Modified binding of proteins from calcitonin-negative tumor cells to the neuroendocrine-specific CANNTG motif of the calcitonin gene

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ABSTRACT
Transcription of the calcitonin (CT) gene in the medullary thyroid carcinoma (MTC) cell line TT is modulated by a neuroendocrine-specific enhancer fragment (nucleotides – 965 to – 905) containing two CANNTG motifs (E2 and E3) and an Ets-like response element. To determine the cell-specific component of this fragment, oligonucleotides containing the individual elements were inserted in front of a minimal CT promoter and tested for reporter protein production in CT-positive (TT) and -negative (RO-D81 and HeLa) cells. In TT cells, using two copies of E2 or four copies of Ets increased minimal promoter activity a 20–40 fold. Using two copies of E3 had no effect on minimal promoter activity. In CT-negative MTC cells (RO-D81), the Ets response element was active but the two copies of E2 were not. Similar results were obtained with the non-neuroendocrine cell-line HeLa. I therefore concluded that E2 was the cell-type-specific component of the enhancer. An E2-specific binding protein was detected in both MTC cell lines but not in HeLa. This protein had different mobility and DNA-binding specificity in CT-positive TT cells and CT-negative RO-D81 cells. In conclusion, the CAGCTG motif of E2 modulated the cell-specific transcription of the CT gene, and its inactivation in CT-negative MTC cells correlated with modifications in its binding proteins.

INTRODUCTION
Calcitonin (CT) is produced by a restricted population of neural crest-derived endocrine cells [1]. Most CT-producing cells are located in the parafollicular region of the thyroid (the C cells) [2]. A scattered population of C-like cells is also present in other organs such as lung, thymus and prostate. Malignancies of CT-producing neuroendocrine cells may develop in the thyroid (medullary thyroid carcinoma [MTC]) and lung (small-cell lung carcinoma [SCLC]) as well as in the colon, breast, and prostate [2]. Progression of MTC from focal, microscopic carcinoma to metastatic tumors is often characterized by a transition from pronounced CT expression to weak and patchy expression [3].

Several cell-specific transcription factors also regulate cell growth and differentiation [4–8]. Downregulation of CT gene transcription in metastatic cells may therefore reflect the inactivation of such factors and explain subsequent aberrant, unregulated cell growth. To identify these factors it was necessary first to identify cell-specific enhancer elements of CT gene transcription. Several studies have dealt with the factors that modulate CT gene transcription in human MTC and SCLC cells [9—11]. There is a body of evidence that suggests that constitutive, neuroendocrine-specific CT gene transcription in these cells is modulated by an enhancer approximately 1 kb upstream from the transcription start site [9,10]. It has been shown that there were several functional CANNTG motifs (E boxes) in this enhancer. However, because separating these elements abolished enhancer activity [11], it was not possible to show which was the cell-specific component of the enhancer. Furthermore, analysis of protein binding to the CANNTG motifs did not reveal cell-specific qualities [11].

In the study reported here, my goal was to identify the cell-specific component of the human CT enhancer. For that I dissected the human CT enhancer and identified autonomous enhancer elements. These were tested in slowly dividing CT-positive and in rapidly dividing CT-negative MTC cells, which I have chosen as representatives of two stages of tumor development, a differentiated and a metastatic one. I showed that three motifs were essential for enhancer function in the differentiated CT-positive MTC cells: two CANNTG motifs and an Ets-like motif. One of the CANNTG elements was the cell-specific component of the human CT enhancer because it was not functional in the CT-negative MTC cells or in non-neuroendocrine cells. Protein binding to this element was different in CT-positive and CT-negative cells.

MATERIALS AND METHODS
Cell culture and transfections
TT cells were maintained in RPMI 1640 supplemented with 10% fetal bovine serum. RO-D81 and HeLa were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum.

Twenty four hours before transfection, cells were plated in 35-mm dishes at a density of 3×10^5/dish (for TT) or 10^5/dish (for RO-D81 and HeLa) in DMEM and 10% fetal bovine serum. The cells were transfected with 8 µg of plasmid DNA per dish by the DEAE dextran method [9] and then briefly treated (1 min)
with 10% dimethyl sulfoxide. Medium samples were collected 3–5 days after transfection. Growth hormone production by the reporter gene was measured by a two-site IRMA assay as described by the manufacturer (Nichols Institute, San Juan Capistrano, CA).

**Plasmid construction**

Plasmid pCTGH was prepared by subcloning a BamHI to MboI fragment (nucleotides −129 to +91 of the CT gene) into the BamHI site at the transcription start site of a plasmid containing the growth hormone gene. Synthetic double-stranded oligonucleotides or fragments synthesized by the polymerase chain reaction were inserted immediately upstream from nucleotide −129 of the CT gene in plasmid pCTGH. Each construct was characterized by restriction mapping and by DNA sequencing.

**Electrophoretic mobility shift assays (EMSA)**

Fragments containing E2 (nucleotides −965 to −920) or E3 (nucleotides −935 to −877) were labeled with $^{32}$P to a specific activity of $3 \times 10^4$ cpm/ng DNA. Each binding reaction contained 0.5 ng of DNA probe, 10 μg of nuclear proteins prepared as described by Dignam et al. [12], and 1–2 μg of poly (dIC)(dIC) as nonspecific competitor DNA. The binding reactions were performed at room temperature for 30 min. The complexes were then resolved by electrophoresis through 4% polyacrylamide gels at 4°C. For competition experiments the same conditions were used, except that specific oligonucleotides and non-specific DNA were added to the binding reactions before addition of the probe. The sequence of the E3 oligonucleotide was 5′-AAACGGCTCAGGCAGGTGATGGATGGCAG-3′; the sequence of the E2 oligonucleotide was 5′-GGAAGCAAAGGGGCAGCTGTGCAAACGG-3′. The sequence for E2m was the same as E2 except that the underlined CAGCTG was mutated to CTTCGG.

**RESULTS**

Identification of autonomous elements in the human CT enhancer of constitutive transcription

Previous studies in this laboratory showed that there is a constitutive, neuroendocrine-specific enhancer of the human CT gene between nucleotides −1060 and −905 upstream of the CT transcription start site [9]. A shorter sequence, nucleotides −1020 to −920, also had enhancer activity. This shorter fragment contained only one of the two CANNTG (E motif) sequences that were later shown by Ball et al. [11] to be essential for the transcriptional activity of the constitutive enhancer. In their study, the E motifs, one which mapped at nucleotides −940 to −930 and the other at nucleotides −920 to −910, could not function independently; their separation completely abolished enhancer activity. To resolve this discrepancy and to identify any autonomous components of the cell-specific enhancer, I created a series of deletions and point mutations in fragment −1060 to −905, attached each new fragment to pCTGH, and tested each construct in TT cells.

Figure 1 shows that deleting nucleotides −1060 to −966, which removed a CANNTG motif previously called the upstream element (USE, [9]), and now called E1, reduced transcription by 30 to 50%. An additional deletion of nucleotides −921 to −905, which contained a CANNTG sequence termed E3, reduced transcription by another 40%. To determine if the remaining CAGCTG motif (termed E2), was essential for enhancer activity, I inserted point mutations that converted it to CTTCGG. These

![Figure 1](image-url)
mutations abolished the transcriptional activity of enhancer fragment -965 to -920. Further deletion of the enhancer fragment to nucleotides -948 to -920 completely abolished its transcriptional activity. These results led me to conclude that in human TT cells the sequence -965 to -920 contained at least two transcriptionally active motifs. One overlapped the CAGCTG motif (E2) and the other was upstream from the first between -965 and -948.

Since neither element could function on its own, I examined the transcriptional activity of multiple copies of each. Four copies of oligonucleotide -965 to -935 or two copies of oligonucleotide -948 to -920 acted as a powerful enhancer. Because they overlap, it was important to determine the functional component in each enhancer fragment. In the CAGCTG sequence (E2 motif) in fragment -948 to -920, I inserted point mutations that modified it to CTTCCG. These point mutations completely abolished the transcriptional activity of multiple copies of E2 (Figure 1). The other enhancer fragment (nucleotides -965 to -935) contained the sequence CCGGAAGC, which is homologous to the Ets 1 and Ets 2 responsive elements (CC/AGGAA/TGC/T) [13]. A mutation in this Ets-like binding site that changed the GGAA to CTAC abolished the transcriptional activity of the duplicated motif. Finally, because E3 was shown to be essential for human CT enhancer activity by Ball et al. [11], I examined transcriptional activity of this element. Neither one nor two copies of a short oligonucleotide (-925 to -895) containing the E3 motif CAGGTG had autonomous transcriptional activity. I therefore concluded that between nucleotides -965 and -905 there were only two autonomous enhancer elements. One was the E2 motif CAGCTG, and the other was an Ets-like responsive element GGAA. In these experiments, E3 augmented the activity of the enhancer to some extent, but it was not essential for enhancer function as been previously reported.

To reconcile the difference between my findings and the report by Ball et al. [11], I considered the possibility that the individual elements of the basal enhancer may act differently in their normal position in respect to the promoter. Therefore I reexamined the transcriptional activity of Ets, E2, and E3 by inserting point mutations into them without changing their distance from each other and from the promoter and the transcription start site. Figure 2 shows that mutation of E2 or E3 abolished the transcriptional activity of the fusion gene. Point mutations in Ets reduced transcription remarkably but did not abolish it. In conclusion, it seems that E3 was important for basal transcription only when it was located at its normal distance from the promoter. However, when the enhancer elements were moved closer to proximal promoter elements and the transcription start site, only the autonomous Ets and E2 motifs were essential for enhancer activity.

Identification of the cell-specific component in the CT basal enhancer

Once the elements of the cell-specific enhancer were mapped and autonomous motifs were identified, it was possible to determine which was inactivated in CT-negative cells. Starting with a fragment containing both E2 and Ets (nucleotides -965 to -920), I examined enhancer activity in the following cell lines: TT, a slowly-dividing (doubling time of 83 hours [14]) CT-positive MTC cell line; RO-D81, a rapidly-dividing (doubling time of 24 hours [15]) CT-negative MTC cell line; and HeLa, a CT-negative, non-neuroendocrine line. Fragment -965 to -920 (containing Ets and E2) enhanced transcription by 6 fold in TT cells, 5 fold in RO-D81 and 1.5 fold in HeLa (Figure 3). Mutating E2 in that fragment, but leaving the Ets site intact abolished its enhancer activity in TT cells but had no effect in RO-D81 and HeLa. Using four copies of the Ets element activated transcription in all three cell lines but less so in HeLa cells.
to the probes I added various unlabeled competitors to the R0-D81 cells. To determine the binding specificity of the proteins enhancer elements, I performed electrophoretic mobility shift assays. To detect and characterize protein binding to these factor was cell-specific was by protein —DNA interaction trans-criptional activity, the only way to determine if its acting was inactive without E3. Since E3 had no autonomous enhancer because it was active in all three cell lines (though to different degrees). On the other hand, the 28-bp fragment containing the E2 (CAGCTG) motif was a cell-specific enhancer element. Because E3 was not an autonomous enhancer element, it was not possible to determine by transfection experiments whether it was also cell specific.

Characterization of protein binding to the cell-specific E-motif

I hypothesized that transcription induction by the cell-specific enhancer of the human CT gene requires trans-acting factors specific to CT-producing cells; these proteins may be missing or inactive in the CT-negative cells. To examine this hypothesis, it was necessary to test protein binding to the cell-specific enhancer elements. Of the three elements described above (Ets, E2, and E3), E2 was the most logical to test because it functioned in vivo failed to block formation of this particular complex.

Finally, two copies of E2 were active only in CT-positive TT cells and not in RO-D81 or HeLa.

From these results I concluded that the Ets site is a ubiquitous enhancer because it was active in all three cell lines (though to different degrees). On the other hand, the 28-bp fragment containing the E2 (CAGCTG) motif was a cell-specific enhancer element. Because E3 was not an autonomous enhancer element, it was not possible to determine by transfection experiments whether it was also cell specific.

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Figure 4. Binding of nuclear factors from CT-positive and -negative cells to E3. Crude nuclear extracts from TT, RO-D81, and HeLa cells were incubated individually with a 32P-labeled fragment (nucleotides —935 to —877) containing the E3 element (CAGGTG) or a mutated version of it (CTTGCT) as described in Materials and Methods. After incubation the samples loaded onto a 4% polyacrylamide/ bisacrylamide gel (19:1) and electrophoresed, separation of the protein-DNA complexes was performed at 4°C. The oligonucleotide competitors used are indicated below each lane. The sequence for muE2 is given in reference [9]. The sequence for E3 is given in Materials and Methods. The arrows indicate the position of the E3-specific complexes. The wild-type probe is shown at the bottom; the bar underneath indicates the sequence overlap between the E3 oligonucleotide and the probe. The autoradiogram shown is of one gel, but lanes 1—5 were exposed for 24 hours and lanes 6—13 were exposed for 72 hours.

Figure 5. Identification of an E2-binding protein in MTC cells. Crude nuclear extracts (10 mg/reaction) from TT, RO-D81, and HeLa cells were incubated with a 32P-labeled fragment containing the E2 sequence (—965 to —920), and electrophoretic mobility shift assay was performed. The type of oligonucleotide competitor and amount used (ng) are indicated above each lane. E2, double-stranded oligonucleotide from —948 to —920; E2m, E2 with the mutation of CAGCTG to CTTCGC. The arrows show the position of the E2-specific complexes. The probe is shown at the bottom; the bar underneath indicates the sequence overlap between the E2 oligonucleotide and the probe. incubation mixtures and examined the resulting complexes by polyacrylamide gel electrophoresis. Figure 4 shows the binding of proteins to the E3 element. Incubation of TT cell extract with a fragment containing nucleotides —935 to —877 resulted in the formation of two distinct complexes, indicated by the arrows. These complexes did not form when extracts were incubated with unlabeled oligonucleotide containing E3 (nucleotides —925 to —895), indicating that the proteins in these complexes bound to a region containing this motif. My coworkers and I have previously shown that TT extracts contained a protein with binding activity to another CANNTG motif of the CT enhancer (E1). Based on competition assays, it seemed that this protein could also bind to the immunoglobulin enhancer element muE2 [9]. To examine the possibility that E3 is also related to muE2, I repeated the competition with unlabeled muE2 oligonucleotide and found that it did not compete with E3. To prove that E3-binding proteins did recognize the CANNTG motif, I prepared another probe in which CAGGTG was mutated to CTTGCG; these mutations destroyed the transcriptional activity of E3 in the transfection assays. Figure 4 lane 4 shows that neither complex formed with the mutated E3; these complexes, therefore, contained a CAGGTG-binding protein or proteins.

I then examined whether E3-binding proteins were present in CT-negative cells. Figure 4 lanes 6—13 shows that both HeLa and RO-D81 cells contained proteins with the same mobility and binding specificity as the E3-binding proteins in TT cells.

My next step was to examine protein binding to a fragment containing the cell-specific E2 motif (nucleotides —965 to —920). Incubation of TT cell extract with this enhancer fragment resulted in the formation of four complexes (Figure 5). The formation of one of these complexes was blocked by an unlabeled oligonucleotide that contained the E2 motif (nucleotides —948 to —920). A similar competitor (Em) with three point mutations (CAGCTG to CTTCGC) that destroyed transcriptional activity of E2 in vivo failed to block formation of this particular complex.
These results indicated that specific binding of proteins to E2 depended on the integrity of the CAGCCTG motif.

E elements function as binding sites for transcription factors of the helix-loop-helix (HLH) protein family [16]. To examine the relationship of the MTC-cell E2 binding proteins to HLH proteins, competitors containing the immunoglobulin enhancer elements \( \mu E1, \mu E2, \) and \( kE2 \) [17] were also tested. \( \mu E2 \) and \( \mu E1 \) function as binding sites for the E2A gene products Pan 1 and Pan 2 [18,19]. \( kE2 \) functions as a generic binding site for synthetic heterodimers of E2A gene products with myoD or with Drosophila's achaete scute gene products [20]. None of these sequences inhibited the binding of E2-specific proteins to DNA (Figure 6 and data not shown). These results suggest that the E2-binding protein in TT cells had unique binding characteristics not shared by these HLH proteins.

HeLa extracts did not contain detectable E2-binding activity (Figure 5). These results support the hypothesis that the cell-specific transcription factor is either absent or inactivated in these cells. Interestingly, the CT-negative MTC cell line RO-D81 also contained a protein that bound specifically to the E2 element: its binding was abolished by competition with the E2 oligonucleotide but not by competition with the mutated E2 oligonucleotide. However, the electrophoretic mobility of this E2 complex was different from that of the E2-specific complex formed in TT cell extracts (Figure 5). Further characterization of this complex by competition assays (Figure 6) showed that \( \mu E1 \) oligonucleotide inhibited the formation of the E2-protein complex in RO-D81 extracts but not in TT cell extracts. These results suggest the E2-binding protein in CT-negative RO-D81 cells differed from the protein in CT-positive TT cells in size and DNA binding specificity.

I concluded that the E3-binding proteins were ubiquitous whereas E2-binding activity was restricted. Since E2-binding activity was not detectable in non-neuroendocrine CT-negative cells, the absence of a neuroendocrine-specific E2-binding protein may account for the lack of enhancer activity in these cells. On the other hand, the modifications in this protein may account for its inactivation in the metastatic, CT-negative MTC cell line RO-D81.

**DISCUSSION**

The principal components of the neuroendocrine-specific enhancer of the human CT gene are three CANNTG motifs and an Ets-like responsive element. In this study I found that one CANNTG motif, E2, was cell specific. Because E2 was inactive in the rapidly dividing CT-negative MTC cells and because its mutation abolished transcriptional activity of transgenes containing CT 5′ flanking DNA, I hypothesized that its inactivation plays a key role in the downregulation of CT gene transcription in metastatic tumors.

The CANNTG motif in E2 is also present in response elements for transcription factors of the HLH family. For example, the E2A gene products (ubiquitous HLH proteins cloned from B-cell and insulinoma cells) [16,21]; the MyoD, a muscle-specific protein that induces myogenesis [8,20]; Drosophila achaete scute T3, a protein involved in differentiation of the peripheral nervous system [7,20]; and c-myc, a proto-oncogene implicated in the regulation of cell growth [22], all bind to and regulate transcription via CANNTG motifs. Although this common hexamer sequence is presently the only link between the CT gene trans-acting factors and the HLH family, it is tempting to speculate that the E2-binding protein is a member of the HLH family. The possible relationship of the CT trans-acting factor to a family of transcription factors that regulate growth and differentiation increases the significance of the inverse correlation between CT gene transcription and tumor progression.

In rat MTC cells, an 18-bp fragment containing a sequence identical to E2 was sufficient to induce transcription from homologous and heterologous promoters [23]. On the other hand, in the human MTC cell line TT, a single copy of E2 required the cooperation of two additional elements: E3, which also contains a CANNTG motif and is located immediately downstream from E2, and an Ets-like response element, which is located immediately upstream from E2. The explanation for these functional differences may be that the transactivator of E2 is different in rat and human MTC cell lines. Further characterization of the rat element by introducing point mutations and protein–DNA interaction assays will clarify this point.

The importance of E3 was established previously by Ball et al. [11]. In their experiments, a mutation that selectively destroyed E3 without changing the position or distance of E2 from the promoter and transcription site completely abolished basal transcription. I confirmed these results but found that when E3 was brought closer to a minimal promoter, it had only a supportive effect on overall enhancer activity. It somewhat augmented the transcriptional activity of an enhancer fragment containing Ets and E2, but this enhancer was still active when E3 was deleted. Moreover, unlike Ets and E2, E3 could not
function as an autonomous enhancer element, even when duplicated. Since its activity was primarily position dependent, I speculate that its only role is to coordinate looping of the DNA to facilitate the interaction of the distal basal enhancer binding proteins with downstream promoter elements and the transcription complex. This hypothesis is supported by a recent report from this laboratory that E3 coordinated the synergism between the distal basal enhancer and the downstream cAMP-responsive elements located between nucleotides —252 and —129 [24].

When E2 was near the promoter, its activity completely depended on the Ets-like response element located immediately upstream. Since the Ets motif is also present in the rat gene [9], I believe that it is fundamentally important for the regulation of CT gene transcription. The members of the Ets family of proteins are oncogenes that regulate transcription, cell transformation, and development [25—27]. That an Ets-like motif was highly active in MTC cells implies that Ets-related oncogenes play an important role in neuroendocrine cell transformation. The activity of members of the Ets family is modulated by other oncogenes, including ras [28,29]. Because ras has been previously shown to upregulate transcription of the CT gene in TT cells [30], it is possible that part of the function of ras is to upregulate the transcriptional activity of the CT Ets response element.

The mechanism for inactivation of E2 in CT-negative MTC cells and the reason for its inactivity in non-neuroendocrine cells are unknown. However, this study is the first to show a difference in binding to E2 in CT-positive and CT-negative cells; E2 binding activity was not detectable in HeLa cells. More importantly, it was modified in the CT-negative MTC cells. My interpretation of these results is that the E2-binding protein was probably absent in the non-neuroendocrine cells, whereas in the metastatic MTC cells its transcriptional activity was compromised by an unknown modification that changed its size and binding specificities. Further characterization of the E2-binding proteins will be required to determine if the differences between the transcriptionally active and inactive proteins are due to interaction with other transcription factors or to a posttranscriptional modification.

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