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# RREB-1, a Novel Zinc Finger Protein, Is Involved in the Differentiation Response to Ras in Human Medullary Thyroid Carcinomas

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**An activated *ras* oncogene induces a program of differentiation in the human medullary thyroid cancer cell line TT. This differentiation process is accompanied by a marked increase in the transcription of the human calcitonin (*CT*) gene. We have localized a unique Ras-responsive transcriptional element (RRE) in the *CT* gene promoter. DNase I protection indicates two domains of protein-DNA interaction, and each domain separately can confer Ras-mediated transcriptional inducibility. This bipartite RRE was also found to be Raf responsive. By affinity screening, we have cloned a cDNA coding for a zinc finger transcription factor (RREB-1) that binds to the distal RRE. The consensus binding site for this factor is CCCCAAACCACCCC. RREB-1 is expressed ubiquitously in human tissues outside the adult brain. Overexpression of RREB-1 protein in TT cells confers the ability to mediate increased transactivation of the *CT* gene promoter-reporter construct during Ras- or Raf-induced differentiation. These data suggest that RREB-1 may play a role in Ras and Raf signal transduction in medullary thyroid cancer and other cells.**

Ras proteins are critical regulators of normal cell proliferation and differentiation. Activated forms of Ras are involved in a wide range of human malignancies (3, 7). In recent years, through a remarkable convergence of biochemical assays and genetic analyses, several cytoplasmic components of the Ras signaling pathway have been elucidated (15, 17, 30, 39, 44, 49). It is now clear that Ras proteins function as intermediates between upstream tyrosine kinases and a downstream cascade of protein kinases, including the mitogen-activated protein kinases (MAPKs) (27, 37), the stress-activated protein kinases or the Jun kinases (SAPKs/JNKs) (59), and the p38/HOG1 kinase (21, 43).

While several nuclear transcription factors have been implicated as substrates of activated MAPKs and the SAPKs, the exact nuclear targets, *trans*-acting factors, and effectors of Ras-triggered signaling still remain elusive. The best-characterized example and the first class of transcription factors shown to be activated by the Ras cascade is the AP-1 complex, a heterodimer composed of c-*fos* and c-*jun* proto-oncoproteins (47, 51, 53). The binding site is an AP-1 motif [TGA(C/A)TCA] or, in some cases, the related CREB/ATF binding site (TGACGT CA) (22, 38, 47, 58). Subsequently, AP-1-independent Ras-responsive elements (RREs) were identified. Some of these include a consensus binding site of GGGACTTTCCG for the NF-κB families of transcription factors (2) or an Ets site in the proximal RRE region (reviewed in reference 11). The Ets family of DNA binding motif proteins recognizes a DNA sequence which includes a purine-rich core, 5′-GGAA-3′ (20, 28). Ets-related factors are known to cooperate synergistically, independent of AP-1 (56). The only other *ras*-responsive factor that has been identified so far is a 120-kDa nuclear protein, RRF1, which has been found to be present in human and mouse cells (41). RRF1 has also been found to recognize the Ets motif CAGGATATGA in the transforming growth factor-β1 and NVL-3 enhancers and may have some relationship with the Ets family of transcription factors (42).

Ras is known to regulate several classes of important genes. These include genes associated with the mitogenic response (e.g., c-*fos* and c-*jun*), growth factors (e.g., transforming growth factor-β), extracellular matrix proteases (e.g., stromelysin and collagenase), and tumorigenesis and metastasis (e.g., metalloproteases, cysteine proteases, and their specific inhibitors, tissue inhibitors of metalloproteases and cystatins), and viral genes (e.g., the VL-30 element) (reviewed in references 6 and 11). Therefore, it is important to identify the downstream nuclear components, the transcription factors critical for Ras function.

While the phenotypic consequence of a constitutively activated *ras* pathway frequently results in increased growth and cell transformation, some cultured cell types respond to *ras* activation with increased cellular differentiation. These cell types include PC12 pheochromocytoma cells (36), neuroblastoma cells (32), lymphoblastoid cells (48), TT human medullary thyroid cancer (MTC) cells (34), and 3T3-L1 fibroblasts (4). It remains unclear why the *ras* signaling cascade in these systems elicits cellular differentiation, instead of transformation, responses. In either event, Ras signal transduction proceeds through activation of one or more signal transduction cascades and culminates in the alteration of transcription of specific genes. We have used our tissue culture model system for human MTC, in which an activated v-*ras*$^{H}$ oncogene (34) or activation of a c-*raf*-1 gene (10) induces neuroendocrine differentiation, to understand the differentiation program controlled by Ras or Raf. Since a prominent feature of the differ-

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entiated phenotype in these cells is increased expression of the calcitonin (*CT*) gene (34), we have studied the regulation of *CT* gene expression as a model for transcriptional regulation by Ras and Raf. Here, we describe two separate Ras- and Raf-responsive transcriptional elements (RREs) in the calcitonin gene promoter. We report the cloning of a gene for a novel zinc finger transcription factor which can specifically bind to one of the RREs of the *CT* gene and can augment the Ras and Raf transcriptional response of the *CT* gene.

## MATERIALS AND METHODS

**Cell culture.** Culture of the TT line of human MTC cells and infection with Harvey murine sarcoma virus (HaMSV) have been described previously (34). TT:ΔRaf-1:ER cells were cultured in phenol red-free medium, and the c-*raf*-1:ER gene was activated by treatment of the cells with 1 μM β-estradiol, as described previously (10).

**Plasmid construction.** For all expression studies, the plasmids discussed in detail in Results were constructed as follows. Plasmids pCT731CAT (formerly termed CT750CAT), pCT367CAT (formerly CT380CAT), pCT252CAT, and pCT132CAT, containing the indicated lengths of the *CT* gene 5′ region (the numbers in these constructs reflect the lengths) as well as 88 bp of exon 1 inserted upstream from a chloramphenicol acetyltransferase (CAT) reporter gene, have been previously described (13). pCT215CAT was derived from pCT731CAT by *Bal* 31 nuclease deletion. pCT252Δ14CAT was derived from pCT731CAT by *Bal* 31 nuclease deletion from the *Bam*HI site at position −132 upstream to −206, followed by removal of *CT* gene sequences from the *Aat*II site at position −252 to −731 by restriction with *Aat*II and *Hin*dIII. pCT132/1-2FCAT, pCT132/1-2RCAT, pCT132/7-8FCAT, and pCT132/7-8RCAT were constructed by cloning oligonucleotides 1-2 and 7-8 (described below) in the forward (F) or reverse (R) orientation, upstream of the *CT* gene sequences in pCT132CAT. pCT132/1-2B and pCT132/7-8B were constructed by cloning oligonucleotides 1-2B and 7-8B in reverse orientation in the same position of pCT132CAT. All constructs were confirmed by sequencing.

**Transient-transfection assays.** TT cells were studied by using transient CAT expression assays 10 days after infection with HaMSV. Transfections of reporter plasmids by electroporation and assay of CAT activity (19) were done exactly as described previously (13). For studies of *raf* activation of *CT* gene expression, uninduced TT:ΔRaf-1:ER cells were transfected with reporter plasmids by electroporation. Twenty-four hours later, cells were treated with 1 μM β-estradiol to activate the c-*raf*-1:ER gene or, as a negative control, with 0.1% ethanol carrier. After a further 24 h, the cells were harvested for CAT assays.

**Transient-transfection assays for RREB-1 function.** The full-length RREB-1 cDNA was subcloned downstream of the long terminal repeat promoter between the *Xba*I and *Eco*RI sites of the mammalian retroviral expression vector pMV7p1 (24). The recombinant expression plasmid is referred to as pMV7p1-371. The CAT reporter plasmids pCT132CAT and pCT252CAT have been described previously (13), and pCT132/1-2FCAT is described above. A CAT reporter plasmid (1 or 2 μg) and various amounts (2 to 4 μg) of pMV7p1-371 or pMV7p1 alone were cotransfected into TT:ΔRaf-1:ER cells by using Lipofectamine (Life Technologies) according to the manufacturer's protocol. Briefly, about $10^6$ cells per well were seeded in six-well tissue culture plates, 6 days prior to transfection, in 4 ml of phenol red-free RPMI–16% fetal calf serum–100 U of penicillin per ml–100 μg of streptomycin per ml–200 μg of G418 per ml. Twenty-four hours after Lipofectamine treatment, 4 ml of this medium was added to the wells. This medium contained either 1 μM β-estradiol or ethanol as a carrier. All transfections were carried out in duplicate. The cells were harvested 48 h after treatment with β-estradiol and assayed for CAT activity as described previously (13).

**Gel mobility shift assays.** Nuclear extracts were prepared from control TT cells or from TT cells 10 days after HaMSV infection according to a modification (35) of a previously described method (14). Double-stranded oligonucleotides 1-2 (5′-GATCCGGTCCCCCACCATCCCCCGCCATTTCCA-3′), 7-8 (5′-ATCCATTTCCATCAATGACCTCAATGCAAATAC-3′), 1-2B (5′-GATCCGGTCCCCCACCATCCCCCGCCA-3′), and 7-8B (5′-GATCTCAATGACCTCAATGCAAATAC) and an oligonucleotide containing a binding site for AP-1 (5′-GATCAGCTTGATGATGAGTCAGCCCG-3′) were synthesized on an Applied Biosystems 380A DNA synthesizer, with a GATC 5′ overhang on each strand. Double-stranded oligonucleotides containing binding sites for AP-2 (5′-GATCGAACTACCGCCCGCGGCCCGT-3′), AP-3 (5′-CTAGTGGGACTTTCCACAGATC-3′), CREB (5′-GATTGGCTGACGTCAGAGAGCT-3′), GRE (5′-GATCAGAACACAGTGTTCTCTA-3′), NF1/CTF (5′-ATTTTGGCTTGAAGCCAATATG-3′), NF-κB (5′-GATCGAGGGGACTTTCCCTAGC-3′), OCT1 (5′-GATCGAATGCAAATCACTAGCT-3′), and Sp1 (5′-GATCGATCGGGGCGGGGCGATC-3′) were obtained from Stratagene. Oligonucleotides 1-2, 7-8, and AP-1 were 3′ end labeled with Klenow DNA polymerase and [α-$^{32}$P]dCTP.

Gel mobility shift assays were done as previously described (35). Approximately 1.0 ng of double-stranded oligonucleotide was bound to 3 μg of TT or TT*ras* nuclear extract at 0°C in 20 μl of 50 mM KCl–10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid)–5 mM EDTA–10% glycerol (pH 7.9) containing 2 μg of poly(dI-dC). In those experiments using competitor oligonucleotides, the competitor was added just prior to the labeled oligonucleotide. Samples were incubated on ice for 10 min and then loaded on a 5% polyacrylamide gel and electrophoresed at 4°C for 2 h.

**DNase I protection assay.** DNase I footprinting was done according to a method previously described (25). A restriction fragment containing *CT* gene sequences downstream from the *Dde*I site at bp −272 was 3′ end labeled by filling in with Klenow DNA polymerase and [α-$^{32}$P]dCTP. Binding reaction mixtures containing $10^4$ cpm of end-labeled fragment, 2 μg of poly(dI-dC), and 10 μg of TT or TT*ras* nuclear extract in 20 μl of 25 mM Tris-HCl (pH 7.9)–6.25 mM $MgCl_2$–10% glycerol–50 mM KCl were incubated on ice for 15 min and then at 22°C for 2 min. An equal volume of freshly diluted DNase I (4 U/ml) in 10 mM $MgCl_2$–5 mM $CaCl_2$ was added and incubated for an additional 2 min at 22°C. The reaction was stopped with 40 μl of 200 mM NaCl–20 mM EDTA–1% sodium dodecyl sulfate (SDS)–250 μg of tRNA per ml and then phenol extracted and ethanol precipitated. Samples were run on an 8% polyacrylamide sequencing gel, with a Maxam-Gilbert G (dimethyl sulfate) reaction of the same restriction fragment as a marker.

**Isolation of recombinant clones encoding RREB-1.** A lambda gt11-cDNA expression library was made by random hexamer priming of poly(A)$^+$ RNA from the human MTC cell line TT. A total of 200,000 plaques were screened by DNA affinity cloning (50, 55). The catenated DNA probe of oligonucleotide $(1\text{-}2)_n$ that contained the *CT* gene RRE (5′-GATCCGGTCCCCCACCATCCCCCGCCATTTCCA-3′) was labeled by nick translation with [α-$^{32}$P]dCTP, and $10^6$ cpm/ml was used for the binding reaction. During secondary and tertiary screening of the expression library, 100 mg of sonicated calf thymus DNA (sheared to ca. 1 kb, heat denatured 10 min at 99°C, and quenched on ice) was used as a nonspecific competitor in a 50-ml binding reaction mixture with the labeled catenated DNA probe. Filter lifts, binding reactions, and washes were carried out on the same day. A positive clone termed lambda gt11-24.1 was picked for further characterization. This 2.16-kb partial cDNA was subcloned after PCR amplification of the lambda gt11-24.1 recombinant by using lambda gt11 primers (GT1 and GT2; New England Biolabs).

To obtain the full-length cDNA, we screened the gt11 TT cDNA library with the 2.16-kb cDNA. We subsequently screened this library and an SW480 colon cancer cell library (Clontech) with probes for the most 5′ cDNA clone. Filters were hybridized at 60°C overnight in BLOTTO (5× SSPE [1× SSPE is 0.18 M NaCl, 10 mM $NaH_2PO_4$, and 1 mM EDTA {pH 7.7}], 10% formamide, 1.0% SDS, 6.0% polyethylene glycol 6000, 200 μg of salmon sperm DNA per ml, 5.0 mg of powdered milk per ml), washed sequentially in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.5% SDS at room temperature for 10 min and in 0.1× SSC–0.5% SDS at 65°C for 20 min, and exposed to film at −70°C. Following *Eco*RI digestion of the recombinant phage DNA, the cDNAs were subcloned directly into pBluescript. Nucleotide sequences were determined by double-stranded DNA sequencing by the chain termination method (Sequenase 2.0; United States Biochemicals). All cDNA clones were confirmed to be nonchimeric by reverse transcription-PCR on RNA from TT cells or Caco2 cells, using an anchored primer within confirmed sequences and a paired primer from the most 5′ clone. Comparisons were made with existing nucleotide and amino acid sequences from GenBank/EMBL by Blast searching.

**DNA-protein binding assays. (i) DNA binding specificity.** The lambda gt11-24.1 recombinant clone (containing the three C-terminal zinc fingers of RREB-1) was plated at a density of about 250 plaques per 15-mm-diameter petri dish with top agarose containing 10 mM IPTG (isopropyl-β-D-thiogalactopyranoside). After plaque formation, a nitrocellulose filter (BAS; Schleicher & Schuell) was overlaid on this dish for 4 h at 37°C. The filter was cut into three equal parts and was subjected to the DNA binding assay (50, 55) in three separate petri plates. One of the triplicates was incubated with the oligonucleotide $(1\text{-}2)_n$ catenated probe (actual binding site probe), the second was incubated with the oligonucleotide $(7\text{-}8)_n$ probe (a downstream region of the *CT* promoter), and the third was incubated with a catenated oligonucleotide $(5\text{-}6)_n$ probe (a randomer, similar in size and base composition to oligonucleotide 1-2). The binding reactions were carried out in the presence of the calf thymus DNA, and the filters were processed and washed as described above under library screening and exposed to film.

**(ii) Southwestern (DNA-protein) blot analysis.** Bacterially produced glutathione *S*-transferase (GST)–RREB-1 fusion protein, TT nuclear extract, and GST protein alone were electrophoresed on an SDS-polyacrylamide gel and transferred to a nitrocellulose filter. This filter was probed for sequence-specific DNA binding activity (33). A catenated oligonucleotide $(1\text{-}2)_n$ probe was prepared as described above under library screening. Sonicated salmon sperm DNA was used as a nonspecific competitor. The filters were blocked with 5% (wt/vol) nonfat dry milk in binding buffer (10 mM HEPES [pH 7.9], 50 mM KCl, 1 mM EDTA, 1 mM dithiothreitol) for 1 h at 4°C, transferred to binding buffer supplemented with 0.25% dry milk and $10^5$ cpm of α-$^{32}$P-labeled DNA probe per ml, and incubated for another hour at 4°C. Filters were washed in three 100-ml changes of binding buffer–0.25% dry milk at 4°C, blotted on 3 MM Whatman paper, and exposed to film at −70°C.

**(iii) Gel mobility shift assays.** Mutant oligonucleotides with various base substitutions in the wild-type oligonucleotide 1-2 (see Fig. 5a) were purchased from Life Technologies. Oligonucleotides were made double stranded with the appropriate reverse primer (R1 [5′-TGGAAATGG-3′] or R2 [5′-TGGAAAGT

CA-3′]) and Klenow DNA polymerase and labeled with [α-$^{32}$P]dCTP. Gel mobility shift assays were done as previously described (35) with 3 μg of TT nuclear extract or 500 ng of purified GST–RREB-1.

**Northern (RNA) blot analysis.** Total RNA was extracted from the TT and TT*ras* cells by the acid phenol-guanidium isothiocyanate method (12). Cell culture and virus infection were as previously described (34). Ten μg of poly(A)$^+$ RNA from TT or TT*ras* cells was electrophoresed on a 1.5% formaldehyde gel, transferred to Zetaprobe filters, and hybridized by using a nick-translated 2.16-kb fragment of the RREB-1 cDNA. The blot was rehybridized to a human β-actin cDNA probe as a control for loading. Hybridization signals were quantitated with a PhosphorImager (Molecular Dynamics) and analyzed with ImageQuant software. A Northern blot containing 2 μg of poly(A)$^+$ RNA from various adult human tissues was purchased from Clontech and hybridized to a 1.4-kb cDNA of RREB-1 labeled with [α-$^{32}$P]dCTP by nick translation.

**Binding site selection.** Affinity-purified GST–RREB-1 fusion protein was used to isolate binding sites from a degenerate oligonucleotide, according to a modified CASTing (cyclic amplification and selection of target sequences) method (40, 57). The degenerate oligonucleotide consisted of a 60-mer beginning with the sequence 5′-GAGATATTAGAATTCTACTC followed by a stretch of 23 random bases and ending with the sequence 5′-GGTACATATACTCGAGT. Underlined are the *Eco*RI and *Xho*I sites designed in the 60-mer to facilitate subcloning of the selected fragments.

The oligonucleotide was made double stranded by hybridization with primer LL-*Xho*I-A (5′-TGCACTCGAGTATATGTACC-3′) and elongation for 30 min at 30°C with Klenow DNA polymerase. This double-stranded oligonucleotide (0.25 μg) was bound to 5 μg of the dialyzed fusion protein in 100 μl of buffer [20 mM HEPES, 40 mM KCl, 2 μg of poly(dI-dC), 2 mM $MgCl_2$, 0.1 mM $ZnSO_4$, 1 mM dithiothreitol, 5% glycerol, 0.2 mg of bovine serum albumin (BSA) per ml]. The protein-DNA complexes were bound to a 50% slurry of glutathione-Sepharose, equilibrated in low-salt buffer (50 mM Tris [pH 8.0], 10 mM EDTA, 0.2 mg of BSA per ml, 0.1% Nonidet P-40), and washed twice with low-salt buffer and twice with low-salt buffer lacking Nonidet P-40. The specific DNA was eluted by four successive rounds of heating at 100°C for 2 min in high-salt buffer (1 M NaCl, 50 mM Tris [pH 8.0], 10 mM EDTA) and ethanol precipitated. The pellet was resuspended in LTE (10 mM Tris [pH 7.5], 1 mM EDTA), and PCR was performed with primers LL-*Eco*RI-A and LL-*Xho*I-A (29) in a 50-μl reaction volume containing 200 μM each deoxynucleoside triphosphate and 100 μCi of [α-$^{32}$P]dCTP. The PCR was hot started at 94°C for 5 min, *Taq* DNA polymerase (Boehringer Mannheim) was added, and cycles were at 95°C for 30 s and 60°C for 30 s for 30 cycles with a final extension at 72°C for 5 min. The PCR product was purified on a TE-30 column (Clontech). The amplified oligonucleotides were subjected to four additional cycles of binding, selection, amplification, and purification. GST protein was used as a negative control for the same kind of selection and amplification. The selected fragments were digested with *Eco*RI and *Xho*I, gel purified, and cloned into pBluescript (Stratagene). Individual clones were isolated and sequenced. To confirm the specificity of selected binding site clones, a gel mobility shift assay was performed exactly as above.

**Nucleotide sequence accession number.** The GenBank accession number for RREB-1 is U26914.

## RESULTS

**Localizing an RRE in the *CT* gene promoter.** The stimulation of *CT* gene transcription in TT cells by v-*ras*$^H$ in our earlier studies (34) prompted us to search for an RRE. The human *CT* gene 5′ sequences, from position −731 to +88, were placed upstream of the CAT reporter gene. This construct and constructs with deletions within the upstream calcitonin gene sequences were tested for expression and *ras* inducibility by electroporation into uninfected TT cells and into TT cells infected with HaMSV (TT*ras* cells). A summary of the results obtained from these experiments is outlined in Fig. 1a and b. Introduction of v-*ras*$^H$ stimulated expression from constructs containing at least 215 bp of calcitonin gene 5′ sequences but not from the construct containing 132 bp. Deletion of sequences from position −206 to −132 from a *ras*-inducible construct (pCT252CAT) which contained 252 bp of the 5′ sequences of the *CT* gene rendered the resultant construct (pCT252Δ14CAT) refractory to *ras* induction (Fig. 1a). Therefore, an RRE in the calcitonin gene appears to reside within the sequences from position −215 to −132.

To further delineate and characterize the sequences responsible for RRE function in the human *CT* gene, we first looked for known transcription factor binding motifs within the region from position −215 to −132 (Fig. 1c). Within this sequence, a sequence related to a TPA-responsive element (TRE) from position −168 to −161 and an octamer sequence from position −161 to −154 have been noted (8). In addition, we noted a direct decamer repeat, TCCCCCPuCCA, from position −199 to −180 and an imperfect 27-base palindrome, which includes two putative octamers similar to homeodomain binding sequences, from position −180 to −154. A sequence comparison of the 5′ region (position −300 to −60) of the human *CT* gene with that of the rat gene revealed a high level of conservation, suggesting important functional regulatory roles.

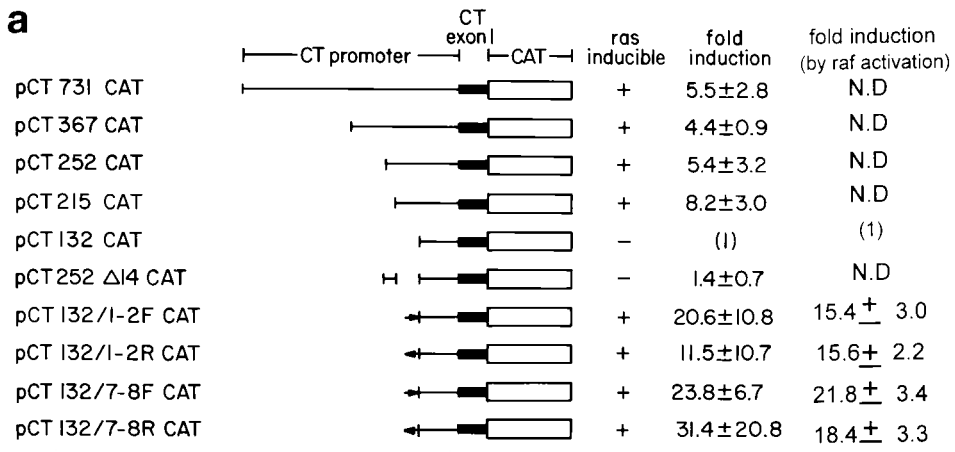
**Defining the RRE.** Within the upstream human *CT* gene candidate RRE region, DNase I footprinting with nuclear extracts from TT or TT*ras* cells showed two areas of partial protection (Fig. 2a). One of these areas, from position −199 to −180, included the decamer repeat, and the other, from about position −175 to −110, included the 27-base palindrome, the putative TRE, and the octamer sequences. No differences in protection between the patterns resulting from the use of TT or TT*ras* nuclear extract were apparent.

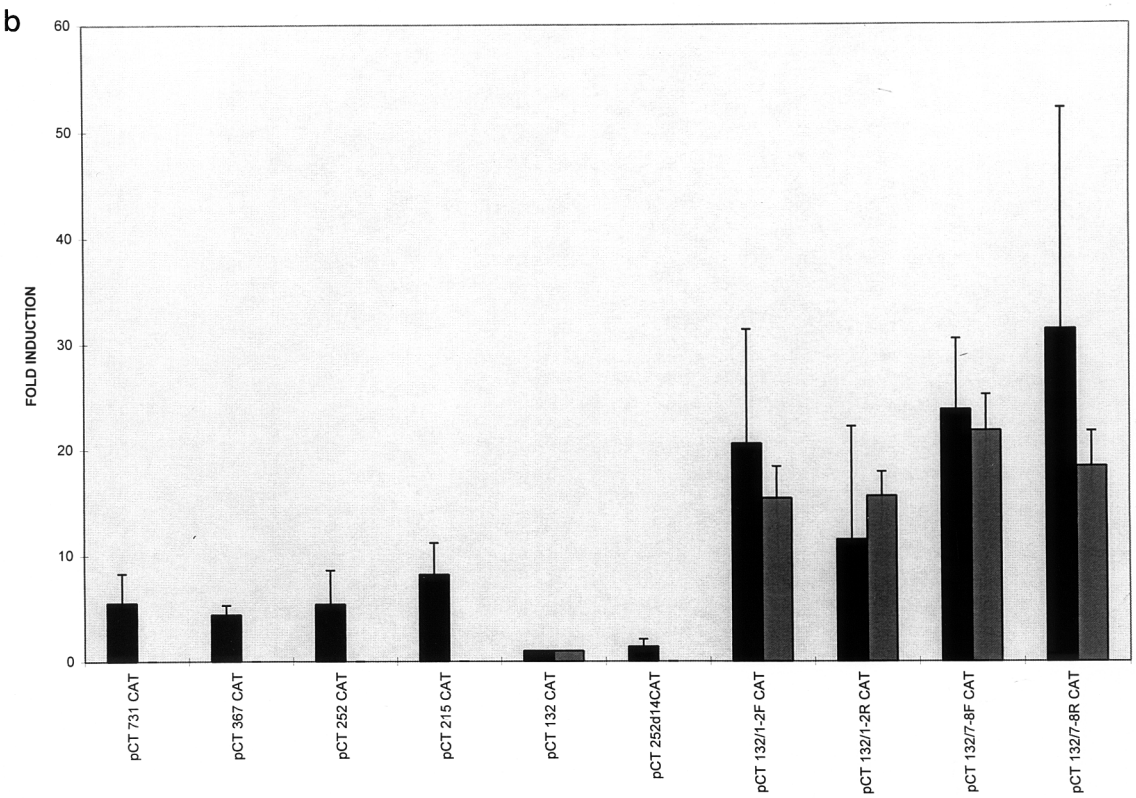
These protected sequences were examined further by gel mobility shift assays. Double-stranded oligonucleotides corresponding to each of the above-described partially protected areas were synthesized. Oligonucleotide 1-2, containing the C-rich decamer repeat, extended from base −201 to base −174, and oligonucleotide 7-8, containing the 27-base palindrome, extended from base −182 to base −152 (Fig. 1c). The interaction of these oligonucleotides with either a TT or TT*ras* cell nuclear extract resulted in the formation of specific DNA-protein complexes for each of the sequences analyzed (Fig. 2b). The DNase I protection assay as well as the gel mobility shift assay showed no apparent differences between TT and TT*ras* nuclear extracts, indicating the potential involvement of a similar factor(s) in the assembly of these nuclear protein complexes.

**Two distinct complexes bind the RRE.** At least some of the proteins responsible for the complexes formed with oligonucleotides 1-2 and 7-8 are unique. The complexes formed with oligonucleotide 1-2 are not efficiently inhibited by competition with excess oligonucleotide 7-8 (Fig. 2b, panel A, lane 5). Moreover, the complexes formed with oligonucleotide 1-2 are very sensitive to the sulfhydryl blocker methylmethane thiosulfonate, while those formed with oligonucleotide 7-8 are relatively insensitive to this agent (data not shown), further suggesting that these complexes are not identical. However, complexes formed by oligonucleotide 7-8 are inhibited by oligonucleotide 1-2 (Fig. 2b, panel B, lane 4), suggesting that some of the proteins involved in binding of these two oligonucleotides may be shared.

We have previously shown that TT*ras* cells exhibit induced expression of c-*jun* and contain proteins which will bind to a AP-1/Jun consensus sequence (35). Since oligonucleotide 7-8 contains sequences related to the AP-1 consensus binding site, we examined the possibility that AP-1/Jun might be involved in binding to oligonucleotide 7-8. An oligonucleotide containing a consensus sequence for AP-1/Jun failed to effectively compete for binding to oligonucleotide 1-2 or 7-8 (Fig. 2b, lanes 6). These data indicate that the protein complexes binding to oligonucleotides 1-2 and 7-8 are distinct from the AP-1 family.

The protein complexes formed with oligonucleotides 1-2 and 7-8 also do not appear to be related to transcription factor AP-2, AP-3, NF1/CTF, or Sp1. To demonstrate this, we attempted to inhibit the binding of oligonucleotide 1-2 or 7-8 by competition with excess oligonucleotides containing a consensus sequence for these transcription factors. In addition, protein complex formation with oligonucleotide 1-2 was also tested for competition with excess oligonucleotides containing consensus sequence for transcription factors CREB, GRE,




c

-367 GATCAGA GTTGGAAGAG TCCCTACAAT CCTGGACCCT TTCCGCCAAA TCGTGAAACC

-310 AGGGGTGGAG TGGGGCGAGG GTTCAAAACC AGGCCGGACT GAGAGGTGAA ATTCGCCATG

-250 ACGTCAAACT GCCCTCAAAT TCCCGCTCAC TTTAA**GGGCG TTACTTGTTG GTCCCCCACC**

-190 **ATCCCCCGCC ATTTCCATCA ATGACCTCAA TGCAAATACA AGTGGGACGG TCCTGCTGG**

FIG. 1. Transient transfections of *CT* gene promoter-reporter constructs. (a) The CT-CAT plasmids contain various 5′-flanking regions of the *CT* promoter and the first 88 bp of the coding region of the first exon inserted in front of the CAT gene (open boxes) as shown. In the plasmid constructs listed, F refers to oligonucleotide 1-2 or 7-8 cloned in the forward orientation and R refers to these oligonucleotides cloned in the reverse orientation. *CT* gene-CAT fusion constructs were introduced into TT*ras* cells by electroporation. Results for fold induction by v-*ras*$^{H}$ activation for each construct represent the averages and standard errors from at least four experimental points. Values are normalized as fold CAT expression compared with introduction of pCT132CAT in TT*ras* cells in the same experiment. Fold induction by *raf* activation (in TT:ΔRaf:ER cells after treatment with 1 μM β-estradiol) also represents the averages and standard errors from three independent experiments done in duplicate. N.D, not determined. (b) Values for fold induction by v-*ras*$^{H}$ activation (solid bars) and *raf* activation (shaded bars) (from panel a) presented as a bar graph. (c) Sequence of the human *CT* gene from position −367 to −132. The RRE is from position −215 to −132 (boldface), the oligonucleotide 1-2 region (−201 to −174) is underlined, and the oligonucleotide 7-8 region (−182 to −152) is overlined.

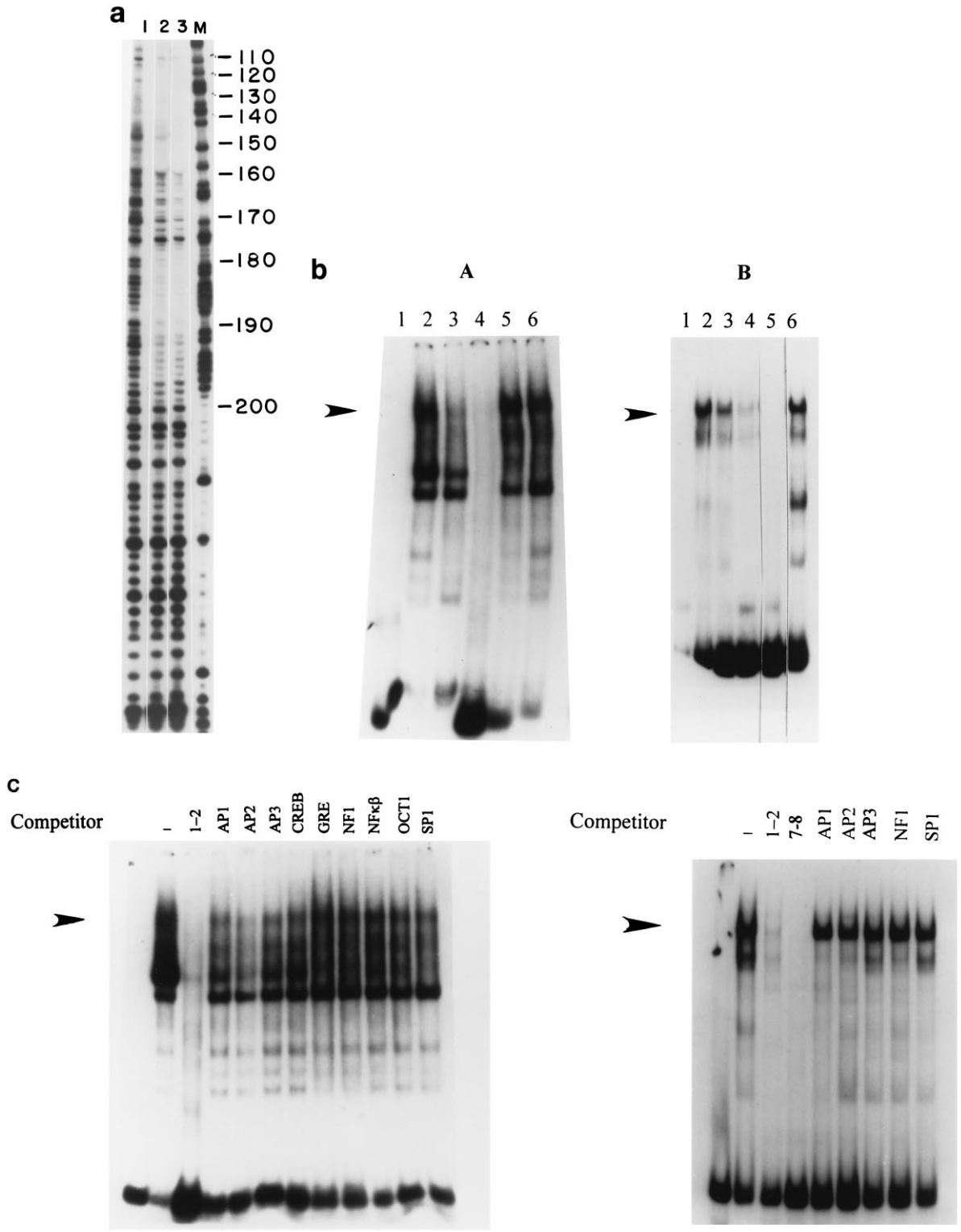



FIG. 2. (a) Comparison of DNase I footprinting patterns in the *CT* gene promoter with nuclear extracts from TT cells and TT*ras* cells. A *Dde*I DNA fragment of the *CT* gene promoter was end labeled at base −272, purified, and subjected to DNase I footprint analysis. Lane 1, no protein; lane 2, 10 μg of TT nuclear extract; lane 3, 10 μg of TT*ras* nuclear extract; lane M, Maxam-Gilbert G reaction markers. Numbers on the right are positions (in bases). (b) Gel mobility shift with TT and TT*ras* nuclear extracts and oligonucleotide 1-2 (A) or oligonucleotide 7-8 (B). Distinct protein-DNA complexes are seen with the two different oligonucleotides, and these are identical for TT or TT*ras* nuclear extract (lanes 2 and 3). The major specific bands are indicated by arrowheads. (A) Lane 1, free oligonucleotide; lane 2, 3 μg of TT extract; lane 3, 3 μg of TT*ras* extract; lanes 4, 5, and 6, 3 μg of TT nuclear extract subjected to competition with a 200-fold excess (100 ng) of unlabeled oligonucleotide 1-2, 7-8, or AP-1, respectively. (B) Same as panel A except with 10 μg of protein per lane. (c) Competition of the protein-DNA complex with cognate oligonucleotides and with oligonucleotides for selected known transcription factors. Protein binding to oligonucleotides 1-2 (left) and 7-8 (right) from a TT nuclear extract is shown.

NF-κB, and OCT1. None of these consensus sequence oligonucleotides were able to inhibit the binding to either oligonucleotide 1-2 or 7-8 (Fig. 2c).

**The *CT* gene RRE is bipartite.** We examined whether the domains of the *CT* gene represented by oligonucleotides 1-2 and 7-8 can function separately in *ras*-mediated enhancement of *CT* gene transcription. Oligonucleotides 1-2 and 7-8 were separately cloned into the *ras*-noninducible construct pCT132CAT, which contains 132 bp of *CT* gene 5′ sequences but not the RRE. Each of these oligonucleotides, in either orientation (forward [F] or reverse [R]), rendered the pCT132CAT construct *ras* inducible (Fig. 1a and b). These results suggested that the RRE is bipartite and that either domain can independently confer Ras responsiveness. However, since oligonucleotides 1-2 and 7-8 contain nine overlapping bases, it was still possible that the shared nucleotides constituted a single RRE. To test this possibility, we cloned the truncated oligonucleotides 1-2B and 7-8B, from which these nine overlapping bases were removed, into pCT132CAT. These constructs were also Ras responsive (data not shown), indicating that the two domains in the *CT* gene RRE can function independently. One possible reason for the large variation in these CAT assays and hence the large error bars could be variable expression of v-*ras*$^H$, since a new HaMSV infection was necessary for each experiment. Therefore, we examined an inducible system for TT cell differentiation, employing an activatable c-*raf*-1 gene, as described below.

We have recently shown that the differentiation program induced by v-*ras*$^H$ in TT cells can also be induced by activation of c-*raf*-1 (10), which acts downstream of *ras* in many cell systems. A retroviral construct encoding an activated c-Raf-1 protein fused to the hormone binding domain of the estrogen receptor (ΔRaf-1:ER) (46) was introduced into the TT cell line. In these cells (TT:ΔRaf-1:ER), c-*raf*-1 can be activated by 1 μM estradiol. Upon addition of estradiol, the cells cease proliferation and manifest the same changes in morphology and changes in *CT* gene expression as seen in the v-*ras*$^H$ insertion studies. In the current study, we have transfected these *raf*-inducible TT cells with pCT132CAT, as well as pCT132CAT constructs additionally containing oligonucleotides 1-2 and 7-8. The activation of c-*raf*-1 enhanced the activity of the CAT constructs with oligonucleotide 1-2 or 7-8, indicating that these RREs are also Raf responsive (Fig. 1a and b).

**Cloning of RREB-1, a zinc finger protein.** DNA affinity cloning was utilized to isolate a cDNA clone for a protein, RREB-1, with DNA binding specificity for the RRE represented by oligonucleotide 1-2. By screening 200,000 primary plaques of a TT cell cDNA library in lambda gt11, we initially isolated one cDNA clone which encoded a DNA binding domain specific for oligonucleotide 1-2. Overlapping clones were isolated, by hybridization, from this library and a cDNA library made from a colon cancer cell line SW480. The complete coding sequence predicts a protein of 755 amino acids (89 kDa), with four tandem, $C_2H_2$-type zinc fingers (Fig. 3). RREB-1 does not exhibit any striking homologies, except for the zinc finger domains, to other genes or proteins, as determined by Blast and Prosite searches. Several putative phosphorylation sites were identified by comparing the amino acid residues with known phosphorylation sites. This included one (PESP) for MAPK (1) near the predicted C terminus of RREB-1.

**DNA binding characteristics of RREB-1.** A DNA binding assay was performed in order to confirm and further define the sequence specificity of RREB-1, and a Southwestern blot analysis and a gel shift assay were performed to confirm binding of RREB-1 to its cognate oligonucleotide, 1-2. For the DNA binding assay, the plaque lift assay originally used to isolate

MEPIDLSIPKNFRKGDKDLATPSERKKPEEEAGSSEQPSPCPAPGPSLPV
TLGPSGILESPMAPAPAATPEPPAQPLQGPVQLAVPIYSSALVSSPPLVG
SSALLSGTALLRPLRPKPPLLLPKPPVTEELPPLASIAQIISSVSSAPTL
LKTKVADPGPASTGSNTTASDSLGGSVPKAATTATPAATTSPKESSEPPA
PASSPEAASPTEQGPARTSKKRGRKRGMRSRPRANSGGVDLDSSGEFASI
EKMLATTDTNKFSPFLQTAEDNTQDEVAGAPADHHGPSDEEQGSPPEDKL
LRAKRNSYTNCLQKITCPHCPRVFPWASSLQRHMLTHTDSQSDAETAAAA
GEVLDLTSRDREQPSEGATELRQVAGDAPVEQATAETASPVHREEHGRGE
SHEPEEEHGTEESTGDADGGRGRVEQPEPGPGLRHQAHGLQAGGGRRRGR
PGGAASQEQKLACDTCGKSFKFLGTLSRHRKAHGRQEPKDEKGDGATTAE
EGPSPAPEQEEKPPETPAEVVESAPGAGEAPAEKLAEETEGPSDGESAAE
KRSSEKSDDDKKPKTDSPKSVASKADKRKKVCSVCNKRFWSLQDLTRHMR
SHTGERPYKCQTCERTFTLKHSLVRHQRIHQKARHAKHHGKDSDKEERGE
EDSENESTHSGNNAVSENEAELAPNASNHMAVTRSRKEGLASATKDCSHR
EEKVTAGWPSEPGQGDLNPESPAALGQDLLEPRSKRPAHPILATADGASQ
HVGME

FIG. 3. The predicted primary structure of RREB-1 contains four zinc fingers. The putative translation initiation codon of RREB-1, indicated by the methionine at position 1, is located at nucleotide 253 in the cDNA. The zinc fingers are indicated in boldface, and the putative MAPK site (PESP) is in boldface and underlined.

RREB-1 was repeated on a confluently lysed plate of phage lambda gt11-24.1 (producing the β-galactosidase–RREB-1 fusion protein containing the zinc finger domains II, III, and IV [numbering of the finger domains is from N to C terminal]). This assay showed that the β-galactosidase–24.1 fusion protein bound strongly to oligonucleotide 1-2 but not to oligonucleotide 7-8 or to a randomer, oligonucleotide 5-6 (Fig. 4a). The GST–RREB-1 fusion protein (GST-24.1) showed binding to oligonucleotide 1-2 in Southwestern blotting and gel mobility shift assays (Fig. 4b and c). Taken together, these data are consistent with a role for this protein as a sequence-specific DNA-binding protein.

To examine whether RREB-1 corresponds to any of the DNA binding activities detected in the cell extracts, and also to define the nucleotides within oligonucleotide 1-2 that are critical for RREB-1 protein association, the relative affinities of the cellular protein and the recombinant protein for a set of oligonucleotides related to oligonucleotide 1-2 but mutated in specific bases (Fig. 5a) were tested. As shown in Fig. 5b, the protein-DNA complexes formed with the mutant oligonucleotides had migration patterns different from that of the complexes formed with oligonucleotide 1-2. The recombinant protein, GST–RREB-1, showed no binding to the mutant oligonucleotides M1, M2, M3, M4, and M5 (Fig. 5b). These data indicate that the pattern of DNA binding specificity shown by RREB-1 is consistent with that seen for binding of oligonucleotide 1-2 in TT cell nuclear extracts.

**Expression of RREB-1 mRNA in cells and tissues.** The expression of RREB-1 in TT cells and TT*ras* cells and in a variety of normal human tissues was evaluated by Northern blot analysis (Fig. 6). A 9.0-kb mRNA transcript was observed in both cell lines and in all tissues, with the exception of adult brain. The levels of RREB-1 mRNA in these tissues appeared to be similar. During *ras*-induced differentiation, steady-state expression of RREB-1 mRNA appeared to be increased twofold, raising the possibility that RREB-1 expression may be regulated in part at the mRNA level by Ras.

**A consensus binding site for RREB-1.** The consensus binding sequence for RREB-1 was determined by CASTing (57). DNA sequences which could bind tightly to RREB-1 were selected by five rounds of binding of random DNA sequences to GST–RREB-1 followed by PCR amplification (see Materials and Methods). The final pool of selected DNA sequences was cloned in pBluescript and sequenced. The RREB-1 bind-

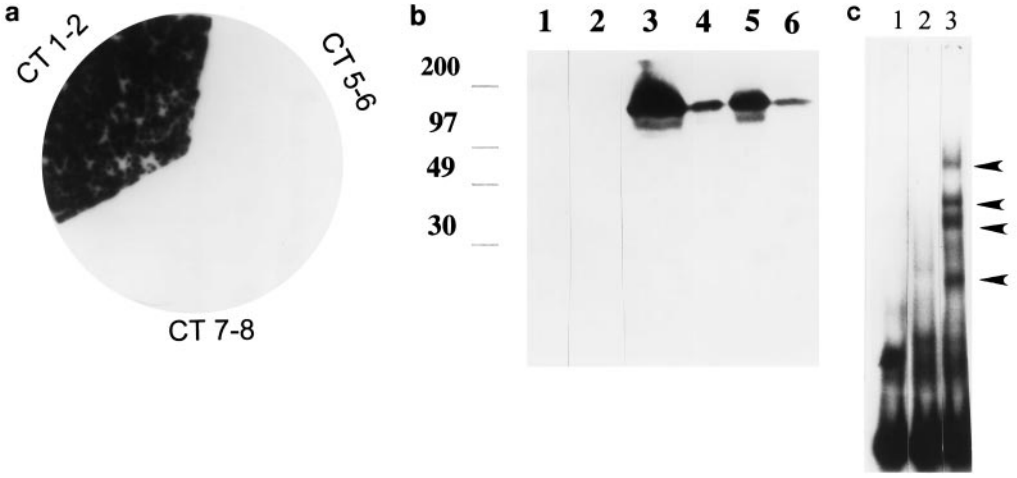



FIG. 4. DNA binding assays of the original binding site clone, identified by screening a lambda gt11-TT cell cDNA expression library. (a) To analyze the sequence-specific binding of the original cDNA clone, lambda gt11-24.1, a binding assay similar to the plaque lift assay was used (see Materials and Methods). A nitrocellulose replica filter was prepared from plating the β-galactosidase fusion protein specified by the bacteriophage lambda gt11-24.1 recombinant. This filter was cut into three equal parts and probed with oligonucleotide 1-2, 7-8, or 5-6 (randomer sequence) in the presence or absence of calf thymus DNA as a nonspecific competitor. All probes were labeled to the same specific activity, and each was used at a concentration of $10^6$ cpm/ml. Shown are the blots probed in the presence of the calf thymus DNA. (b) Southwestern blot analysis of the protein specified by bacteriophage lambda gt11-24.1 cloned into the pGEX3 plasmid in order to produce a GST fusion protein, GST-24.1, upon induction with IPTG. Bacterial cell (Topp 2 cells; Stratagene) extracts, purified GST fusion protein, and TT nuclear extract were electrophoresed on an SDS–10% polyacrylamide gel and transferred to a nitrocellulose membrane as described in Materials and Methods. Lane 1, Topp 2 cells induced with IPTG; lane 2, Topp 2 cells transformed with pGEX3 vector producing GST and induced with IPTG; lane 3, Topp 2 cells transformed with pGEX3-24.1 induced with IPTG, producing the GST-24.1 fusion protein; lane 4, purified GST-24.1 (6 μg); lane 5, purified GST-24.1 (50 μg); lane 6, TT nuclear extract (6 μg). The filter was then subjected to a denaturation and renaturation process as described in Materials and Methods and bound to the catenated oligonucleotide 1-2 probe. No binding was evident in the bacterial extract with GST alone (lane 2), but strong binding was seen with bacterial cell extracts containing the GST-24.1 fusion protein (lane 3) and the purified GST fusion protein (lanes 4 and 5) and to the native protein present in TT nuclear extract (lane 6). Coomassie blue staining patterns of the GST-24.1 and the purified fusion protein (not shown) corresponded to the bands seen on the Southwestern blot. Numbers on the left indicate molecular weights (in thousands). (c) Gel mobility shift assay. Bacterial cell extracts from Topp 2 cells transformed with pGEX3 plasmid alone or with pGEX3-24.1 and induced with IPTG (as described for panel b) were used in a mobility shift assay with labeled *CT* 1-2 oligonucleotide. Lane 1, free oligonucleotide; lane 2, bacterial cell extracts (1 μl) from bacteria transfected with the pGEX3 plasmid alone and induced with IPTG; lane 3, bacterial cell extracts (1 μl) from bacteria transfected with the pGEX3-24.1 plasmid and induced with IPTG. Specific protein DNA complexes (arrowheads) are seen in bacterial extracts expressing the GST-24.1 fusion protein but not in bacterial extracts expressing GST alone.

ing sites of 11 independent cloned DNA fragments, selected by CASTing, are shown in Fig. 7a. Significant homologies among these fragments were apparent by inspection. The core consensus binding site was found to be $C^{73}/A^{27}$, $C^{100}$, $C^{91}/A^{9}$, $C^{100}$, $A^{100}$, $A^{82}/C^{18}$, $A^{73}/C^{27}$, $C^{73}/A^{18}/T^{9}$, $C^{64}/A^{36}$, $A^{64}/C^{36}$, $C^{73}/A^{27}$, $C^{82}/A^{18}$, $C^{73}/A^{27}$, $C^{64}/A^{18}/G^{9}/T^{9}$. This sequence is closely related to the sequence CCCCACCATCCCC, found within the *CT* gene RRE. A bias toward C and A is also seen in the bases flanking this 14-base core consensus site (Fig. 7a), suggesting that the consensus may be a 9- to 11-base repeat. This would be similar to the 10-bp repeat seen in the *CT* gene RRE. Such a repeating pattern might suggest that two molecules of RREB-1 are necessary for strong binding to this sequence, and it would be consistent with the results of the gel shift experiments described above (Fig. 5), in which mutation of bases in either repeat within oligonucleotide 1-2 interfered with binding.

The individual binding sequences of selected clones were confirmed by gel mobility shift assay to form specific DNA-protein complexes with the TT nuclear extracts (Fig. 7b). Included in this assay as a negative control was a fragment selected by CASTing with the GST protein alone (GGTACA TATTTCGAGTCGATGA), and the *CT* gene RRE oligonucleotides 1-2 (a 33-mer) and dsAT1 (a 60-mer containing the sequence of oligonucleotide 1-2, described in Materials and Methods) were included as positive controls. Oligonucleotide 1-2 produced three major protein-DNA complexes with the TT nuclear extract. A protein-DNA complex with a mobility similar to that of the most slowly migrating protein-DNA complex was present in all of the CASTing-selected binding site clones and the dsAT1 positive control fragment, but this specific protein-DNA complex was not seen with the fragment selected with GST protein alone. Thus, the RREB-1 consensus binding site is similar to the motif found in the *CT* gene RRE, and similar binding activity is present in TT cell nuclear extracts.

**Transcriptional activation of the *CT* gene by RREB-1.** The ability of RREB-1 to directly activate gene expression through its DNA binding site in the *CT* gene was tested with the TT:ΔRaf-1:ER cells. These TT cells, which stably express ΔRaf-1:ER, are morphologically identical to parent TT cells. However, when exposed to estradiol, activation of ΔRaf-1:ER rapidly induces a differentiation response identical to that seen following infection of TT cells with v-*ras*$^{H}$, including activation of *CT* gene expression (10). Since TT:ΔRaf-1:ER cells provide an inducible system in which differentiation can be easily manipulated, we chose to examine RREB-1 function in these cells. Reporter plasmid pCT132CAT (lacking the RRE), pCT252CAT (containing both of the RREs), or pCT132/1-2FCAT (containing only the distal RRE) and a Moloney murine leukemia virus long terminal repeat-driven eukaryotic expression vector encoding RREB-1 were cotransfected into TT:ΔRaf-1:ER cells. These cells then either were left untreated or were treated with estradiol to activate the Raf:ER protein kinase. Cotransfection of pCT252CAT, containing both RREs, with the RREB-1 expression plasmid resulted in a threefold increase in the reporter gene expression upon β-estradiol treatment compared with the cotransfection of the pCT252CAT reporter gene with the expression vector alone (Fig. 8). Cotransfection of pCT132/1-2FCAT, containing the distal RRE, with the RREB-1 expression plasmid increased inducible CAT activity by fivefold in the reporter gene expression upon β-estradiol treatment

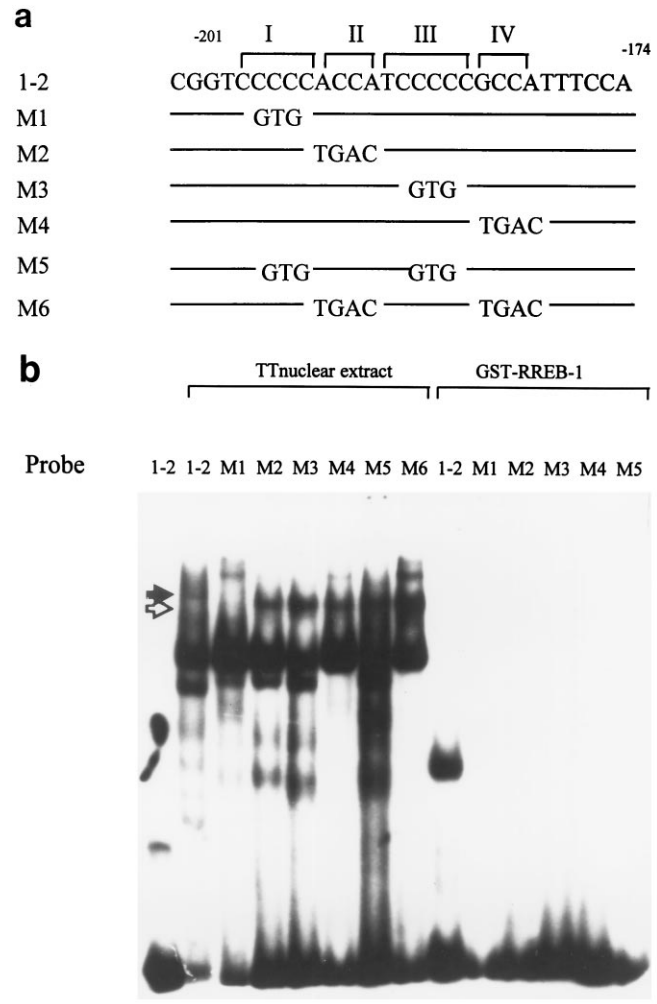



FIG. 5. Mutations within the sequence from position −174 to −201 affect DNA-protein interactions. (a) Specific mutations (M1 to M6) were generated within the context of the wild-type oligonucleotide from bp −174 to −201. (b) Gel shift assay with mutant oligonucleotides as probes. TT nuclear extract (3 μg) or purified GST–RREB-1 (500 ng) was used in each binding reaction with 1 ng of labeled oligonucleotide.

compared with cotransfection of the pCT132/1-2FCAT reporter gene with the expression vector alone (Fig. 8). No difference in gene expression was observed for untreated TT:ΔRaf-1:ER cells cotransfected with pCT252CAT and RREB-1 or with pCT132/1-2FCAT and RREB-1. Expression of pCT132CAT, lacking the RRE, was not augmented by cotransfection of RREB-1 into TTΔRaf-1:ER cells, even upon estradiol treatment. These cotransfection experiments showed that RREB-1 activated the expression of the CAT reporter gene adjacent to its cognate binding site (RRE), and this activation was Raf dependent. In addition, these results indicate that RREB-1 cannot activate gene expression from other sequences within the 132-bp basal *CT* promoter.

## DISCUSSION

The ability of a mutated *ras* gene to differentiate TT cells in culture affords an excellent in vitro model to dissect the transcriptional components of the Ras intracellular signaling cascade and also to identify the gene(s) which may be involved in the normal differentiation of the thyroid C-cells, the progenitor cells of MTC. Our data show that in vitro protein-DNA binding to *CT* gene RRE sequences is unchanged by an activated v-*ras*$^{H}$ gene. Several known transcription factors bind DNA equally well under basal or induced conditions. Induction of transcription via these factors depends on phosphorylation. These transcription factors include yeast heat shock factor (52), cyclic AMP responsive element-binding protein (CREB) (18), Oct-2 (54), and Sp1 (23). Similarly, transcriptional activation from the *CT* gene RRE may also require phosphorylation or other posttranscriptional modification of the proteins involved in the binding to oligonucleotides 1-2 and 7-8.

Three parallel pathways of protein kinase cascades, activated by Ras, have been described. In these three pathways, the signal is propagated by the sequential activation of Raf, MEK, and MAPK families of protein kinases; of MEKK, SEK/MKK-4, and JNK protein kinases; and of p38/HOG1 protein kinases, respectively. RREs in a number of other genes have been described previously. In many of these genes, the RREs appear to be related to consensus binding sequences for AP-1, Ets-, or NF-κB-related transcription factors. The activation of some of these transcription factors is dependent on phosphorylation by *ras*-activated protein kinases (26). For example, c-Jun is phosphorylated by JNK protein kinases, and this stimulates its activity as part of the AP-1 complex. Similarly, the Ets family member Elk-1 can be phosphorylated by MAPKs, thereby stimulating its activity as part of the ternary complex which activates the c-*fos* promoter. Finally, either c-Raf-1 or JNK can phosphorylate the NF-κB inhibitor protein IκB, resulting in the release and nuclear translocation of active NF-κB (9).

The fact that *CT* gene transcription is responsive not only to activated *ras* but also to activated *raf* suggests that Ras may stimulate the *CT* gene through the Raf-MEK-MAPK pathway. Further evidence for this hypothesis is derived from the observation that RREB-1 is able to augment the Raf-mediated transcriptional response of the *CT* promoter. These observations together suggest that RREB-1 may be activated through phosphorylation by a MAPK, although it is possible that crosstalk

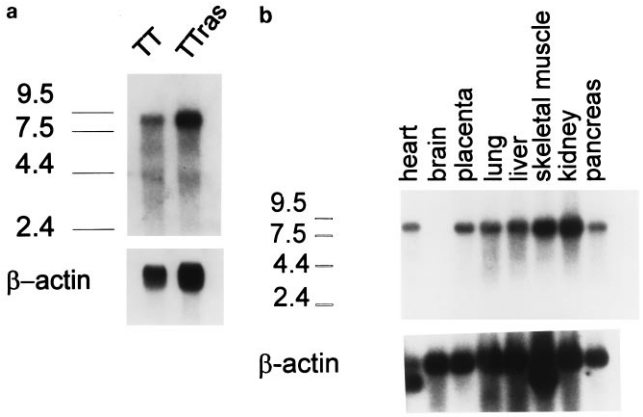



FIG. 6. (a) Northern blot analysis of RREB-1 mRNA in TT cells and in TT cells infected with Ha-MSV (TT*ras*). Left lane, 10 μg of poly(A)$^{+}$ RNA from uninfected TT cells; right lane, 10 μg of poly(A)$^{+}$ RNA from TT cells differentiated by the v-*ras*$^{H}$ oncogene. The RNA was electrophoresed through a 1.0% formaldehyde gel, transferred to a nylon filter, and hybridized to a RREB-1 probe. A 9.0-kb message is detected in both TT and TT*ras* cells. The blot was stripped and subsequently hybridized to a β-actin probe to control for loading (lower panel). (b) A Northern blot for RREB-1 expression in normal human tissues, containing 2 μg of poly(A)$^{+}$ RNA in each lane, was hybridized with an RREB-1 probe and subsequently with β-actin (lower panel). Numbers on the left of each panel indicate molecular sizes (in kilobases).

a

| | | | |
|---|---|---|---|
| FP 9 | aa | CCCCAAACACCCCC | acaaaa |
| FP 12 | ac | CCCCAAACCACCCA | ccaccc |
| FP 15 | ca | CCCCAAACCAACCC | caccca |
| C 2 | actcacc | CCCCAACACCCCCT | ttggta |
| C 16 | tgctca | CCACAAATCACCCC | acccgg |
| D 16 | act | CCCCACACCACCAC | aacacc |
| R 2 | ggaacaac | CCCCAAACAACACC | cacctc |
| FP 16 | | CCCCAAACCACCAG | ctgagc |
| FP 22 | aca | ACCCAACACAACCA | ccaccc |
| FP 162 | ccaacc | ACCCACCCACCAAC | acac |
| FP 5 | acaca | ACCCAAACACACCC | acac |

| | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| G | - | - | - | - | - | - | - | - | - | - | - | - | - | 1 |
| A | 3 | - | 1 | - | 11 | 9 | 8 | 2 | 4 | 7 | 3 | 2 | 3 | 2 |
| T | - | - | - | - | - | - | - | 1 | - | - | - | - | - | 1 |
| C | 8 | 11 | 10 | 11 | - | 2 | 3 | 8 | 7 | 4 | 8 | 9 | 8 | 7 |
| Consensus | C | C | C | C | A | A | A | C | C | A | C | C | C | C |

| | | | |
|---|---|---|---|
| CT 1-2 | ggtc | CCCCACCATCCCCC | ccat |

b

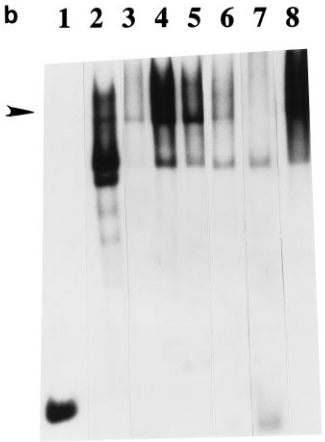



FIG. 7. (a) Definition of a consensus binding site for RREB-1. Sequences present after five cycles of CASTing were digested with *Xho*I and *Eco*RI and cloned into pBluescript. Individual clones were picked and sequenced. The RREB-1 binding sites of 11 independent cloned DNA fragments determined by CASTing are shown. Nucleotides in boldface represent identity of the oligonucleotide sequence to the core consensus. Sequences surrounding the consensus sequence are shown in lowercase, and the frequency with which any residue is found in a given position is also shown. The *CT* gene RRE corresponding to the *CT* 1-2 region is shown at the very bottom. (b) Confirmation of selected binding site clones. Four of the 11 independent clones shown in panel a (FP9 [lane 3], FP12 [lane 4], FP15 [lane 5], and FP22 [lane 6]) were picked and analyzed in a gel mobility shift assay with TT nuclear extract. Included in this assay were oligonucleotide 1-2 and dsAT1 as positive controls. dsAT1 is a 60-mer synthesized with the oligonucleotide 1-2 sequence surrounded by bases corresponding to the flanking sequences of the degenerate oligonucleotide used in CASTing. A randomly picked clone isolated by CASTing with GST alone was also included in this assay (described in Results). The arrowhead indicates a specific DNA-protein complex present in all the clones selected with GST–RREB-1 by CASTing and in the two positive controls (lanes 2 and 8) but absent in the clone selected with GST alone (lane 7).

among signal transduction pathways could be operative in this system. Furthermore, within the predicted amino acid sequence of RREB-1, we have identified a candidate site for phosphorylation by MAPK.

Several reported RREs are bipartite, having at least two motifs for binding of transcription factors, and have been shown to require both domains for RRE function, suggesting that synergistic effects of the transcription factors are necessary (16). We have shown that the *CT* gene RRE is bipartite; however, its function can be mediated independently by either of the two DNA binding motifs we have identified. This suggests that each of the bound transcription factors in the *CT* gene RRE can independently mediate a transcriptional signal, without the synergistic interaction of a second factor. The biological significance of a bipartite RRE in the *CT* gene, in which each domain can function independently, is unclear. However, one could imagine, for example, that the two domains act synergistically in vivo, although in our transient-expression assays in vitro, such synergy was not evident. Alternatively, the transcription factors which interact with these two domains may be activated separately by different types of stimuli. These stimuli might induce different programs of cell response, each of which might mediate increases in *CT* gene transcription.

In cultured MTC cells, Ras induces a program of neuroendocrine differentiation (34). In vivo, human MTC tumors also

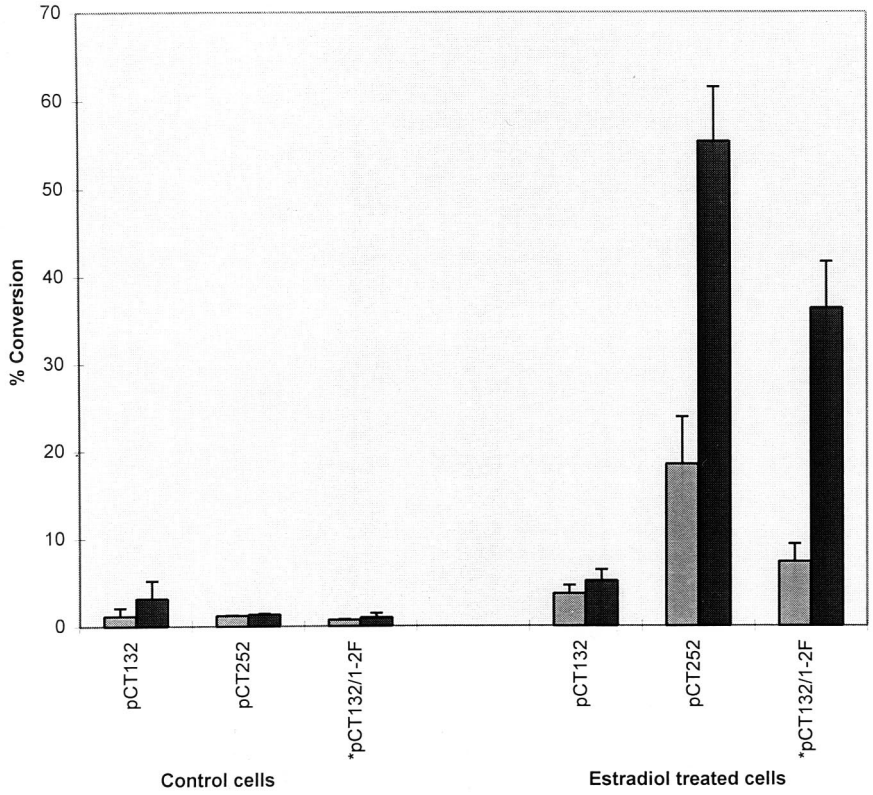



FIG. 8. Activation of *CT* gene expression by RREB-1. A representative of three experiments, each performed in at least duplicate, is shown. TT:ΔRaf-1:ER cells were transiently cotransfected with the following CT-CAT reporter constructs: Ras-nonresponsive pCT132CAT, Ras-responsive pCT252CAT or pCT132/1-2FCAT, the Moloney murine leukemia virus long terminal repeat-driven pMV7p1 expression vector alone, or this vector containing the full-length RREB-1 cDNA. The fold induction and the standard errors for the CAT assay of the control cells, in which TT:ΔRaf-1:ER cells were treated with ethanol (carrier) for 48 h posttransfection, and for the CAT assay of TT:ΔRaf-1:ER cells treated with β-estradiol for 48 h posttransfection to activate c-*raf*-1 were quantitated by PhosphoImager analysis. The asterisks indicate that in the CAT assays with the pCT132/1-2FCAT constructs, only 12.5 μl of the cell extract was used, compared with 100 μl of cell extract used with the other reporter constructs. Shaded bars, vector; solid bars, RREB-1.

display a phenotype which suggests that maintenance of neuroendocrine differentiation may be important in the course of the disease. Usually, these tumors are slowly growing and well differentiated, marked by homogeneously high levels of calcitonin by immunohistochemistry. However, in occasional MTC patients, calcitonin immunohistochemical staining may be heterogeneous or absent. This latter subset of MTC is frequently accompanied by rapid tumor growth and a poor prognosis (5, 31, 45). One may speculate that disruption of the biochemical pathways influenced by Ras in TT cells, including the factor(s) interacting with the RRE reported here, may be involved in the tumor progression observed in vivo. The functional roles of RREB-1 and other transcription factors which interact with the RRE elements defined in this study will be important not only for understanding basic aspects of transcriptional regulation of the *CT* gene but also for understanding the differentiation behavior of C-cells and MTC.

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