments—We gratefully acknowledge Bill Giersch, Paul Durham, Shannon DeRaad, and Lois Tverberg for their discussions and generous assistance with these studies.

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