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THE HUMAN CALCITONIN GENE IS LOCATED ON THE SHORT ARM OF CHROMOSOME 11

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SUMMARY: By molecular hybridization of human calcitonin cDNA probes to DNA from human-rodent hybrid cells containing identified human chromosomes, we have mapped the human calcitonin gene to the short arm of chromosome 11. This location has been confirmed by in situ hybridization, which further localized the calcitonin gene to region 11p13-15. The significance of this region regarding gene linkage and possible markers for inherited cancers is discussed.

INTRODUCTION: Calcitonin (CT) is a small polypeptide hormone produced, in mammals, by the C-cells of the thyroid (1). This hormone is thought to be active in the regulation of calcium metabolism. Medullary thyroid carcinoma (MTC) is a tumor of the C-cells. In 20% of patients with MTC, the disease is inherited as an autosomal dominant trait (2,3). The levels of calcitonin produced by the tumor are high, suggesting that the tumor maintains the differentiated phenotype of the progenitor C-cells. However, in some cases of MTC, the tumor cell populations are heterogeneous for production of calcitonin, and calcitonin levels can actually drop precipitously in some patients with advancing disease. Such individuals have a poor prognosis (4). The basis of this fall in cellular calcitonin during the course of the disease is unknown. In order to study the molecular events underlying the variable expression of the calcitonin gene in MTC, and possibly to have insight into the molecular basis of both sporadic and inherited MTC, we have

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investigated the chromosomal location of the human calcitonin gene, and now report its localization to the p13-15 region of chromosome 11.

METHODS:

Cells and somatic cell hybrids: Culture line OH-3, a human small cell lung carcinoma, was used as a source for standard human DNA. This DNA demonstrates the same restriction map using a calcitonin cDNA probe as does DNA from several normal human placentas. CHL cells and 3T3 cells were used to isolate hamster and mouse DNAs, respectively. Human-mouse and human-Chinese hamster somatic cell hybrids were constructed by PEG fusion and HAT selection as previously described (5,6) and were assayed for the presence of all human chromosomes except Y by isoenzyme analyses (7).

Molecular probes: A cDNA clone library was prepared using mRNA from TT cells, a human medullary thyroid carcinoma cell line which produces high levels of CT (8), by dG:dC tailing into the Pst I site of pBR322 using published procedures (9,10). The library was screened by colony hybridization (11), using a 456 base pair Sin I fragment of pCal, a rat calcitonin cDNA clone (12). Three positive human cDNA clones were thus obtained; their characterization will be fully detailed elsewhere. pTT42 contains a 675 base pair insert which contains preprocalcitonin cDNA sequences. It hybridizes specifically to 5' sequences of the rat preprocalcitonin cDNA clone pCal. In addition pTT42 hybridizes to the same size restriction fragments as the rat pCal clone on Southern blots of human or rat genomic DNA, using several restriction enzymes, including the Bam HI 3.0 kilobase pair employed in the present fragment study. pTT42 and pCal also hybridize to the same size mRNA from TT cells upon Northern hybridization (data not shown). Plasmids were prepared by alkaline lysis (13). Inserts were recovered after Msp I digestion (releasing the entire insert along with 110 base pairs of flanking pBR322 DNA) and electrophoresis, by either NaI-ethanol precipitation (14) or electroelution. For Southern hybridizations, the inserts were labeled with [^{32}P] dCTP (Amersham, 3000 Ci/mmol) to specific activities of $5X10^8$-$1.5X10^9$ cpm/ug by random priming with Klenow DNA polymerase (14). For in situ hybridization to chromosomes, the whole pTT42 plasmid was partially digested with Msp I, boiled and labeled to $1.0X10^8$ cpm/ug by random priming, using [^{3}H] dATP (50 Ci/mmol) and [^{3}H] TTP (97 Ci/mmol).

Southern blot hybridization: DNA was prepared from cells by the method of Blin and Stafford (15) or Gross-Bellard et al (16). Aliquots of DNA were digested overnight with 3 units of restriction enzyme/ug DNA in conditions suggested by the suppliers (BRL, New England Biolabs, Promega Biotec). Twelve micrograms of DNA were electrophoresed through agarose gels and transferred to nitrocellulose (17). Hybridization conditions were 60°C in 1 M NaCl, 50 mM Na phosphate, 2 mM EDTA, pH 6.8, containing 1% SDS, 10X Denhardt's reagent (1XDenhardt's reagent=0.02% bovine serum albumin, 0.02% polyvinylpyrrolidone, 0.02% Ficoll 400), and 0.1 mg/ml sonicated salmon sperm DNA, with 10^7 cpm of [^{32}P] dCTP labeled probe as described above. Hybridization was performed over 1-2 days, after which the filters were rinsed at room temperature in 2XSSC (1XSSC=0.15M NaCl, 0.015M Na citrate), 0.5% SDS, then washed extensively at 60°C in 0.5XSSC, 0.5% SDS. The filters were then autoradiographed with Kodak XAR-1 film and Dupont Cronex Lightning Plus intensifying screens for 16 hr-5 days at -70°C.

In situ chromosome hybridization (18): Chromosome preparations were made from human peripheral blood samples incubated with PHA for 72 hrs and synchronized with methotrexate, followed by mitotic arrest with Colcemid. The cells were harvested, treated with hypotonic (0.075 M) KCl, fixed with ethanol-acetic acid (3:1), and dropped onto clean microscope slides. The slides were treated with boiled RNase A (100 ug/ml) in 2XSSC for 60 minutes at 37°C, then treated with 70% formamide, 2XSSC for 4 minutes at 70°C. Tritium labeled probe, prepared as described above, was suspended at 0.2 ug/ml in 50% formamide, 10% dextran sulfate, 2XSSC, 40 mM Na phosphate, 1X

Denhardt's reagent, 0.2 mg/ml sonicated salmon sperm DNA. The probe was denatured by heating at 70°C for 5 minutes. Thirty microliters of this hybridization solution were applied to each slide. The slides were covered with coverslips and incubated for 16 hr at 37°C, after which they were rinsed in 50% formamide, 2XSSC at 39°C and exposed to Kodak NTB2 nuclear track emulsion for one week at -70°C. They were then developed with Kodak Dektol. Metaphase cells were stained with Giemsa and photographed; the same cells were then stained and photographed with quinacrine fluorescence to show Q-bands. Between one and five grains were associated with chromosomes in each metaphase spread scored. The background grain count was within this same range and, in most of the spreads, was lower than the number of grains associated with chromosomes. The in situ data was obtained independently by one of us, who was unaware of the results obtained with the somatic cell hybrids.

RESULTS: We have screened several series of rodent-human hybrids, including a total of 58 hybrids, for the presence of human calcitonin gene sequences. To do so, we digested DNA from the hybrids with restriction endonuclease Bam HI, electrophoresed the DNA through agarose gels, and transferred the DNA to nitrocellulose filters for Southern hybridization analysis. Bam HI leaves a 3.0 kilobase pair CT specific restriction fragment in human DNA (Figure 1A, lane 1). The Bam HI fragments in hamster and mouse DNA are in excess of 10 kilobase pair and do not hybridize well with the human CT probes under the hybridization conditions employed here (Figure 1A, lanes 2 and 3). Analysis of the chromosomes found within these hybrids showed high levels of discordance for all human chromosomes except chromosome 11 (Table 1 and Figure 1B). The short arm of chromosome 11 was discordant with the presence of the 3.0 kilobase pair fragment in only 4 of 58 hybrids. In three of these four, the isoenzyme marker for chromosome 11 (lactate dehydrogenase A, ref. 7) was weakly positive, suggesting that many of the cells in the hybrid clone did not contain human chromosome 11. The remaining fraction containing chromosome 11 may have been below the limit of sensitivity of the hybridization probe.

Two of the hamster-human hybrids examined contained the long arm but not the short arm of chromosome 11 (5). This was determined by the presence of the human isoenzyme for esterase A4 which is located on the long arm of chromosome 11, and absence of ras^H DNA sequences and the human isoenzyme for lactate dehydrogenase A, both located on the short arm. These hybrids

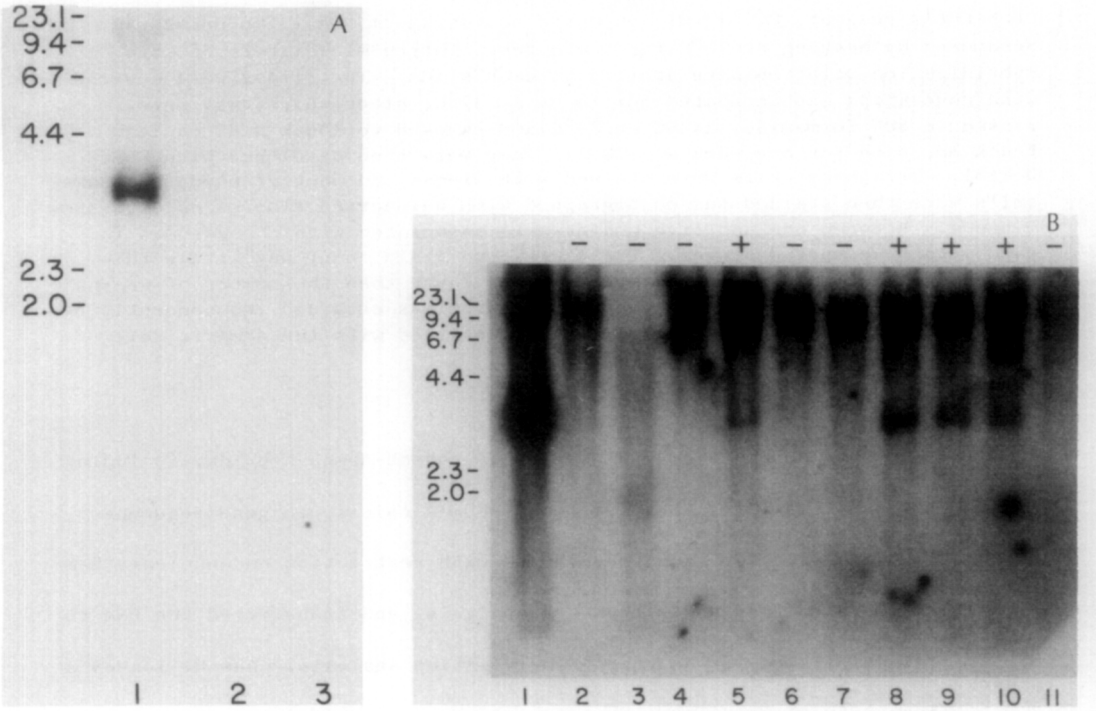


Figure 1: Hybridization of human calcitonin cDNA probe to human, rodent and human-rodent hybrid DNA. Twelve micrograms of genomic DNA were restricted with 36 units of Bam HI overnight, electrophoresed through 0.8% agarose gels, and transferred to nitrocellulose filters. Filters were hybridized for two days with 1.5×10^7 cpm of pTT42 (human calcitonin cDNA) insert, as described in Materials and Methods. A, lane 1, human (OH-3) DNA; lane 2, Chinese hamster (CHL) DNA; lane 3, mouse (3T3) DNA. B, lane 1, human (OH-3) DNA; lanes 2-11, human-mouse hybrid DNA. Hybrid DNAs are marked + or - to indicate presence or absence of human CT gene sequences. Molecular weight markers, bacteriophage DNA digested with Hind III, are shown in kilobase pairs.

did not contain human CT DNA sequences. These results strongly indicate that the human calcitonin gene is located on the short arm of chromosome 11.

In order to confirm these results and better localize the CT gene, we hybridized the human CT probe to normal metaphase chromosome spreads in situ (Figs. 2 and 3). Of 62 grains scored from 26 metaphases, 5 grains (8.1%) clustered in an area of the short arm of chromosome 11, between bands 11p13 and 11p15. All of the grains on chromosome 11 were in this region. The grains were scored for statistical significance by Poisson distribution, as described by Morton et al (19). The cluster on 11p13-15 is significant at p=.0006. Grain clusters centered on chromosome locations 3q27, 12q24, 16q22, and Xp21 were significant only at .01 p .05. Taken together with the hybrid

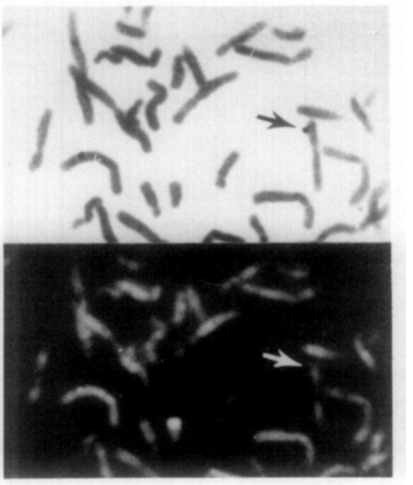
Table 1: Discordance of human chromosomes with presence of human calcitonin sequences. Human-rodent hybrids were assayed for the presence of human chromosomes by isoenzyme analysis. Discordance represents the presence of a chromosome and the absence of CT gene sequences, or the absence of a chromosome and the presence of CT gene sequences.

Chromosome	% Discordance
1	29
2	36
3	19
4	34
5	36
6	28
7	38
8	29
9	31
10	34
11p	7
11q	10
12	33
13	43
14	40
15	47
16	33
17	38
18	40
19	31
20	21
21	41
22	24
X	60

cell data, these results provide preliminary evidence for the regional localization of the human calcitonin gene as 11p13-15.

DISCUSSION: The human calcitonin gene is located in an area of the genome, 11p, which is unusually well characterized for linkage of various genes (20-22). For example, molecular hybridization analysis has established the order and position of the genes for parathyroid hormone, oncogene ras^H, non-alpha globin family and insulin on 11p (20). Our data will now allow us to determine the linkage of CT with these other genes.

With respect to medullary thyroid carcinoma, a dominantly heritable cancer, we are intrigued by the location of CT on 11p13-15. In a cell line derived from a patient with virulent MTC (8), we have detected a chromosomal rearrangement in this region (J.R.T. et al, unpublished). We raise the possibility that a rearrangement of genetic material might modulate CT expression, as is seen in virulent cases of MTC. It is also interesting that another autosomal dominant cancer, Wilms' tumor, a childhood renal cancer, frequently exhibits a deletion at 11p13 (23). Very speculatively, a



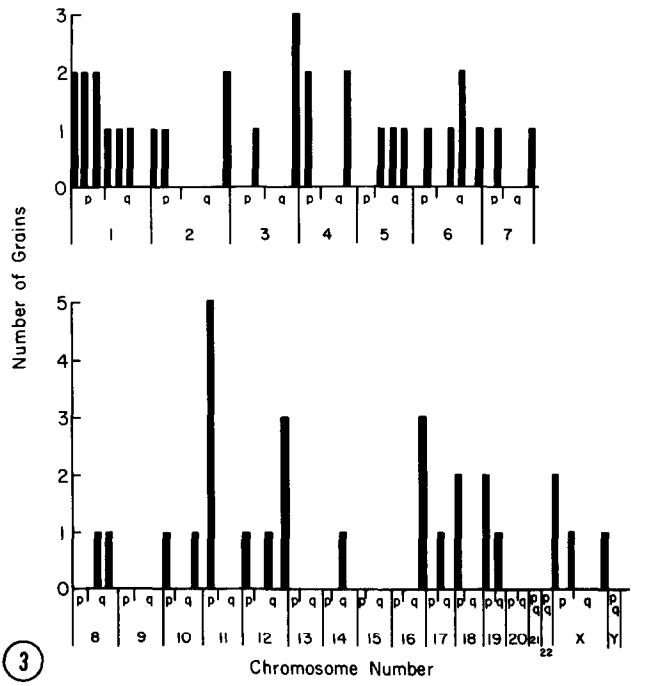


Figure 2: In situ hybridization of human calcitonin cDNA probe to a normal human metaphase chromosome spread. Hybridization, autoradiography, and staining were accomplished as described in Materials and Methods, using tritium labelled pTT42. Giemsa stain (top); quinacrine (Q) banding (bottom). A grain can be seen at 11p14-15 (arrow).

Figure 3: Histogram of grain distribution from in situ chromosome hybridization using pTT42 probe. Significant clustering of grains (p=.0006) is seen only at 11p13-15 with less prominent clusters at 3q27, 12q27, 16q22, and Xp21.

rearrangement or deletion in this area may activate a gene sequence responsible for transformation of the progenitor cells for either MTC or Wilms' tumor. Pertinent to this possibility is the location of oncogene ras^H on 11p (5,24-27). The ras oncogene family has been implicated as transforming genes in numerous human cancers (28-34); ras^H as the first human transforming gene identified (28-30). Our findings suggest studies to determine the possible linkage of CT and ras^H to the deletion in Wilms' tumor and the rearrangement in the cultured MTC cells, and to investigate the frequency of this rearrangement in MTC tumor tissue.

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REFERENCES

1. DeLellis, R.A., and Wolfe, H.J. (1981) Pathobiol. Annual 16, 25-52.
2. Schimke, R.N., and Hartmann, W.H. (1965) Ann. Intern. Med. 63, 1027-1037.
3. Keiser, H.R., Beaven, M.A., Doppman, J., Wells, S.A., Jr., and Buja, L.M. (1973) Ann. Intern. Med. 78, 561-579.
4. Lippman, S.M., Mendelsohn, G., Trump, D.L., Wells, S.A., Jr., and Baylin, S.B. (1982) J. Clin. Endocrinol. Med. 54, 233-240.
5. McBride, O.W., Swan, D.C., Santos, E., Barbacid, M., Tronick, S.R., and Aaronson, S.A. (1982) Nature, 300, 773-774.
6. McBride, O.W., Swan, D.C., Tronick, S.R., Gol, R., Klimanis, D., Moore, D.E., and Aaronson, S.A. (1983) Nucleic Acids Research 11, 8221-8236.
7. Swan, D.C., McBride, O.W., Robbins, K.C., Keithley, D.A., Reddy, E.P., and Aaronson, S.A. (1982) Proc. Natl. Acad. Sci. USA 79, 4691-4695.
8. Leong, S.S., Horoszewicz, J.S., Shimaoka, K., Friedman, M., Kawinski, E., Song, M.J., Zeigel, R., Chu, T.M., Baylin, S., and Mirand, E.A. (1981) in Advances in Thyroid Neoplasia 1981, (Andreoli, M., Monaco, F., and Robbins, J., eds.), pp. 95-108, Field Educational Italia, Rome, Italy.
9. Buell, G.N., Wickens, M.P., Payvar, F., and Schimke, R.T. (1978) J. Biol. Chem. 253, 2471-2482.
10. Wickens, M.P., Buell, G.N., and Schimke, R.T. (1978) J. Biol. Chem. 253, 2483-2495.
11. Hanahan, D., and Meselson, M. (1980) Gene 10, 63-67.
12. Amara, S.G., Jonas, V., Rosenfeld, M.G., Ong, E.S., and Evans, R.M. (1982) Nature 298, 240-244.
13. Marko, M.A., Chipperfield, R., and Birnboim, H.C. (1982) Anal. Biochem. 121, 382-387.
14. Feinberg, A.F., and Vogelstein, B. (1983) Anal. Biochem. 132, 6-13.
15. Blin, N., and Stafford, D.W. (1976) Nuc. Acids. Res. 4, 2303-2307.
16. Gross-Bellard, N., Oudet, P., and Chambon, P. (1973) Eur. J. Biochem. 36, 32-38.
17. Southern, E.M. (1975) J. Mol. Biol. 98, 503-517.
18. Harper, M.E., and Saunders, G.F. (1981) Chromosoma 83, 431-439.
19. Morton, C.C., Taub, R., Diamond, A., Lane, M.A., Cooper, G.M., and Leder, P. (1984) Science 223, 173-175.
20. Antonarakis, S.E., Phillips, J.A., III, Mallonee, R.L., Kazazian, H.H., Jr., Fearon, E.R., Waber, P.G., Kronenberg, H.M., Ullrich, A., and Meyers, D.A. (1983) Proc. Natl. Acad. Sci. USA 80, 6615-6619.
21. Puck, T.T. (1983) Banbury Reports 14, 205-213.
22. Sparkes, R.S., ed. (1983), In press, International Human Gene Mapping Workshop VII, Univ. of Calif., Los Angeles.
23. Riccardi, V.M., Sujansky, E., Smith, A.C., and Francke, U. (1978) Pediatrics 61, 604-610.
24. de Martinville, B., Giacalone, J., Shih, C., Weinberg, R.A., and Francke, U. (1983) Science 219, 498-501.
25. O'Brien, S.J., Nash, W.G., Goodwin, J.L., Lowy, D.R., and Chang, E.H. (1983) Nature 302, 839-842.
26. Ryan, J., Barker, P.E., Shimizu, K., Wigler, M., and Ruddle, F.H. (1983) Proc. Natl. Acad. Sci. USA 80, 4460-4463.
27. Jhanwar, S.C., Neel, B.G., Hayward, W.S., and Chaganti, R.S.K. (1983) Proc. Natl. Acad. Sci. 80, 4794-4797.
28. Goldfarb, M., Shimizu, K., Perucho, M., and Wigler, M. (1982) Nature 296, 404-409.
29. Shih, C., and Weinberg, R.A. (1982) Cell 29, 161-169.
30. Pulciani, S., Santos, E., Lauver, A.V., Long, L.K., Robbins, K.C., and Barbacid, M. (1982) Proc. Natl. Acad. Sci. USA 79, 2845-2849.
31. Lane, M.A., Sainten, A., and Cooper, G.M. (1982) Cell 28, 873-880.
32. Pulciani, S., Santos, E., Lauver, A.V., Long, L.K., Aaronson, S.A., and Barbacid, M. (1983) Nature 300, 539-542.
33. McCoy, M.S., Toole, J.J., Cunningham, J.M., Chang, E.H., Lowy, D.R., and Weinberg, R.A. (1983) Nature 302, 79-81.
34. Cooper, C.S., Blair, D.G., Oskarsson, M.K., Tainsky, M.A., Eader, L.A., and Vande Woude, G.F. (1984) Cancer Res. 44, 1-10.