Regulation of the Calcitonin/Calcitonin Gene-related Peptide Gene by Cell-specific Synergy between Helix-Loop-Helix and Octamer-binding Transcription Factors*

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Lois A. Tverberg‡ and Andrew F. Russo§
From the Department of Physiology and Biophysics, University of Iowa, Iowa City, Iowa 52242

The calcitonin/calcitonin gene-related peptide (CGRP) gene is transcribed in thyroid C-cells and a subset of neurons. We have localized sequences required for cell-specific enhancement of calcitonin/CGRP transcription in rat thyroid C-cell lines. An 18-base pair element approximately 1 kilobase pair upstream of the transcriptional start site stimulated expression of a luciferase reporter gene 50-fold in 44-2C C-cells. There was less than 2-fold stimulation in HeLa and Rat-1 cells, which do not express the endogenous calcitonin/CGRP gene. The enhancer contains potential binding sites for helix-loop-helix (HLH) and octamer transcription factors based on sequence homologies. The functional significance of these sites was shown by point mutations in the HLH and octamer motifs and by separation of the two motifs, all of which decreased enhancer activity greater than 10-fold. The involvement of HLH proteins was further shown by co-expression of the mammalian achaete-scute homologue-1 HLH protein, which activated the enhancer severalfold in HeLa cells. Electrophoretic mobility shift analyses revealed several DNA-protein complexes containing HLH and octamer-binding proteins. One octamer-binding complex (OB1) most likely contains the ubiquitous Oct-1 protein, whereas a second complex (OB2) was cell-specific. In contrast to OB1, OB2 had lower affinity for a consensus octamer motif, and its DNA binding was not affected by addition of antiserum that recognizes Oct-1 and Oct-2 proteins. In addition, we observed a large complex that appears to contain both an HLH protein and OB2. These results demonstrate that calcitonin/CGRP enhancer activity is controlled by a cell-specific synergistic activation involving HLH and octamer-binding factors.

The calcitonin/calcitonin gene-related peptide (CT/CGRP)* gene is expressed in specific cell types of both the endocrine and nervous systems. The gene is alternatively spliced to yield mRNA encoding calcitonin in thyroid C-cells or the neuropeptide CGRP in a subset of central and peripheral neurons. Expression of this gene is regulated by a number of hormones and second messengers that can stimulate or repress transcription. Agents that activate protein kinases A and C have been shown to increase transcription (deBustros et al., 1986), whereas transcription is repressed by vitamin D and retinoic acid (Naveh-Many and Silver, 1988). Glucocorticoid hormones can either stimulate or repress transcription in a cell-specific manner (Tverberg and Russo, 1992).

Promoter mapping experiments have revealed that the basal enhancer regions of both rat and human CT/CGRP genes contain consensus helix-loop-helix (HLH) sites which are necessary for cell-specific expression (Stolarsky-Fredman et al., 1990; Peieg et al., 1990; Ball et al., 1992). In earlier studies we localized an enhancer necessary for cell-specific basal expression of the rat CT/CGRP gene to an 18-bp element between -1025 and -1043 bp upstream from the transcriptional start site (Tverberg and Russo, 1992). The CT/CGRP enhancer increases expression of a thymidine kinase promoter reporter gene 50-fold in the 44-2C thyroid C-cell line. In contrast, it stimulates activity less than 2-fold in the heterologous HeLa cervical carcinoma cell line. Interestingly, transcriptional repression by glucocorticoids and by retinoic acid are both localized to this site (Tverberg and Russo, 1992). Sequence analysis of the enhancer reveals that the element contains regions homologous to consensus binding sites for HLH and octamer transcription factors.

Many members of both the HLH and octamer transcription factor families are expressed in a restricted tissue pattern and function as cell-specific transcriptional activators. For example, the MyoD family of HLH factors activate myogenesis by stimulating transcription from muscle-specific enhancers (Edmondson and Olson, 1993; Weintraub al., 1991). In a similar manner members of the achaete-scute HLH family are involved in neurogenesis in Drosophila (Ghysen and Dambly-Chaudiere, 1988). In general, tissue-specific HLH factors form dimers with ubiquitously expressed HLH factors such as E12 in order to bind DNA with high affinity (Murre et al., 1989). Octamer binding factors are members of the POU homeodomain family and have a role in both ubiquitous and cell-specific gene transcription (Scholer, 1991). Although the Oct-1 protein is widely expressed and directs expression of many housekeeping genes, a number of Oct factors are tissue-specific transcription factors (He et al., 1989; Schreiber et al., 1990; Scholer, 1991). In particular, Oct-2 has been found to direct expression of B-lymphocyte-specific genes (Singh et al., 1986; Scheideiret et al., 1988; Zelenik-Le et al., 1992). As

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§ To whom correspondence should be addressed: Dept. of Physiology and Biophysics, University of Iowa, Iowa City, IA 52242. Tel.: 319-335-7872; Fax: 319-335-7330.

The abbreviations used are: CT/CGRP, calcitonin/calcitonin gene-related peptide; HLH, helix-loop-helix; TK-LUC, thymidine kinase-luciferase; MASH1, mammalian achaete-scute homologue-1; CMV, cytomegalovirus; bp, base pair; kb, kilobase pair.

discussed below, HLH and octamer binding motifs are both commonly found close to other transcription factor sites, and the activity of both appears to be dependent on the presence of adjacent binding factors (Weintraub et al., 1990; Sartorelli et al., 1990; Poellinger et al., 1989; Roebuck et al., 1990).

Because the CT/CRP enhancer element contains potential HLH and octamer-binding sites, we set out to confirm that these sites are required for enhancer activity and to characterize the proteins binding to these sites. Our approach was to first show by mutation analyses that the HLH and octamer motifs are both required for enhancer activity. The involvement of HLH proteins was further shown by activation of the element upon co-transfection of an HLH protein. Finally, we describe the proteins bound to the HLH and octamer motifs using electrophoretic mobility shift assays. Based on these studies, we conclude that a cell-specific combination of HLH and octamer-binding transcription factors synergistically activate the enhancer to determine the restricted expression pattern of the CT/CRP gene.

EXPERIMENTAL PROCEDURES

Cell Culture and Transfection Assays—The 44-2C, CATT, and HeLa cells were maintained and transfected by electroporation as previously described (Tverberg and Russo, 1992). The cells were transfected during exponential growth phase and incubated 18-24 h before harvest for luciferase assays. Co-transfection experiments included 20 μg of luciferase reporter plasmid with 20 μg of CMV-MASH1 expression vector. Multiple plasmid preparations were used to insure that results were not dependent on the DNA preparation. Luciferase assays were performed using the Promega Luciferase Assay System (Promega, Madison, WI). Emitting light units were quantified by using a Monolight 2001 luminometer (Analytical Luminescence Laboratories, San Diego, CA). Assays were normalized to 50 pg of protein as determined by the Bradford protein assay (Bio-Rad) using bovine serum albumin as a standard. Although transfection efficiencies were approximately the same within each experiment, absolute luciferase activity varied severalfold between experiments due to variations in cell growth state and transfection survival. In order to compare results from different experiments, activities were normalized to an internal standard as indicated in the figure legends.

Reporter Fusion Genes and Oligonucleotides—Fusion genes were constructed in a vector containing the thymidine kinase promoter and luciferase reporter gene (TK-LUC) as described previously (Tverberg and Russo, 1992). Complementary oligonucleotides containing the 18 bp CT/CRP/O enhancer reporter gene were synthesized and, by the methods of Weintraub and Russo (1992), the sites were staggered and ligated into the BamHI site of TK-LUC in the same manner. The annealed oligonucleotides had staggered ends cohesive for BamHI sites to facilitate cloning and radiolabeling. The sequences inserted into the 5- and 10-bp insertion mutants repeat the same nucleotides (1-3 bp) at the adjacent motifs in an attempt to minimize any disruptive effect of changing the nucleotides flanking the sites. All constructs were sequenced prior to transfection. The sequences of oligonucleotides used for these studies are given in Fig. 1. The sequences shown in Fig. 1 include the terminal restriction enzyme sites that have been made blunt-ended using Klenow DNA polymerase.

Electrophoretic Mobility Shift Assays—Nuclei were harvested from approximately 108 cells using a previously described minienzyme preparation protocol (Lee and Green, 1990), with one modification. Instead of dialyzing the extract to remove excess NaCl, the extract was diluted 1:1 with extract buffer lacking NaCl. Aliquots were stored at −70 °C. Annealed oligonucleotide duplexes (10 pmol) containing recessed 3′ termini were labeled for use as probes by incorporation of [α-32P]dATP (25 μCi, 3000 Ci/mmol) using Klenow DNA polymerase. Both strands of blunt-ended oligonucleotides (10 pmol) were labeled separately using T4 polynucleotide kinase and [γ-32P]ATP (20 μCi, 3000 Ci/mmol), then annealed. Unlabeled oligonucleotides used for competition assays were annealed and then treated with Klenow DNA polymerase if the ends were not already blunt. All probes and competitor DNAs were purified over Sephadex G50 columns.

The binding reaction (20 μl) contained approximately 3 μg (1-2 μl) of nuclear extract, 0.02 pmol of [32P]labeled DNA probe (50,000-100,000 cpm), 10 mM Tris-HCl, pH 7.5, 5% glycerol, 50 mM NaCl, 1 mM EDTA, and 1 mM dithiothreitol. Also, 0.1 μg of poly(dI-dC) (Boehringer Mannheim) and 0.1 pmol of an unrelated double-stranded polynucleotide oligonucleotide (GATCCTCTTAGACATGGGATC) were added as nontarget competitors. Unlabeled competitors were preincubated with nuclear extracts 10 min on ice prior to addition of probe. OCT-2 antisera was generously provided by Drs. T. Kristie and P. A. Sharp (Kristie et al., 1989). Nuclear extracts were incubated for 30 min on ice with antisera prior to addition of probe. After addition of probe, the reaction was incubated for 15 min on ice, then 2.5 μl of 50% glycerol dyes were added, and the samples were immediately loaded on a 6% polyacrylamide gel (1:28 acrylamide) in 0.25× TBE (22.5 mM Tris-HCl pH 8.5, 28 mM boric acid, 0.7 mM EDTA). The gel was prerun for 1 h prior to electrophoresis of the samples for 3 h at 200 V at 4 °C. The gel was transferred to blotting paper, dried, and exposed to x-ray film for 12-18 h with an intensifying screen or for 3-4 days without a screen.

RESULTS

Cell-specific Enhancement Requires Adjacent HLH and Octamer Motifs—We identified two sites (CAGCTG and GTGCAAT) within the CT/CRP enhancer that are homologous to the consensus HLH (CANNTO) (Weintraub et al., 1991) and octamer (ATGCAAT) (Scholer, 1981) motifs (Fig. 1A). These two sites are adjacent to each other and overlap by a single nucleotide. In addition to the HLH and octamer motifs, there is some homology with a composite GRE/AP-1 motif from the proliferin gene (Diamond et al., 1990), a second messenger response element from the interleukin-6 gene (Ray et al., 1990), and a half-site for the glucocorticoid receptor (Beato, 1989). The homologies with the proliferin and interleukin-6 elements may be coincidental, since we cannot detect binding of AP-1 to the enhancer, and the enhancer is not responsive to phorbol esters or cAMP.3 We have not tested whether the glucocorticoid receptor binds to the half-site, although it is intriguing that glucocorticoid repression has been mapped to the enhancer (Tverberg and Russo, 1992).

To test the hypothesis that both HLH and octamer sites are necessary for transcriptional activation, a series of oligonucleotides were synthesized that contain the enhancer element with mutations in either the HLH or octamer sites (Fig. 1B). The mutant oligonucleotides were annealed and inserted upstream of the thymidine kinase promoter linked to TK-LUC. Each construct was transiently transfected into 44-2C thyroid C-cells by electroporation. After a 24-h incubation, cell extracts were prepared and assayed for luciferase activity. One copy of the wild type enhancer element stimulated reporter gene activity approximately 50-fold over that of TK-LUC. In contrast, mutations in either the HLH or octamer sites essentially abolished enhancer activity (Fig. 24). The HLH mutant and octamer mutant enhancers stimulated activity less than 3- and 5-fold, respectively. These results demonstrate two points. First, the mutations establish the functional significance of the HLH and octamer motifs. Second, the 50-fold enhancement of the wild type enhancer containing both the HLH and octamer motifs is greater than predicted from the activity of the mutants containing only one of the motifs. Consequently, the requirement for both HLH and octamer sites suggests that factors that bind these sites activate transcription in a synergistic manner.

We then asked whether separation of the HLH and octamer motifs would affect enhancer activity (Fig. 24). The sites were separated by inserting 5 or 10 bp, corresponding to about a half or full helical turn, respectively. Transfection analyses showed that both the 5- and 10-bp insertions greatly reduced enhancer activity, having less than a 2-fold stimulatory effect.

3 L. A. Tverberg and A. F. Russo, unpublished observations.
A. Calcitonin/CGRP Enhancer Element

![Diagram](https://example.com/diagram.png)

**Fig. 1.** Sequence of the CT/CGRP enhancer and other oligonucleotides. A. Schematic of the 18-bp CT/CGRP enhancer showing the HLH and octamer motifs aligned with consensus HLH and octamer sequences. B. Oligonucleotides containing wild-type CT/CGRP (HLH) and mutant sequences. The light bar indicates the HLH motif and the dark bar the octamer motif. Shaded nucleotides indicate mutant nucleotides. Lowercase nucleotides are the terminal BamHI sequences. C, oligonucleotides containing HLH and octamer sites from other genes. The Pit-1 oligonucleotide contains AuaI terminal sequences as indicated by lowercase nucleotides.

on TK-LUC activity. These data indicate that the HLH and octamer motifs must be in close proximity to activate transcription. However, an important consideration, addressed below, is that although the motifs were preserved, the insertions may still have affected binding of the HLH and/or octamer factors.

To determine whether the HLH or octamer motifs of the CT/CGRP enhancer might act independently in non-C-cell lines, we transfected the wild type and mutant enhancer reporter genes into HeLa cervical carcinoma cells. We have shown previously that the CT/CGRP gene enhancer is not active in these cells (Tverberg and Russo, 1992). The rationale for this experiment was partially based on a recent report that removal of an HLH-containing motif required for cell-specific tyrosine hydroxylase enhancer activity led to increased activity of an adjacent AP-1 element only in heterologous cells (Yoon and Chikaraishi, 1992). In the case of the CT/CGRP enhancer, both the wild type and mutant forms had similar activities in HeLa cells (Fig. 2B). This is consistent with the results seen with the 44-2C C-cells and supports the model discussed below that the enhancer requires cell-specific factors for both the HLH and octamer motifs.

**Co-expression of an HLH Transcription Factor Can Activate the Enhancer Element**—We tested whether the CT/CGRP enhancer could be activated by an HLH factor as predicted by the mutation studies. The approach was to co-transfect the mammalian achaete-scute homologue-1 (MASH1) HLH transcription factor along with the CT/CGRP enhancer. This factor was chosen since MASH1 RNA has been detected in thyroid C-cell lines (Johnson et al., 1990). The activities are reported per 50 μg of extract. Mutations are indicated as asterisks and stippled boxes. B, HeLa cells were transfected with luciferase fusion genes and assayed as in A. The activities are shown as means ± S.E. from four to five independent experiments normalized to the average activity of TK-LUC.

**Fig. 2.** Enhancer activity of wild type and mutant CT/CGRP enhancers in 44-2C and HeLa cell lines. A, 44-2C cells were transfected with luciferase fusion genes containing a wild type or mutant CT/CGRP (H/O) enhancer and incubated 18 to 24 h, then assayed for luciferase activity. The activities are shown as means ± S.E. from three to eight independent experiments normalized to the average activity of TK-LUC. The activities are reported per 50 μg of extract.
CT/CGRP enhancer. We co-transfected the luciferase reporter with an expression plasmid containing the MASH1 gene linked to a cytomegalovirus (CMV) promoter into the cells. Control plates were co-transfected with the CMV-β-galactosidase fusion gene to maintain a constant amount of DNA in the transfections and to control for possible nonspecific effects of the strong CMV promoter on transcription. Co-transfection with the CMV-β-galactosidase vector did not have any detectable effect on TK-LUC reporter gene activity.

We observed that co-transfection of the MASH1 expression vector activated the CT/CGRP enhancer element approximately 4-fold in HeLa cells (Fig. 3). As a control for specificity of MASH1 action, co-expression of MASH1 had little or no effect on the TK-LUC reporter gene lacking the CT/CGRP enhancer. Likewise, MASH1 stimulation was not observed with the reporter genes containing mutant forms of the CT/CGRP enhancer. A somewhat unexpected observation was that MASH1 did not activate the enhancer even when the mutation was in the octamer binding site, and the HLH site was unaffected. The lack of enhancement of the mutants supports the prediction that enhancement requires both the HLH and octamer motifs. Co-transfection of the MASH1 expression vector in Ca77 cells yielded a smaller degree of stimulation (2-fold), and no significant effect could be detected in the 44-2C cells (data not shown). One explanation for the diminished effect of co-transfected MASH1 in the C-cell lines is that MASH1 (or another HLH protein) is not limiting in these cells. However, it must be emphasized that it remains to be established whether normal endogenous levels of MASH1 act on the CT/CGRP enhancer (see "Discussion"). In either case, the transactivation results demonstrate that the CT/CGRP enhancer can be transactivated by HLH transcription factors.

Identification of CT/CGRP Enhancer DNA-binding Proteins—DNA-protein complexes on the CT/CGRP enhancer were identified using the electrophoretic mobility shift assay. We observed several complexes with nuclear extracts prepared from the 44-2C cells using a radiolabeled oligonucleotide containing the 18-bp CT/CGRP enhancer as a probe (Fig. 4). The significance and identity of five complexes were shown using the functionally inactive point mutants as radiolabeled probes and as unlabeled competitors in the binding assay. The complexes were further characterized using oligonucleotides containing known HLH and octamer binding sites as competitors.

The wild type enhancer probe bound five complexes, designated as HB1, HB2, OB1, OB2, and H/OB for CT/CGRP enhancer HLH or octamer-binding factors. As discussed below, the H/OB complex appeared to contain HLH and octamer-binding proteins and was often obscured by the OB1 complex. Other complexes were not consistently observed and may possibly represent nonspecific DNA-protein complexes. The CT/CGRP enhancer oligonucleotide probe containing a mutation in the HLH motif (HLH Mut) gave a different DNA-protein complex profile than seen with the wild type enhancer (Fig. 4A). The HLH mutant oligonucleotide does not bind either the HB1 or HB2 factors. The identity of the new complex seen with this probe is not known. The HLH mutant probe did bind both the OB1 and OB2 complexes. The binding of the octamer complexes in the absence of HLH binding indicates that these two classes of factors can bind the enhancer individually, at least in vitro. Interestingly, binding of OB2 to the HLH mutant oligonucleotide was consistently much greater than seen with the wild type enhancer. The potential significance of this is discussed below. These results demonstrate that the HLH motif is required for HB1 and HB2, but not for OB1 and OB2 complex formation.

The same approach was done using the two different CT/CGRP enhancer oligonucleotides containing mutations in the octamer motif (Oct Mut1 and Oct Mut2). Both octamer mutations reduced the binding of OB2 complex, although binding was not completely abolished (Fig. 4A). As with OB2, OB1 complex formation was also greatly reduced on the Oct Mut2 oligonucleotide probe. However, the Oct Mut1 probe binds OB1 more strongly than the wild type enhancer. Because we know that the Oct Mut1 mutation decreased enhancer activity about 10-fold, this indicates that binding of OB2 complex is likely to be necessary for enhancer activity. Both the octamer mutants bound the HB1 and HB2 complexes, indicating that the reduced octamer binding activity did not significantly affect HLH binding. This observation is consistent with the results seen with the HLH mutant oligonucleotide mentioned above. These results indicate that the OB1 and OB2 complexes interact with the octamer half of the CT/CGRP enhancer and furthermore that the binding specificity of these two factors may differ.

Competition binding assays using excess unlabeled oligonucleotides were done to confirm the specificity and identity of the complexes seen on the CT/CGRP enhancer (Fig. 4B). Self-competition with the wild type CT/CGRP enhancer diminished the HB1, HB2, OB1, and OB2 complexes. Competition with the oligonucleotide containing the CT/CGRP enhancer HLH mutation did not remove HB1 and HB2, yet did reduce binding of OB1 and OB2. This is consistent with the above conclusion that the HB1 and HB2 complexes bind to the HLH motif. Conversely, oligonucleotides containing mutations in the CT/CGRP enhancer octamer motif did not remove the OB1 and OB2 complexes to same extent as the wild type or HLH mutant oligonucleotides. The partial competition for OB1 and OB2 complexes is expected since these mutations did not completely abolish binding, as shown previously (Fig. 4A). As expected, the octamer mutant oligonucleotides did reduce the HB1 and HB2 complexes. These

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4 K. Zimmerman and D. Anderson, unpublished observations.
Tide probes containing wild type or mutant CT/CGRP (H/O) enhancer sequences were incubated with 44-2C nuclear extracts and the complexes were incubated with 44-2C cell extracts preincubated with 25-fold molar excess of the indicated unlabeled competitor oligonucleotide (see Fig. 1B) prior to incubation with radiolabeled H/O enhancer probe and resolution of the resulting complexes on a polyacrylamide gel. The H/OB complex is not detectable in these autoradiograms because it co-migrates with OB1.

results confirm that the OB1 and OB2 complexes form on the octamer motif.

The enhancer mutants containing a 5- or 10-bp insertion were used as probes and as competitors to test whether the complexes could form on the elements when they were separated. As competitors, the 5- and 10-bp insertion mutants were almost as effective as the wild type enhancer (Fig. 4B). As probes both the 5- and 10-bp insertion mutants bound OB1 and HB1 about as well as the wild type enhancer, but the 5-bp insert probe had increased HB2 and OB2 complex formation, and the 10-bp insert probe bound even less of these proteins (data not shown). Consequently, separating the motifs apparently decreased, but did not abolish, binding of factors to the enhancer. Although these results unfortunately do not clearly discern whether the inactivity of the insertion mutants was due to decreased binding, they do support the hypothesis that the HLH and octamer motifs must be in close proximity for enhancer activity.

Competition of the CT/CGRP Enhancer Complexes with Other HLH and Octamer Motifs—We then tested whether the complexes identified by their affinity for motifs on the calcitonin/CGRP enhancer would bind to known octamer and HLH transcription factor binding sites (Fig. 5). These other sites are flanked by sequences unrelated to the CT/CGRP enhancer (Fig. 1C). The HB1 and HB2 complexes were reduced by oligonucleotides containing either the AP-4 HLH binding site (Mermod et al., 1988) or the Pan HLH binding site (Nelson et al., 1990). The OB1 and OB2 complexes were relatively unaffected by these oligonucleotides. Conversely, the OB1 complex was selectively removed by competition with either the consensus octamer binding site (Scholer, 1991) or the related Pit-1 POU protein binding site (Nelson et al., 1988). After competition of the OB1 complex, the HLH and octamer-containing H/OB complex can be more easily detected (indicated by the arrow). Higher concentrations of these competitors were required to remove OB2 and the H/OB complex (discussed further below). It has been reported previously that octamer proteins bind to Pit-1 sites with only 3-5-fold less affinity than octamer consensus sites (Elsholtz et al., 1990). The fact that the less related Pit-1 site removes the OB1 and OB2 complexes supports the conclusion that the complexes are octamer-binding proteins. These experiments indicate that the complexes that form on the CT/CGRP element are most likely HLH and octamer-binding proteins.

The H/OB complex was often obscured by OB1, but could be observed when the mobility shift assays were electrophoresed for longer times or when the Pit-1 or octamer oligonucleotides were included as competitors. This complex is specifically removed by competition with the wild type enhancer or with the enhancer containing mutations in either the HLH or octamer binding sites. This suggests that the H/OB complex may contain factors that bind in both HLH and octamer motifs. Low amounts of Pit-1 or octamer competitor oligo-

![Fig. 4. Mobility shift analysis of complexes binding the CT/CGRP enhancer. A, radiolabeled oligonucleotide probes containing wild type or mutant CT/CGRP (H/O) enhancer sequences were incubated with 44-2C nuclear extracts and the complexes were incubated with 44-2C cell extracts preincubated with 25-fold molar excess of the indicated unlabeled competitor oligonucleotide (see Fig. 1B) prior to incubation with radiolabeled H/O enhancer probe and resolution of the resulting complexes on a polyacrylamide gel. B, 44-2C cell extracts were preincubated with unlabeled H/O enhancer probe and resolution of the resulting complexes on a polyacrylamide gel. The H/OR complex is not detectable in these autoradiograms because it co-migrates with OB1.

![Fig. 5. Competition of enhancer binding complexes by consensus HLH and octamer binding sequences. Mobility shift assay in which 44-2C cell extracts were preincubated with no competitor (lane 1), 25-fold excess (lanes 2, 4, 6, 8, and 10) or 50-fold excess (lanes 3, 5, 7, 9, and 11) of the indicated unlabeled competitor oligonucleotide (see Fig. 1C) prior to incubation with the radiolabeled H/O enhancer probe and electrophoresis. The H/OB complex is indicated both on the left and right sides by an arrow.](https://example.com/image.png)
nucleotides preferentially removed OB1 relative to OB2 (Fig. 5), suggesting that the H/OB complex contains OB2. Furthermore, the relative migration rates of the complexes suggest that the H/OB complex contains OB2 and HB1 or HB2. In addition, as discussed below, H/OB is only detected in extracts containing OB2 proteins.

The OB1 and OB2 complexes appear to have different affinities for the consensus octamer and the CT/CGRP H/O enhancer as discerned from mobility shift competition assays. As mentioned above, higher concentrations of Pit-1 and octamer consensus competitors were required to remove OB2 than OB1 binding (Fig. 5). We found that as little as 5-fold excess unlabeled enhancer substantially reduced OB2 binding but had little effect on OB1 binding to the CT/CGRP enhancer. In contrast, 5-fold excess of the consensus octamer oligonucleotide removes most of OB1 binding but does not affect OB2 binding to the element (data not shown). We have noticed that it is difficult to fully compete the OB1 protein from the CT/CGRP H/O enhancer using the H/O enhancer as competitor. One possible explanation is that there is a large excess of OB1 protein in the extracts and that it has relatively weak affinity for the H/O enhancer. In agreement with this observation, we saw that when the consensus octamer was used as probe, much more OB1 protein bound to the consensus site compared with the CT/CGRP enhancer (see Fig. 6). Competition with 10-fold excess octamer DNA removed OB1 binding from the octamer more effectively than 250-fold excess CT/CGRP enhancer DNA (data not shown). This indicates that OB1 has at least 25-fold higher affinity for the octamer consensus relative to the CT/CGRP enhancer octamer site. A lower band that co-migrated with the OB2 complex binds very weakly to the octamer site and is removed by 10-fold excess of either oligonucleotide. These results indicate that OB1 protein has higher affinity for the octamer consensus relative to the CT/CGRP H/O enhancer, whereas the OB2 protein has higher affinity for the CT/CGRP H/O enhancer.

Addition of Oct Antiserum Blocks Binding of OB1, but Not OB2, to the CT/CGRP Enhancer—To further characterize the OB1 and OB2 complexes, we used a polyclonal antiserum raised against the POU domain of the human Oct-2 protein (Kristie et al., 1989). This antiserum has been shown to block Oct-1 binding to DNA in mobility shift assays (Kristie et al., 1989). Preincubation with the Oct antiserum prevented OB1 from binding to the CT/CGRP H/O enhancer and to the consensus octamer site (Fig. 6). Interestingly, the antiserum did not affect binding of OB2. The inability of the antiserum to block DNA binding by OB2 suggests that the OB2 protein is significantly different than the Oct-1 and Oct-2 octamer-binding proteins. In fact, a complex corresponding to H/OB was observed following preincubation with the antiserum (Fig. 6, lane 3). This is consistent with the increased detection of H/OB following reduction of the OB1 complex by competition with Pit-1 or consensus octamer oligonucleotides. We used extracts from both 44-2C and HeLa cells to show that the co-migrating OB1 complex seen in each extract had the same property. As seen with 44-2C extracts, the Oct antiserum blocked OB1 formation with both the CT/CGRP H/O enhancer and the octamer consensus probes with HeLa extracts (Fig. 6). The observations that OB1 is present in HeLa extracts (and all other extracts tested), is relatively large by mobility shift assays, and is recognized by the Oct antiserum strongly suggest that OB1 is the Oct-1 protein. Oct-1 is known to be a ubiquitously expressed transcription factor that is larger than any other known octamer factor (Scholer, 1991).

Identification of Cell-specific HLH and Octamer Binding Complexes—To identify whether any of the CT/CGRP enhancer complexes were cell-specific, we used extracts from the CA77, HeLa, and Rat1 cell lines for comparison. The CA77 cell line, like 44-2Cs, is a rat thyroid C-cell line that expresses high amounts of CT/CGRP mRNA (Muszynski et al., 1983; Russo et al., 1992). In the CA77 line the CT/CGRP enhancer stimulates TK-LUC expression about 10-fold (Tverberg and Russo, 1992). In contrast, the HeLa human cervical carcinoma and Rat1 fibroblast lines do not express the CT/CGRP gene and have negligible (less than 2-fold) enhancer activity (Tverberg and Russo, 1992). We used extracts from these cell lines in mobility shift

![Fig. 6. Inhibition of OB1, but not OB2, DNA binding by Oct-2 antiserum.](image-url)

Mobility shift assay following preincubation with Oct-2 antiserum recognizing the POU domain of Oct-1 and Oct-2 proteins. 44-2C extracts were incubated with CT/CGRP H/O enhancer probe (lanes 1–3) or consensus octamer probe (lanes 4–6). HeLa extracts were incubated with CT/CGRP H/O enhancer probe (lanes 7–9) or consensus octamer probe (lanes 10–12). Extracts were preincubated with no antiserum (lanes 1, 4, 7, and 10), 0.1 μl of antiserum (lanes 2, 5, 8, and 11), or 0.5 μl antiserum (lanes 3, 6, 9, and 12).
experiments containing the radiolabeled CT/CGRP enhancer oligonucleotide (Fig. 7A). We identified the HLH and octamer binding complexes by competition with 25-fold excess unlabeled AP-4 HLH or octamer consensus oligonucleotides.

Based on these criteria, all cell lines examined contained complexes that co-migrated with OB1 and HB2. The cell lines containing enhancer activity (44-2C and CA77) also contain the OB2 and HB1 complexes. These complexes were not seen

**Fig. 7. Comparison of CT/CGRP H/O enhancer-binding complexes in CA77 and HeLa cell extracts.** A, mobility shift assay using H/O enhancer probe with nuclear extracts from HeLa cells (which lack enhancer activity) (lanes 2-4) or CA77 cells (which have enhancer activity) (lanes 5-8). For reference, a mobility shift using 44-2C extract is shown (lane 1). Extracts were preincubated without competitor (lanes 1, 2, and 5) or with 25-fold excess unlabeled H/O enhancer oligonucleotide (lane 6), AP-4 HLH oligonucleotide (lanes 3 and 7), or consensus octamer oligonucleotide (lanes 4 and 8). B, summary of CT/CGRP H/O enhancer activity and binding proteins observed in different cells. OB1 is most likely the ubiquitous Oct-1 protein (see text). C, schematic model of the CT/CGRP enhancer activation mechanism. Enhancer activation requires synergism between an HLH protein and a cell-specific octamer-binding protein (OB2). The HLH protein is assumed to be a heterodimer and to be either HB1 or HB2, although we cannot distinguish between these two possibilities.

**A.**

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</tr>
<tr>
<td>HB2</td>
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<tr>
<td>OB2</td>
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**B.**

**Cell-specificity of CT/CGRP Enhancer Complexes**

<table>
<thead>
<tr>
<th>Activity</th>
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<tbody>
<tr>
<td>44-2C</td>
<td>+</td>
</tr>
<tr>
<td>CA77</td>
<td></td>
</tr>
<tr>
<td>HeLa</td>
<td></td>
</tr>
<tr>
<td>Rat1</td>
<td></td>
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</table>

<table>
<thead>
<tr>
<th>OB1 (Oct-1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>H/L</td>
</tr>
<tr>
<td>Oct</td>
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<table>
<thead>
<tr>
<th>OB2</th>
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</tbody>
</table>

**C.**

**Model of CT/CGRP Enhancer Mechanism**

- **SYNERGISM**
  - HLH
  - OB2

- HLH
- Oct
using HeLa nuclear extracts (Fig. 7A) or Rat1 cells (data not shown). The H/OB complex could often be more readily detected in CA77 extracts than 44-2C extracts as a slightly faster migrating complex than OB1. As seen with the 44-2C extracts, H/OB was clearly detected when the OB1 complex was removed by octamer competition (Fig. 7A). The H/OB complex was not seen with HeLa or Rat1 extracts. CA77 extracts had low levels of HB2 which were more visible with longer exposure times or using the Oct Mut2 probe. In addition, the complexes observed with CA77 extracts bound the mutant enhancer probes in a similar manner as that seen with 44-2C extracts (data not shown). In particular, OB2 bound the HLH mutant probe to a greater extent than wild type probe and OB1 bound the Oct Mut1 probe. The cell specificity results are summarized in Fig. 1B. Based on the cell specificity and mutation studies, a model for the mechanism of CT/CGRP enhancer activity is schematically shown in Fig. 7C (see “Discussion”).

**DISCUSSION**

A common theme in eukaryotic gene transcription is combinatorial control by multiple transcription factors. Synergistic interactions between these factors can provide a powerful activation mechanism. In this study we have established that HLH and octamer-binding factors synergistically regulate the rat CT/CGRP enhancer. Mutations in either the HLH or octamer motif essentially abolished enhancer activity and disrupted binding of the corresponding proteins to the enhancer. The mobility shift assays identified two proteins (HB1 and HB2) that bind the HLH motif and two proteins (OB1 and OB2) that bind the octamer motif. The OB2 complex was clearly specific to cell lines containing CT/CGRP enhancer activity, whereas OB1 was found in all cell types studied. We propose that cell-specific activation requires synergy between the OB2 factor and an HLH factor, possibly HB1 (Fig. 7C). The cell-specific OB2 protein must play a critical role, since the functionally inactive Oct Mut1 mutation reduced only OB2 complex formation. OB2 and an HLH factor both appear to be present in a larger complex, designated H/OB. Although it was difficult to characterize because it often co-migrated with the OB1 complex, it was clearly resolved upon removal of the OB1 complex by either addition of competitor oligonucleotides or the Oct-2 antiserum. These results indicate that both an HLH protein and an octamer-binding protein are required for CT/CGRP enhancer activity.

Synergism between HLH and octamer-binding proteins has not to our knowledge been described before, but is consistent with previous studies since both HLH and octamer proteins synergize with other factors at adjacent sites. In the case of HLH proteins, MyoD protein can bind single sites, but it must bind to multiple sites or adjacent to other transcription factors to activate muscle-specific genes (Sartorelli et al., 1990; Weintraub et al., 1990). Recently, tissue-specific expression of the tyrosine hydroxylase gene has been shown to involve synergy between an HLH motif and an adjacent AP-1 site (Yoon and Chikaraishi, 1992). Also, the rat insulin gene is apparently transactivated by synergism between the Pan HLH factor and lmx-1, a home-domain-containing protein (German et al., 1992). Although expression of the lmx-1 protein is restricted to insulin-producing cell lines, Pan is ubiquitously expressed. In the case of octamer factors, Oct-2 has been shown to bind cooperatively to adjacent heptamer and octamer sites to synergistically activate immunoglobulin-heavy chain gene promoters (Poellinger et al., 1989). In addition, Oct-1 synergizes with the glucocorticoid receptor to activate the mouse mammary tumor virus promoter (Bruggemeier et al., 1991) and the herpes viral transactivator VP16 to activate viral promoters (Gerster and Roeder, 1988).

Functional synergy between transcription factors can be the result of either cooperative or independent DNA binding interactions (Poellinger et al., 1989; Weintraub et al., 1990; Pettersson and Schaffner, 1990; Carey et al., 1990). In the case of the CT/CGRP enhancer, the in vitro binding studies indicate that the HLH and octamer-binding proteins bind independently and may even interfere slightly with each other’s binding. Mutation of one motif did not noticeably decrease binding to the other motif, and with the HLH mutation, we consistently observed greater OB2 binding than seen with the wild type enhancer. Although the basis for the increased binding is not known, it is possible that there may be some steric hindrance for binding of both factors since the HLH and octamer-binding motifs overlap. This may limit the ability of the larger ubiquitously expressed Oct-1 protein to coordinately bind with an HLH protein to the CT/CGRP enhancer. The human CT/CGRP enhancer region contains an overlapping HLH and octamer motif that differs from the rat enhancer by only a single nucleotide (CAGCTTG-CAAAAC). It seems likely that a similar mechanism involving HLH and octamer-binding proteins may also contribute to its expression.

The OB1 and OB2 proteins that bind to the CT/CGRP enhancer octamer motif have very distinct properties. The OB1 protein is clearly an octamer-binding protein, since its binding to the CT/CGRP enhancer was greatly reduced by preincubation with either a consensus octamer oligonucleotide or an Oct-2 POU domain antiserum. OB1 is most likely Oct-1 based on its large size, ubiquitous expression, and recognition by the Oct-2 antiserum, which has been shown to block Oct-1 DNA binding activity. Although the cell-specific OB2 protein binds to the CT/CGRP enhancer motif, its binding properties are somewhat different than OB1. The OB1 and OB2 complexes had inverse relative affinities for the CT/CGRP enhancer and consensus octamer sequences. In addition, as mentioned above, the Oct Mut1 mutation reduced OB2, but not OB1, binding to the enhancer as seen with both 44-2C and CA77 cell extracts. The differential binding between the two elements may be partially due to sequence differences between the CT/CGRP octamer motif and the consensus site (one well conserved residue overlapping the HLH motif), although the mechanism by which different octamer factors recognize different sites is not yet clearly understood (Schaffner, 1989; Kemler and Schaffner, 1990). These observations are consistent with earlier reports that cell-specific enhancers that contain octamer binding sites tend to bind Oct-1 weakly (Kemler et al., 1991), and that a cervical cell-specific octamer factor binds a nonconsensus octamer site more strongly than the octamer consensus (Dent et al., 1991). The observation that OB2 was not affected by the Oct-2 POU domain antiserum suggests that OB2 has a significantly different structure than the Oct-1 and Oct-2 proteins or may not be a member of the POU family. It is interesting to note that other cell-specific octamer proteins are not recognized by polyclonal antisera against Oct-1 and Oct-2 (Schreiber et al., 1990).

There are several cell-specific HLH and octamer factors that are potential candidates for regulating CT/CGRP gene transcription. Among HLH proteins, one attractive candidate is MASH1, since it is expressed in thyroid C-cell lines and the CT/CGRP enhancer was activated upon co-transfection with MASH1. Although the co-transfection data demonstrate that an HLH factor can transactivate the CT/CGRP enhancer, it does not necessarily show that MASH1 is this...
factor, since overexpression of MASH1 can also activate muscle-specific genes that are clearly not normally regulated by MASH1 (Johnson et al., 1992a). Furthermore, MASH1 is expressed only transiently during peripheral neurogenesis and is not present in adult tissues (Lo et al., 1991). Whether there is a functionally similar adult homologue of MASH1 that regulates the CT/CGRP gene remains to be determined. With respect to OB2, a number of cell-specific octamer transcription factors have been found in neural tissues (He et al., 1989; Schreiber et al., 1990; Hara et al., 1992). Target genes for many of these factors have not been identified. In particular, the N-Oct-3/Brain-2, Brain-3, and neuronal Oct-2 factors have been reported to be in neuroectoderm-derived cell types where the CT/CGRP gene is expressed (He et al., 1989; Dent et al., 1991; Schreiber et al., 1993). An advantage of synergistic activation using these or other cell-specific octamer and HLH factors is that fewer cell-specific factors would be required for phenotypic determination, since the appropriate combinatorial code would be required for transcription of target genes.

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Cell-specific HLH and Octamer Synergism

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