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Human calcitonin gene regulation by helix-loop-helix recognition sequences

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ABSTRACT

Human calcitonin (CT) gene transcription is regulated by proximal 5′ flanking sequences which mediate cAMP-induced expression, and by a distal basal enhancer region. Using transient expression of CT-CAT constructs, we showed that the basal enhancer is active in a CT-producing small cell lung cancer cell line (DMS53) and the thyroid C cell derived tumor line, TT, but is inactive in non-CT-producing cell lines. In deletional and direct mutational analyses of the distal enhancer region, disruption of two elements resembling recognition sequences for the helix-loop-helix (HLH) family of transcriptional regulatory proteins resulted in a significant loss of basal transcriptional enhancer action. These results suggest that HLH recognition motifs may mediate a significant portion of constitutive CT gene transcriptional activity in these cells. Nuclear protein extracts from DMS53 cells formed specific binding complexes with oligonucleotides containing two of these candidate enhancer sequences. However, proteins capable of binding to these CT gene HLH consensus recognition sites were not restricted to CT-producing cells. We conclude that members of the HLH protein family, some expressed ubiquitously and some expressed or activated in a tissue-restricted fashion, may combine to enhance CT gene transcription in tumor cells of neuroendocrine derivation.

INTRODUCTION

The calcitonin (CT) gene is expressed by diverse cells of neuroendocrine lineage including thyroid parafollicular C cells, pulmonary neuroendocrine cells, and neural tissues (1, 2, 3). A variety of tumors produce CT, including medullary thyroid cancer (MTC) derived from thyroid C cells, and several types of lung cancer including small cell lung cancer (SCLC), large cell neuroendocrine carcinoma, and carcinoid tumors (4, 5, 6). A complex, developmentally-regulated mechanism of alternative RNA splicing generates predominantly CT mRNA in thyroid C cells and calcitonin gene-related peptide (CGRP) mRNA in neural tissues (1, 7). Both immunoreactive CT and CGRP have been identified in scattered neuroendocrine cells present in normal lung epithelia (3).

Expression of the human CT gene is regulated at a transcriptional level by a complex series of 5′ regulatory regions. Proximal 5′ flanking sequences mediate cAMP-induced CT gene expression (8). Basal, tissue-specific expression is enhanced weakly by proximal sequences and more strongly by more distal 5′ enhancer sequences (8, 9). In earlier studies, we showed that cell lines derived from MTC and SCLC utilized these two enhancer regions differently (8). Proximal sequences located between −132 and −252 mediated a dramatic cAMP response in the TT line of MTC, but not in the DMS53 line of SCLC. Distal sequences located between −750 and −2000 exhibited a strong basal enhancer action in DMS53 cells and a weaker, more variable basal action in TT cells.

In the present study, we have further analyzed CT basal enhancer function and have begun characterization of basal enhancer binding proteins in these two CT-expressing tumor lines. In order to compare CT gene enhancer function among several tumor cell lines sharing a common neuroendocrine lineage, we also studied two SCLC lines which lack demonstrable production of CT mRNA. By deletional and mutational analysis of transiently expressed CT-CAT constructs, we have shown that maximal basal enhancer action requires the presence of several motifs of the form NCANNTGN, resembling recognition sequences for the helix-loop-helix (HLH) family of transcriptional regulatory proteins (10). Proteins from nuclear extracts of CT-producing cells bind to these sites in a sequence-specific fashion and may be instrumental in the constitutive transcription of the CT gene in neuroendocrine cells.

MATERIALS AND METHODS

Plasmid Constructs

An initial series of human CT gene 5′ end deletion mutants (shown schematically in Figure 1) was derived by restriction enzyme digestion of pCT2000CAT, which contains sequences from −2000 to +88 of the human CT gene fused upstream of a CAT reporter gene in a pUC18 host plasmid (8). To enable fine resolution mapping of the −1178 to −931 interval of the CT gene, a series of additional 5′ end deletion constructs (shown in Figure 2) was created using the polymerase chain reaction (PCR) with cloned CT gene 5′ flanking fragments used as template. Upstream primers contained a HindIII site adjacent to the desired 5′ end of the CT gene insert; downstream primers

corresponded to CT gene sequences proximal to the BglII site at −728. The series of PCR fragments was digested with HindIII and BglII and ligated in place of the HindIII-BglII (−1178 to −728) segment of pCT1178CAT. Interstitial deletion mutant constructs (shown in Figure 3A) were created using PCR from a linearized pCT960CAT template to generate fragments containing a new BglII site adjacent to the desired 3′ terminus and a HindIII site at −960. These fragments were digested with HindIII and BglII and ligated in place of the HindIII-BglII segment of pCT960CAT. The resulting constructs, pCT960Δ898−728CAT and pCT960Δ916−728CAT, lack bases −728 to −898 and −728 to −916 respectively. Constructs containing selected mutations at candidate enhancer sites were created using PCR primers containing the desired mutation and a 3′ tail complementary to the adjacent wild type sequence (11). These plasmids (shown in Figure 3B) were constructed to be identical to the native CT gene 5′ flanking sequence except that 6 bp candidate enhancer motifs were replaced by ClaI or KpnI restriction sites. The identity of each construct was verified by dideoxy sequencing and compared with the human CT gene sequence derived by us and by Broad *et al.* (12).

Tissue culture and transfection

DMS53 cells (13) were grown in Waymouth's MB 752/1 medium (Gibco) supplemented with 16% bovine calf serum (Hyclone). TT cells (14) were grown in RPMI 1640 (Gibco) with 16% bovine calf serum; NCI H82 (15) and OH3 (16) cells were grown in RPMI 1640 with 10% bovine calf serum. Transfection conditions for TT and DMS53 cells were as previously described (8), using a Gene Pulser (Bio-Rad) electroporation apparatus and 50 μg supercoiled plasmid. H82 and OH3 cells were electroporated at 200V in growth medium using a BioRad capacitance extender set at 960 μFD, following preliminary optimization studies with a control plasmid, pRSVCAT (8). CAT activity was assayed from duplicate plates 48 hours later as described by Gorman *et al.* (17) and quantitated by scintillation counting of acetylated chloramphenicol resolved on thin layer chromatography plates. Results in each experiment were expressed as a percentage of the activity of the pRSVCAT control plasmid or relative to the activity of pCT1178CAT.

Gel mobility shift assays

DMS53 cell crude nuclear protein extracts were prepared according to a modification (18) of the method of Dignam *et al.* (19). The extract was precipitated in 53% saturated NH_4SO_4, resuspended in TM buffer (18), and quantified using the Bradford method (BioRad). TT, H82, and OH3 cell extracts were prepared using the Dignam method. Complementary oligonucleotides were synthesized on a Millipore Cyclone Plus DNA synthesizer, purified using oligo-pak columns, heated together to 65 degrees, allowed to anneal while cooling slowly to room temperature, and 3′ end labelled using Klenow DNA polymerase and α^{32}P-dCTP. We employed three 20 or 21 bp oligonucleotide probes containing the native sequence surrounding the candidate enhancer sites termed H_1, H_2, and H_3, respectively, plus identical cold competitor oligonucleotides or heterologous competitors that were mutated at each six bp candidate enhancer core (see Figure 4A for sequence information). Gel mobility shift binding reactions contained 20,000 cpm of probe at an activity of 4,000 cpm/ng, 2 μg poly dI-dC, 50 mM KCl, 16 mM HEPES, 4 mM Tris-HCl pH. 7.9, 1 mM DTT, 1 mM EDTA, 12% glycerol, 8 μg protein extract, plus or minus competitor oligonucleotide. Incubations were performed at room temperature for 15 minutes. Complexes were resolved on 4% low ionic strength polyacrylamide/bisacrylamide (80:1) gels at 4 degrees.

RESULTS

Cell type specific activation of the CT gene distal basal enhancer

The CT gene is expressed at a high level in the human medullary thyroid cancer line TT (20). Typical small cell lung cancer cells in culture produce little or no CT; for example, the H82 and OH3 lines lack detectable CT mRNA by Northern or RNAase protection assays (21). In contrast, the DMS53 line produces levels of CT peptide and CT mRNA comparable to TT cells and a significantly higher ratio of CT to CGRP than is seen in TT cells (8, 21). We therefore tested whether these cell types utilize candidate 5′ enhancer regions differently. When a series of 5′ end deletion CT-CAT constructs were transiently expressed in DMS53 and TT cells (Figure 1), both cell types exhibited a

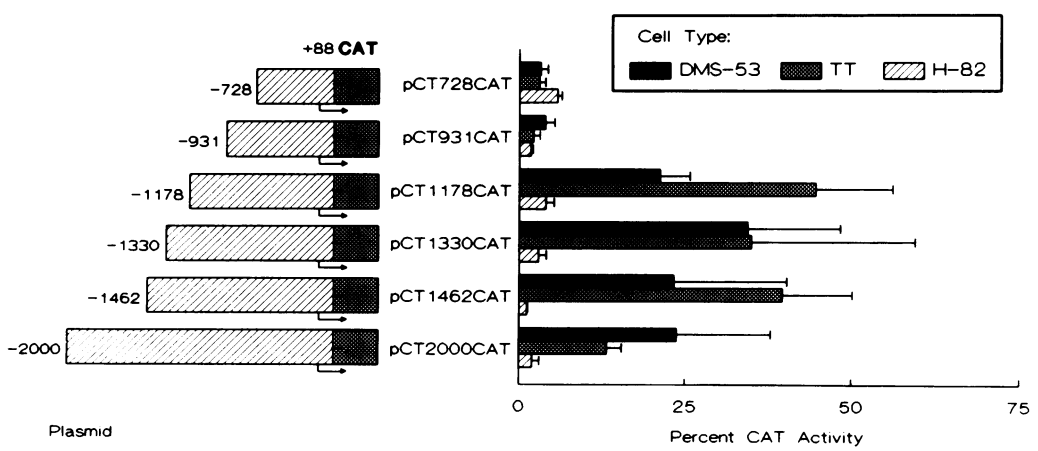

Figure 1. Preliminary assessment of human CT gene basal enhancer activity using CT-CAT constructs transiently expressed in the DMS53 and H82 lines of SCLC, and the TT line of MTC. A 21.2-fold increase in CAT activity was seen for pCT1178CAT compared to pCT931CAT in TT cells; the fold difference was 5.4 in DMS53 cells. No increase was observed in the non-CT expressing H82 line. CAT activity for each construct is expressed relative to the activity of pRSVCAT as the mean +/−SEM of 3−5 experiments.

significant increase in basal CAT activity (5.4 and 21.2 fold, respectively) when regions containing bp −931 to −1178 of the CT gene were included. These data suggest that both cell types may utilize a basal transcriptional enhancer region located within or overlapping this interval.

The enhancer-like effect appears to be limited to CT-producing cells, since no comparable increase was observed in H82 cells, or in a second non-CT-producing SCLC line, OH3 (data not shown). In both non-CT-producing lines, marker gene activity generated by the pRSVCAT control plasmid resembled levels seen in the CT- expressing lines TT and DMS-53. There was no evidence for additional enhancer-like activity in the interval between nucleotides −1178 and −2000. Indeed, TT cells, but not DMS53 cells, exhibited a three-fold decrease in CAT activity over the interval −1462 to −2000 (Figure 1), suggesting the possibility that TT cells may utilize negative regulatory element(s) within this CT gene segment.

Localization of enhancer sequences by 5′ end deletion analysis

In order to more finely map the boundaries of the human CT gene basal enhancer region, we transiently expressed in DMS53 cells a series of PCR-generated 5′ end-deletion mutant constructs. A significant increase in CAT activity (8.5-fold) was seen in a construct extending to −941 (pCT941CAT), compared to pCT931CAT extending to −931 (Figure 2). A smaller two-fold increase was seen in comparison of a construct extending to −1067 vs. one extending to −1040, but this difference was not statistically significant. Interestingly, no difference was seen in marker gene activity between constructs extending to −1040 vs. −990, despite the inclusion of an AP-2 like sequence (22), GGGTGGGGGT, at −1005 to −996. No further significant increase was seen in constructs extending to −1178. These results suggest that in the DMS53 SCLC line, a substantial portion of the basal CT gene transcriptional enhancer activity stems from sequences with a 5′ boundary near nucleotides −941 to −931, with a potential contribution from sequences downstream from position −1067.

Interstitial deletion analysis

In order to estimate the 3′ boundary of the enhancer element(s), we created interstitial deletion constructs which lacked portions of the CT flanking region between −916 and −728 or between −898 and −728. Results shown in Figure 3A demonstrate that deletion of sequences between −898 and −728 (in pCT960Δ898−728CAT) had little effect on reporter gene activity compared to the wild type sequence present in pCT960CAT. In contrast, deletion of bases −916 to −728 (in pCT960Δ916−728CAT) resulted in a 10-fold reduction in CAT activity, comparable to the activity of proximal enhancer sequences alone. The results of these experiments suggest that in DMS53 cells, sequences located between −916 and −898 contribute to CT gene basal enhancer action. Sequences located between −898 and −728 appear not to contribute significantly to basal enhancer action. In combination, the 5′ end deletion and interstitial deletion data suggest that in the DMS53 cells, CT basal enhancer function is largely mediated by sequences located between −941 and −898 with a potential additional contribution from sequences located between −1067 and −941.

Identification of HLH consensus recognition sites in the CT gene basal enhancer

The CT gene 5′ flanking sequence contains three elements of the general form NCANNTGN within the −898 to −1067 span. These sequences resemble E-box recognition sequences for the known family of helix-loop-helix transcriptional regulatory proteins (9, 10). In the above deletion experiments, disruption of any of these motifs was associated with a loss of CAT activity. The most proximal motif GCAGGTGA, termed H_3, is located between −914 and −907. As discussed above, interstitial deletion of 18 bp surrounding this element was associated with a significant loss of enhancer function (compare pCT960Δ898−728CAT vs. pCT960Δ916−728CAT, Figure 3A). Similarly, the central HLH motif, GCAGCTGT (H_2), is located between −935 and −928. Truncation of this candidate enhancer sequence in the construct pCT931CAT was associated with a low level of CAT activity comparable to the activity of proximal sequences alone, whereas preservation of this sequence with only six additional flanking bases in pCT941CAT resulted in an 8.5 fold enhancement (Figure 2). Truncation of the most upstream HLH consensus motif, ACACCTGC (H_1) located between −1042 and −1035, resulted in a more modest decrease in reporter activity (compare pCT1067CAT and pCT1040CAT

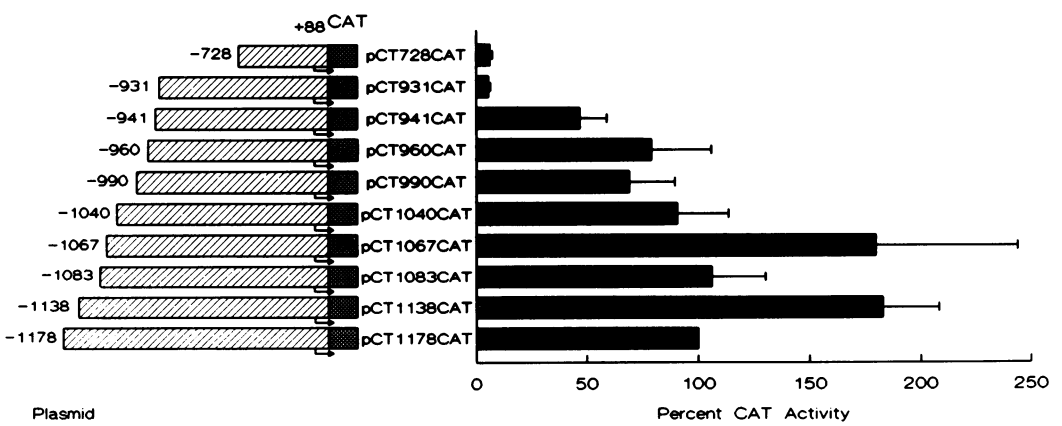

Figure 2. Fine mapping of the interval between nucleotides −931 and −1178 using 5′ end-deletion constructs transiently expressed in DMS53 cells. pCT941CAT generated an 8.5-fold greater CAT activity than pCT931CAT. A two-fold increase could be seen for pCT1067CAT compared to pCT1040CAT. CAT activity for each construct is expressed relative to pCT1178CAT as the mean +/−SEM of 5−8 experiments.

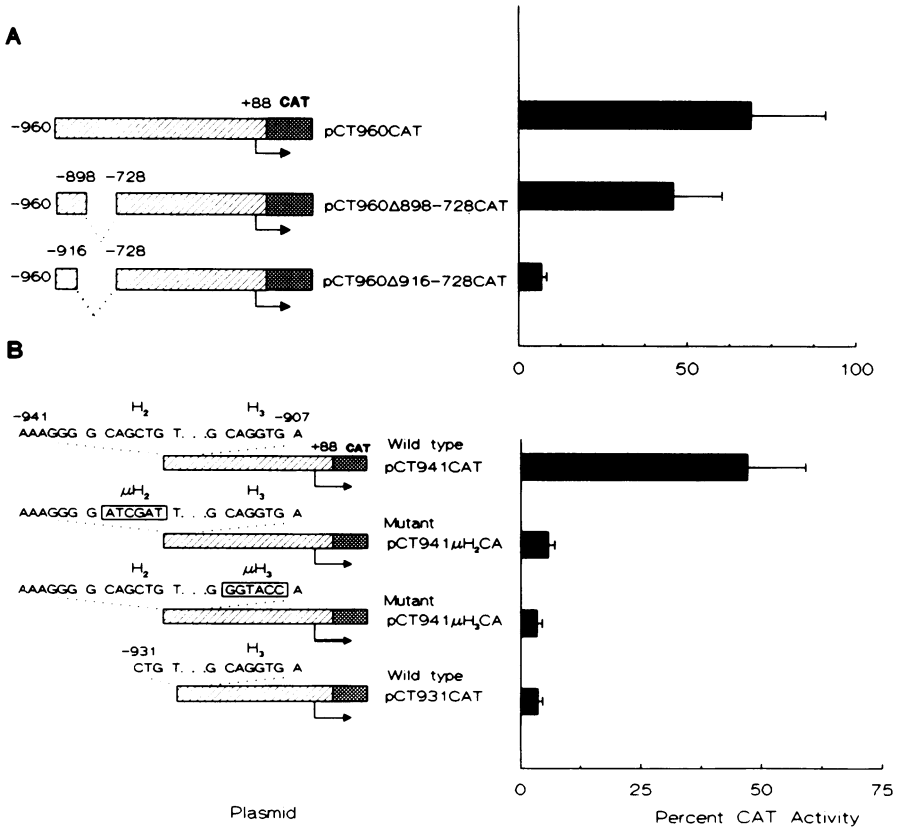

Figure 3. Interstitial deletion and mutational analysis of CT gene candidate enhancer sequence function in DMS53 cells. **A.** Deletion of nucleotides −916 to −728 (pCT960Δ916−728CAT) caused a 10-fold reduction in CAT activity, compared to the wild type pCT960CAT; deletion of nucleotides −898 to −728 (pCT960Δ898−728CAT) caused no significant reduction. **B.** Selective mutation of the H_2 (−934 to −929) or H_3 (−913 to −908) sites reduced CAT activity 8 and 13-fold compared to pCT941CAT. Activity is expressed as a percentage of pCT1178CAT as the mean +/−SEM of 4−8 experiments.

in Figure 2). The 6 base core sequences of the and H_1, H_2, and H_3 sites are not duplicated elsewhere in the proximal 1.5 kb of the CT 5′ flanking sequence.

Mutation of HLH consensus recognition sites inhibits basal enhancer activity

Based on the above evidence that sequences resembling HLH protein recognition motifs exist within candidate enhancer sequences of the CT gene and may play a functional role in the regulation of CT gene expression, we tested whether mutation of such motifs was associated with loss of enhancer function. We chose to analyze the two more proximal motifs (H_2 and H_3), since deletion of DNA segments containing these two elements was associated with the most significant changes in reporter gene activity. As shown in Figure 3B, selective mutation of the six base core of either H_2 or H_3 resulted in a significant 8 to 13-fold decrease in CAT activity compared to the wild type construct containing both intact HLH recognition motifs. The two mutant plasmids generated a similar level of reporter gene activity to the wild type plasmid, pCT931CAT, which contains the H_3 but not the H_2 motif. These results suggest that the presence of both the H_2 and H_3 motifs is necessary for full function of the CT gene basal transcriptional enhancer.

Multiple protein complexes bind to CT gene enhancer sites

In order to begin characterization of nuclear proteins which interact with the CT basal enhancer elements, we tested the ability of oligonucleotides containing either the H_1 (bp −1047 to −1027), H_2 (bp −939 to −919), or H_3 (bp −921 to −900)sites (Figure 4a) to bind to nuclear protein extracts from DMS53 cells in gel mobility shift assays. An oligonucleotide probe containing the H_3 site formed two distinct shifted complexes (see lane 2 in Figure 4B). The specificity of these DNA-protein complexes was investigated by comparing the ability of wild type vs. mutant oligos to compete with the labelled probe for binding to protein extracts. The slow-migrating complex (appearing as the top shifted band in lane 2) was efficiently competed by the corresponding homologous cold competitor (lanes 3−5), but not by an oligonucleotide (μH_3) mutated at the 6 base HLH recognition element enhancer core sequence but otherwise identical to H_3 (lanes 6−8). In contrast, the fast-migrating (lower) complex showed a non-specific competition pattern. In further experiments, the autoradiographic intensity of this band was not saturated at high amounts of labelled oligonucleotide, suggesting that this complex represents a high-capacity, low affinity DNA binding protein (data not shown).

In comparison with the H_3 binding pattern, complexes containing the H_2 oligo appeared fainter and less distinct (Figure 4C). However a qualtitatively similar, sequence-specific competition pattern could be observed for the H_2 complexes. Fainter intermediate-mobility bands could also be seen for both oligo H_2 and H_3. Oligo H_1, in simultaneous comparisons with oligo H_3 using similar labelling, binding, and electrophoresis conditions, formed a much weaker low-mobility complex with

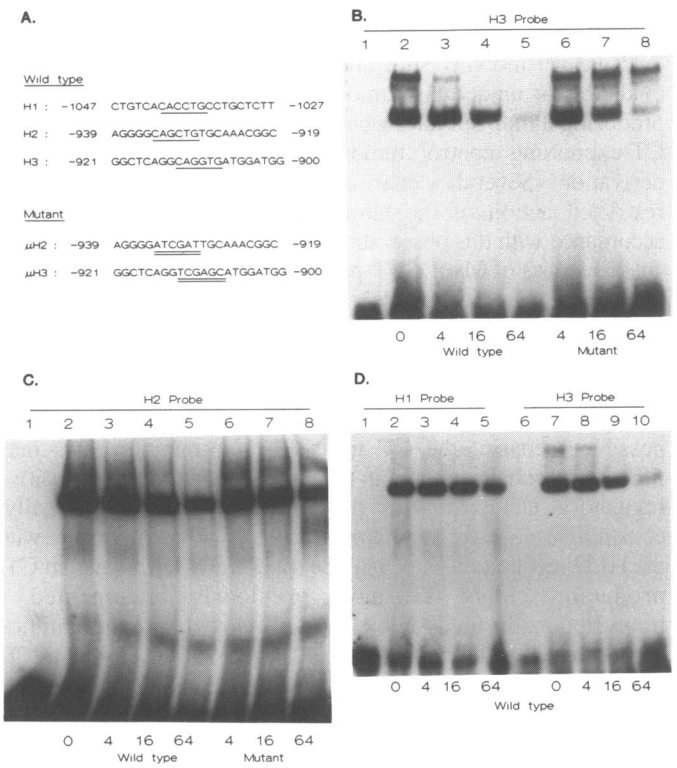

Figure 4. Gel mobility shift analysis of CT gene HLH elements. **A.** Coding strand sequence of probes containing the H_1, H_2, and H_3 elements. Oligos μH_2 and μH_3, compared to oligos H_2 and H_3, are selectively mutated in their respective CAGCTG and CAGGTG core elements. **B.** Lane 1, unreacted H_3 probe; lanes 2–8, DMS53 extract added. Listed under each lane is the fold excess of wild type or mutant competitor DNA. **C.** Lane 1, unreacted H_2 probe; lanes 2–8, DMS53 extract added. **D.** Lanes 1 and 6, unreacted H_1 and H_3 probes, respectively; lanes 2–5 and 7–10, DMS53 extract added to H_1 or H_3 probe. (See results section for analysis of shift patterns).

DMS53 extracts and a similar non-specific high-mobility complex (Figure 4D).

Protein binding to CT gene enhancer sites is not restricted to CT-producing cell types

After observing sequence-specific binding between DMS53 nuclear protein extracts and two CT gene sites resembling HLH protein binding motifs, we questioned whether such binding activity was characteristic of CT-producing cells. We tested the ability of oligo H_3 to bind to extracts from the CT-producing TT line, as well as to extracts from the H82 and OH3 SCLC lines which do not utilize the CT gene basal enhancer. As shown in Figure 5, the TT extract (lane 2) formed complexes with the H_3 probe similar to those seen with an equivalent amount of DMS53 extract (lane 1). With the TT extract, a low-mobility complex was efficiently competed by a 16-fold excess of cold homologous oligo (lane 4). Extracts from H82 (lanes 5–7) and OH3 cells (lanes 8–10) produced comparable shifted complexes, and a similar competition pattern with homologous cold oligonucleotide. This experiment indicates that the H_3 enhancer element is capable of binding to nuclear proteins from several cell lines, and that this activity is not restricted to CT-producing cells. The sensitivity limits of the mobility shift assay, using crude nuclear extracts, may preclude discrimination between subtly different DNA–protein complexes such as those arising from different combinations of heteromeric DNA binding proteins.

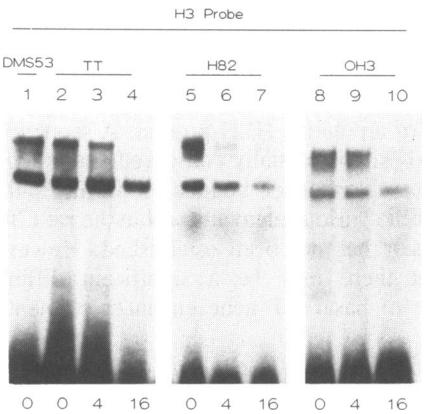

Figure 5. Gel mobility shift analysis of binding to the CT gene H_3 element by nuclear protein extracts from CT-producing and non-CT producing cultured cells. Nuclear protein extracts from CT-producing lines, DMS53 (lane 1) and TT (lane 2), as well as the non-CT producing lines, H82 (lane 5) and OH3 (lane 8), formed specific, slow-migrating complexes with the H_3 probe which are efficiently competed by homologous cold oligo (lanes 3–4, 6–7, and 9–10).

DISCUSSION

Our results demonstrate that, in a CT-producing small cell lung cancer line as well as in a medullary thyroid carcinoma cell line, CT gene basal transcriptional enhancer activity stems from a complex region located approximately 1 kilobase upstream from the CT transcription initiation site. By means of 5′ end deletion and interstitial deletion analysis, we narrowed the principal basal enhancer activity to a region with a 5′ boundary between −931 and −941 and a 3′ boundary between −898 and −916. Mutation of either of two HLH-like recognition motifs within this region was sufficient to abolish the enhancer activity associated with this region. Together, these results provide evidence that consensus HLH recognition motifs mediate a significant portion of constitutive CT gene transcriptional enhancer activity in this cell line.

We had shown earlier that the human CT gene segment between −750 and −2000, containing the basal enhancer region, was sufficient in DMS53 cells to activate expression through a heterologous thymidine kinase promoter (8). This CT gene segment maintained partial enhancer activity in reverse orientation. In the present study, both TT cells and DMS53 cells appeared to utilize enhancer sequences between −931 and −1178; however TT cells also exhibited a decrease in marker gene activity over the interval −1462 to −2000. The presence of a negative regulatory element in the −1462 to −2000 segment could account for the relatively modest basal activity of pCT2000CAT that we have observed in the TT line (8).

Both the rat and human CT genes have been shown previously to utilize orientation and promoter-independent basal enhancers located in similar positions in the distal 5′ flanking region (9, 23). Stolarsky-Fredman *et al.* mapped a rat CT/CGRP gene tissue-specific basal enhancer, using the C cell tumor line CA77, to a region encompassing bases −957 to −1127 (corresponding roughly to base −857 to −1006 in the human sequence) (23). Deletion of either the proximal or distal half of this region resulted in loss of enhancer activity, suggesting a complex enhancer organization with two or more interacting components. In comparison with the human sequence, the rat basal enhancer region has a 50 base conserved segment containing the H_2 site

as well as the AP-2 like sequence. A sequence resembling the human H_3 site is also present within the rat basal enhancer region, but with one base pair alteration, GCAGGCG vs. GCAGGTG in the human, which could potentially interfere with the binding of a putative HLH protein. A sequence resembling the human H_1 site is partially conserved and is located distal to the 5′ rat basal enhancer boundary. To date, the functional relevance of the various elements within the rat CT/CGRP gene basal enhancer has not been established. However, our data suggest that there may be a significant difference in the organization of basal CT gene enhancer elements in the two species.

In the human CT gene, Peleg, *et al.* defined a minimal enhancer sequence located between −1060 and −905 necessary for maximal reporter gene expression in TT cells (9). Simultaneous deletion of base −1060 to −1033 plus bases −920 to −905 from the minimal enhancer resulted in a four-fold decrease in marker gene expression. The relative contribution of these two deleted segments was not directly tested, although a 5′ end deletion from bases −1078 to −1032 led to a comparable four-fold decrease in expression. A series of oligonucleotides containing known E-Box sequences was shown to compete with the wild type CT minimal enhancer sequence for binding to TT nuclear protein extracts. The potential enhancer function of the CT gene E-Box sequences was not directly tested in mutational analyses. Our data extend these findings to directly implicate the two more proximal HLH consensus sequences, H_2 at −935 to −928, and H_3 at −914 to −907, as CT gene transcriptional enhancer elements.

Previously known or novel HLH protein family members are attractive candidates for regulation of constitutive CT expression and participation in the differentiation program of CT-producing neuroendocrine cells. By sequence comparison, the CT gene H_2 site, GCAGCTGT, resembles the immunoglobulin heavy chain μE_2 enhancer, CCAGCTGC (24), while the CT H_3 site, GCAGGTGA, has seven of eight base pairs identical to the light chain KE_2 enhancer motif, GCAGGTGG (24, 25). The upstream H_1 motif, ACACCTGC shares an eight base pair identity with a muscle creatine kinase enhancer site (26).

To date, HLH proteins have been implicated as basal transcriptional enhancer binding proteins for a large group of genes, including muscle specific genes induced by MyoD (27), the immunoglobulin Kappa light chain gene induced by E2A proteins (10, 25), and the rat insulin I gene with NIR and FAR elements responsive to factors not yet fully characterized (28, 29). The organization of multiple HLH recognition sites within an enhancer region appears important for transcriptional activation. For example, MyoD competes with a negative regulator of binding, Id, to form a heterodimeric complex with an E2A protein (27). These MyoD-E2A protein complexes must bind to two or more upstream enhancer elements, frequently located in clusters, to permit transcriptional activation (30, 31). Clustering of elements characterizes the Ig heavy and light chain enhancers as well (24).

For the human CT gene, we showed that nuclear factors present in DMS53 and TT cells are capable of sequence-specific binding to the HLH consensus recognition motifs. Such binding activity might be expected, given that similar motifs are known to bind *in vitro* to the widely expressed E2A gene products E12 and E47 (10). In the protein extraction and binding conditions which we employed, the H_3 and H_2 sites formed more distinct complexes with DMS53 nuclear protein extracts than did the H_1 site. The weaker H_1 complexes that we observed, compared to H_3 and H_2, may correlate with the apparently smaller functional contribution of the H_1 site in transient expression assays.

Using gel mobility shift analyses, we were unable to detect evidence for unique HLH motif binding complexes in the CT-producing tumor cell lines which we tested compared to two non-CT-expressing control tumor lines, also of neuroendocrine derivation. Several scenarios could account for the tissue-restricted action of the human CT gene basal enhancer in accordance with this observation. In analogy to the DNA binding characteristics of MyoD, CT-producing cells, such as the DMS53 line, may contain tissue-specific enhancer binding proteins that heterodimerize with high abundance, ubiquitous proteins and compete for access to the HLH consensus sites. Alternatively, CT-producing cells may allow tissue-specific post-translational activation of CT gene enhancer binding proteins. In a third possible scenario, the CT gene HLH recognition sites may function as enhancers only in the company of additional positive regulatory elements with tissue-restricted activity. Finally, constitutive negative regulatory factors, acting in concert with the HLH recognition sites, may be specifically inactivated in CT-producing cells. We have previously documented a transcriptionally unfavorable 'closed' chromatin configuration and/or hypermethylation of the CT gene in several non-CT-expressing lines (21). However it seems unlikely that unfavorable chromatin or methylation status plays an exclusive role in the transcriptional inactivation of the gene since the two non-CT-expressing cell lines which we examined in this study do not support transient expression of unmethylated synthetic CT-CAT constructs.

In summary, we have shown that human CT gene sequences resembling recognition motifs for the HLH family of transcriptional regulatory proteins have a significant role in mediating basal transcriptional enhancement of the gene. The characterization of factors which interact with the CT gene basal enhancer may lead to elucidation of the mechanisms regulating the tissue-restricted pattern of CT gene expression observed in differentiated neuroendocrine tissues.

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