Point Mutations of Ras Oncogenes are an Early Event in Thyroid Tumorigenesis

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Identifying the nature of the genetic mutations in thyroid neoplasms and their prevalence in the various tumor phenotypes is critical to understanding their pathogenesis. Mutational activation of ras oncogenes in human tumors occurs predominantly through point mutations in two functional regions of the molecules, codons 12,13 (GTP-binding domain) or codon 61 (GTPase domain). We examined the prevalence of point mutations in codons 12, 13, and 61 of the oncogenes K-ras, N-ras, and H-ras in benign and malignant human thyroid tumors by hybridization of PCR-amplified tumor DNA with synthetic oligodeoxinucleotide probes. None of the eight normal thyroid tissues harbored point mutations. Four of nineteen nodules from multinodular goiters (21%), 6/24 microfollicular adenomas (25%), 3/14 papillary carcinomas (21%), and 0/3 follicular carcinomas contained ras point mutations. The predominant mutation was a valine for glycine substitution in codon 12 of H-ras. None of the multinodular goiter tumors known to be polyclonal (and thus due to hyperplasia) had point mutations, whereas one of the two monoclonal adenomas arising in nodular glands contained in H-ras codon 12 valine substitution, which was confirmed by sequencing the tumor DNA. These data show that ras activation is about equally prevalent in benign and malignant thyroid neoplasms, and thus may be an early event in the tumorigenic process. (Molecular Endocrinology 4: 1474–1479, 1990)

INTRODUCTION

There is growing evidence that tumor progression and neoplastic phenotype is determined by multiple genetic lesions, the nature of some of which are now becoming unraveled (1, 2). Activating mutations of protooncogene sequences, through amplification, translocation, or point mutations, have been identified in a variety of human neoplasms. Amplification of N-*myc* and of c-erb-B2/neu are frequent in neuroblastomas (3) and in breast and ovarian cancers, respectively, and in breast cancer copy number correlates with disease prognosis (4). Chromosomal translocations which juxtapose c-*myc* with immunoglobulin promoter sequences are features of B cell lymphoid tumors, and the bcr/abl translocation which gives rise to the Philadelphia chromosome is the hallmark of chronic myelogenous leukemia (1). Point mutations of the ras genes are also associated with tumorigenesis. Some forms of cancer appear to have a predilection for transforming point mutations of a particular member of the ras family: *i.e.* K-ras in lung, colon, and pancreas (5–7), N-ras in acute myeloid leukemia (8). Interestingly, point mutations of K-ras are about equally prevalent in benign colonic adenomas as in colon carcinomas (9), suggesting that this activating mutation may be an early event in the generation of these neoplasms, and that additional genetic damage must occur to render these tumors malignant.

Tumors of thyroid follicular cells provide an attractive model to study the molecular genetics of tumor progression, since they comprise a spectrum of neoplasms which includes the uniformly benign colloid adenomas, the slowly progressive differentiated papillary and follicular carcinomas, and the invariably fatal anaplastic carcinomas (10). These tumors are clonal in nature (11), indicating that they must have arisen from one or more somatic cell mutations. We recently observed that rearrangements of H-ras, predominantly gene amplification, are occasional features of both benign and malignant thyroid neoplasms (12). Although ras gene rearrangements may affect the phenotype of tumor cells, activation of genes in the ras gene family usually occurs through single base substitutions in the guanine nucleotide binding site (codons 12 and 13), or in the effector domain containing the intrinsic ATPase activity (codon 61). In order to better understand the role of ras oncogenes in thyroid tumorigenesis, we screened a large number of benign and malignant thyroid tumors for activating point mutations in the three ras genes by selective hybridization of polymerase chain reaction amplified DNA from the tumors with panels of synthetic oligodeoxinucleotide probes.

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RESULTS

The DNA of normal thyroid tissues (n = 8) and thyroid tumors (n = 60) was sequentially amplified by PCR using primers flanking codons 12/13, or codon 61 of H-ras, K-ras, and N-ras. Blots with the amplified tissue DNAs were then hybridized with panels of oligodeoxinucleotide probes specific for the six to seven possible point mutants in each codon for each gene. As positive controls, we used PCR amplified DNA where one of the primers contained a desired nucleotide substitution. As negative controls, polymerase chain reaction (PCR) amplified DNA from normal thyroid tissue was used (as expected, none of the eight normal thyroid tissue specimens harbored point mutations of the ras genes).

Figure 1 shows a representative blot, in this case of a screen for N-ras codon 61 arginine mutations. A positive control of PCR amplified DNA containing the arginine mutation was blotted in slots 1A–C. These only hybridized with the arginine mutant probe (*right panel*), and not with the wild type probe (*left panel*). The PCR samples from all the tissues in this blot hybridized with the wild type probe, indicating that they all contained at least one normal allele. Only the follicular adenoma DNA in slot 3C hybridized with the N-61 arginine probe, and thus was shown to contain one allele with this point mutation (Fig. 1, *right panel*).

In a similar fashion, the blotted PCR samples from all tissues shown in Fig. 2 hybridized with the N-ras codon 61 wild type oligodeoxinucleotide probe. Only the DNA from the follicular adenoma in slot 3B hybridized with the N-61 glutamine mutant probe (Fig. 2, *right panel*).

The most frequently encountered point mutations in the thyroid tumors we examined were in H-ras codon 12. Shown in Fig. 3 is a slot blot of PCR samples of thyroid tissues hybridized with H-ras codon 12 wild type (*left panel*) or H-12 valine mutant oligonucleotide probes (*right panel*). All but the slots containing the H-12 valine positive control (1A–C) hybridized with the wild type probe. A papillary carcinoma which we previously reported (12) and which was known to contain an H-ras codon 12 T for G substitution also hybridized to the valine mutant probe and served as an additional positive control (2A–C). Other tumors giving a positive signal included 4 large, well circumscribed nodules from multinodular goiters (4C, 5A–C), 4 follicular adenomas (6A–C, 7A), and two other papillary carcinomas (8A–C). We encountered no point mutations of K-ras in any of the thyroid tumors.

The finding that some nodules from multinodular goiters harbor ras point mutations was unexpected, in that these tumors are usually believed to arise as a result of hyperplastic proliferation in response to exogenous goitrogenic stimuli rather than to clonal expansion of a somatically altered cell. To confirm these data, we sequenced the amplified DNA from MNG nodule 8C. To this end we synthesized primers flanking H-ras codon 12/13 which contained *Eco*RI sites in the distal ends, and used them to amplify the tumor DNA. The PCR amplified DNA was packaged into a sequencing vector, and sequenced by the dideoxy chain termination method. As shown in Fig. 4, the sequence of the tumor

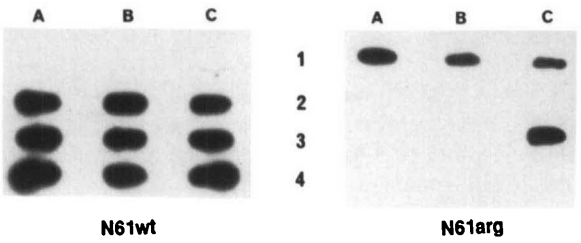

Fig. 1. Slot Blot of Thyroid Tissue DNA Amplified with Primers Flanking N-ras Codon 61 Hybridized with γ ^{32}P-Labeled Oligonucleotides for N-ras Codon 61 Wild Type (N61wt), or Codon 61 Arginine Point Mutant (N61arg)

1A–C: arginine mutant positive control. 2A–C, 3A–C: follicular adenomas. 4A–C: papillary carcinomas.

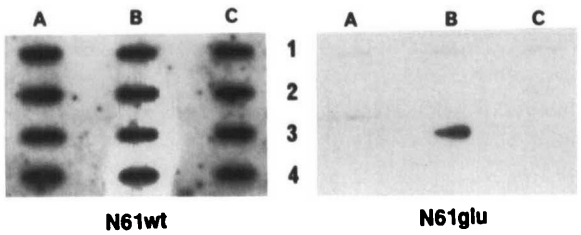

Fig. 2. Slot Blot of Thyroid Tissue DNA Amplified with Primers Flanking N-ras Codon 61 Hybridized with γ ^{32}P-Labeled Oligonucleotides for N-ras Codon 61 Wild Type (N61wt), or Codon 61 Glutamine Point Mutant (N61glu)

1A–C: nodules from MNG. 2A–C, 3A–C: follicular adenomas. 4A–C: papillary carcinomas.

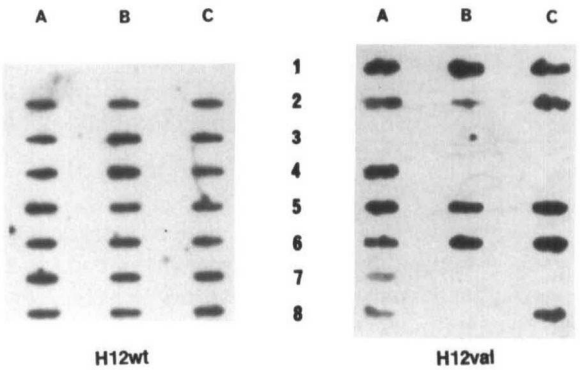

Fig. 3. Slot Blot of Thyroid Tissue DNA Amplified with Primers Flanking H-ras Codons 12,13 Hybridized with γ ^{32}P-Labeled Oligonucleotides for H-ras Codon 12,13 Wild Type (H12wt), or Codon 12 Valine Point Mutant (H12val)

The first two rows were blotted in triplicate. In all other rows each blot corresponds to PCR product of a separate tissue sample. 1A–C: Triplicates of papillary carcinoma (tumor #4). 2A–C: Triplicates of papillary carcinoma (tumor #21). 3A–C: Normal thyroid. 4A–C, 5A–C: Nodules from multinodular goiters. 6A–C; 7A, 7C: Follicular adenomas. 7B: Papillary carcinoma. 8A, 8C: Papillary carcinomas. 8B: Nodule from multinodular goiter.

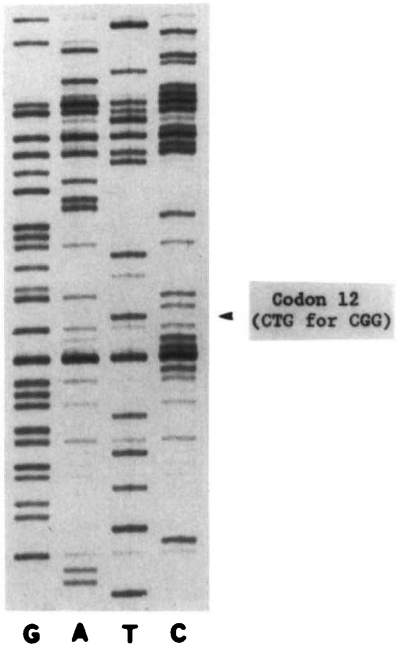

Fig. 4. Sequencing gel of PCR amplified DNA from a tumor obtained from a multinodular goiter

Arrow indicates T for G substitution in codon 12 of H-ras.

Table 1. Prevalence of Ras Point Mutations in Thyroid Neoplasms

Tissue	Number with point mutations	Ras mutation
Normal thyroid	0/8	
Nodules from MNG	4/19 (21%)	H12val (×4)
Microfollicular adenomas	6/24 (25%)	H12val (×4) N61glu N61arg
Papillary carcinomas	3/14 (21%)	H12val (×3)
Follicular carcinomas	0/3	

confirmed the T for G substitution in codon 12 of H-ras.

We recently reported a study on the clonal composition of human thyroid neoplasms performed by X-chromosome inactivation analysis, and observed that occasionally monoclonal neoplasms do arise in multinodular goiters (11). Interestingly, none of the four nodules which we knew to be polyclonal had ras point mutations, whereas one of the two monoclonal tumors arising in these glands contained an H-ras codon 12 valine mutation.

Table 1 summarizes the data on the prevalence of point mutations of ras oncogenes in thyroid tumors according to their histological diagnosis. The DNA of 6/24 microfollicular adenomas (25%), 4/19 nodules from multinodular goiters (21%), and 3/14 papillary carcinomas (21%) harbored activating base substitutions of either H-ras or N-ras.

DISCUSSION

There are major unanswered questions about the manner in which thyroid tumors evolve, and particularly, about the fundamental defects in cellular growth control which may lead to tumors with more malignant potential. In human neoplasia, the sequence of genetic mutations leading to changing tumor phenotypes does not appear to be random, as has recently been shown in glial (13) and colonic neoplasms (9, 14). James *et al.* (13) identified a sequence of genetic alterations in human glial tumors. Chromosome 17 losses appear to be an early event, since they are already present in grade II astrocytomas, whereas amplification of the epidermal growth factor receptor only becomes apparent in grade III astrocytomas. Monosomy for chromosome 10 was present in 28/29 glioblastomas (malignancy grade IV), but no similar losses were observed in any of 22 gliomas of lower malignancy grade. In human colonic neoplasms, activating point mutations of K-ras were present in 9% of class I adenomas, 58% of class II adenomas, and 58% of carcinomas (9). The sequences of chromosome 5q linked to familial polyposis were not lost in the adenomas from patients with polyposis, but were lost in 29–35% of patients with adenomas and carcinomas, respectively, from patients with sporadic colon neoplasia. In contrast, a region of chromosome 18q21.3 was deleted frequently in carcinomas, and in advanced adenomas, and rarely in early stage adenomas. A putative tumor suppressor gene present in this chromosome region, the DCC gene (15), has recently been identified. Finally, chromosome 17p sequences containing the p53 tumor suppressor gene were lost almost exclusively in carcinomas (16).

We have previously shown that structural rearrangements of H-ras, particularly gene amplification, are about equally prevalent in benign and malignant thyroid neoplasms (12). In this paper we examined a large number of thyroid neoplasms for activating point mutations of the three human ras genes. None of the eight normal thyroid tissue samples had ras point mutations. The total number of normal thyroid specimens tested is considerably less than that of the thyroid neoplasms. Normal tissues, however, have not been reported to harbor these activating mutations, which almost certainly occur as somatic events. We and others (17, 18) have also noted that normal tissue does not induce transformation in the NIH3T3 cell focus assay, which has a predilection for ras oncogenes. Point mutations were present in 21% of nodules from multinodular goiters, 25% of follicular adenomas, and 21% of papillary carcinomas, suggesting that these genetic lesions may be one of the initiating events in thyroid tumorigenesis. The most frequently observed mutation was a valine for glycine substitution in codon 12 of H-ras. The observation that some tumors with nodular goiters harbored ras point mutations was unexpected. By relating the present data with our recent findings on the clonal composition of thyroid tumors (11), we observed that none of the polyclonal tumors derived from

Table 2. Oligonucleotide Probes Used to Screen for Ras Oncogene Point Mutations

H-ras 12	GGCGCCGGCGGTGTGGGCAA	(gly)	H-ras 13	GGCGCCGGCGGTGTGGGCAA	(gly)
	AGC	(ser)		AGT	(ser)
	TGC	(cys)		TGT	(cys)
	CGC	(arg)		CGT	(arg)
	GAC	(asp)		GAT	(asp)
	GTC	(val)		GCT	(ala)
	GCC	(ala)		GTT	(val)
K-ras 12	GGAGCTGGTGGCGTAGGCAA	(gly)	K-ras 13	GGAGCTGGTGGCGTAGGCAA	(gly)
	AGT	(ser)		AGC	(ser)
	TGT	(cys)		TGC	(cys)
	CGT	(arg)		CGC	(arg)
	GAT	(asp)		GAC	(asp)
	GCT	(ala)		GTC	(val)
	GTT	(val)		GCC	(ala)
N-ras 12	GGAGCAGGTGGTGTTGGGAA	(gly)	N-ras 13	GGAGCAGGTGGTGTTGGGAA	(gly)
	AGT	(ser)		AGT	(ser)
	TGT	(cys)		TGT	(cys)
	CGT	(arg)		CGT	(arg)
	GAT	(asp)		GAT	(asp)
	GCT	(ala)		GCT	(ala)
	GTT	(val)		GTT	(val)
H-ras 61	ACCGCCGGCCAGGAGGAGTA	(gln)	K-ras 61	ACAGCAGGTCAAGAGGAGTA	(gln)
	GAG	(glu)		GAA	(glu)
	AAG	(lys)		AAA	(lys)
	CGG	(arg)		CGA	(arg)
	CTG	(leu)		CTA	(leu)
	CCG	(pro)		CCA	(pro)
	CAT	(his)		CAT	(his)
	CAC	(his)		CAC	(his)
N-ras 61	ACAGCTGGACAAGAAGAGTA	(gln)			
	GAA	(glu)			
	AAA	(lys)			
	CGA	(arg)			
	CTA	(leu)			
	CCA	(pro)			
	CAT	(his)			
	CAC	(his)			

Full sequence of wild type oligonucleotides is shown. Listed below them are codons with the appropriate single base substitutions. Amino acids coded for by the mutant codons are shown in brackets.

multinodular goiters contained activated ras genes, whereas one of the two monoclonal neoplasms arising in goitrous glands had an H12val mutation (clonal studies were not possible in the three other tumors from multinodular goiters which contained point mutations). It should be emphasized that the prevalence of ras mutants we report for multinodular goiters is likely to be biased by the fact that many patients with this condition were sent to surgery because they harbored a dominant or growing nodule. Our data suggests that ras activation may confer these tumors with a growth advantage, which may be fully expressed in glands exposed to environmental goitrogens. Lemoine *et al.* (17, 18) reported a similar, albeit higher (around 50%), frequency of activating point mutations of ras genes in follicular adenomas and differentiated follicular carcinomas. As we, they found about a 20% prevalence of point mutations in papillary carcinomas (18). There are several potential explanations for the higher prevalence of ras point mutations observed by Lemoine *et al.* (17, 18) in their follicular adenomas. On the one hand, it is possible that there were true differences in the frequency of ras oncogene activation between the two patient populations, as they could have been exposed to diverse environmental mutagens. There is evidence from experimental mammary tumorigenesis that the nature of the carcinogen can determine which of the ras genes becomes activated (19). These observations have recently been extended to the thyroid (20), with H-ras becoming activating in nitrosomethylurea-induced tumors, and K-ras in those induced by ionizing radiation. Alternatively, the methodological conditions used in the two studies may have contributed to the different prevalence of ras mutations observed. Lemoine *et al.* (17, 18) amplified DNA from selected sections of paraffin blocks, and thus could have had less contamination with normal tissue than may have been present in our fresh frozen tissues (although we took great care to sample from within the tumor specimen). Furthermore, they amplified the material for 50 cycles whereas we did so for 30, and therefore are theoretically more likely to have detected a mutated allele even if it was present in a small subpopulation of cells. On the other hand, we applied more stringent controls in our experiments to avoid false positive and false negative results due to background hybridization (*i.e.* by blotting PCR-amplified DNA containing desired points mutations, and by sequencing the tumor DNA of critical samples which gave a positive result by oligodeoxinucleotide hybridization). The conclusions of both studies are however quite similar, that ras point mutations are found with about equal prevalence in benign and malignant thyroid tumors, and thus may be an early event in the development of these neoplasms.

These data indicate that besides activation of ras genes, additional somatic mutations must occur to explain the different phenotypic characteristics of thyroid neoplasms, and more specifically, to render some of these tumors with the ability to invade lymphatic (papillary) or blood vessels (follicular carcinomas), or to become dedifferentiated (anaplastic). Bongarzone *et al.* (21) have reported that papillary carcinomas have a high frequency of activation of tyrosine kinase oncogenes such as trk, and ret (21). This latter oncogene resulted from the intrachromosomal rearrangement of an unknown amino-terminal sequence to the tyrosine kinase domain of the ret protooncogene (22). The relative roles of these and other oncogenes, as well as of the inactivation of putative tumor suppressor genes in thyroid tumor progression remains to be elucidated.

MATERIALS AND METHODS

Tissue Material

Human thyroid tissue material was obtained at surgery, and immediately frozen in liquid N_2 until assayed. In most cases, samples were obtained of both the thyroid tumor and of normal thyroid. Eight normal thyroid tissue samples and 60 thyroid tumors were studied: 19 nodules from patients with multinodular goiter, 24 follicular adenomas, 14 papillary carcinomas, and three follicular carcinomas.

Nucleic Acid Extractions

Tissues were ground under liquid N_2 using mortar and pestle. DNA was extracted from a cesium chloride ultracentrifugation gradient (23). The DNA layer was immediately precipitated in 2.5 vol ethanol, and recovered by spooling. The DNA pellet was then rinsed in 10 ml 80% ethanol, and recovered by centrifugation at 3000 × *g* at room temperature. DNA was then digested at 37 C overnight with 1 mg/ml proteinase K in a buffer containing 150 mM NaCl, 10 mM Tris, pH 7.5, 10 mM EDTA, and 0.4% sodium dodecyl sulfate (SDS). The mixture was the phenol-chloroform extracted. Sodium acetate (final concentration, 0.3 M) was added to the aqueous layer, which was then ethanol precipitated. DNA was pelleted, air dried, and resuspended in H_2O. After quantification by absorption at 260 nm, extracts were stored at 4 C until assayed.

PCR Amplification of Thyroid Tissue DNA (24)

Genomic DNA (1 μg) was amplified for 30 cycles with 2.5 U Taq polymerase (Perkin Elmer-Cetus, Norwalk, CT) and 1 μM oligonucleotide primers flanking codons 12/13 or codon 61 for either H-ras, K-ras, or N-ras (six separate amplification reactions were carried out for each tumor DNA sample) (Clontech Laboratories, Palo Alto, CA) in a total vol 100 μl. The following pairs of oligonucleotide primers were used: H-ras 12/13: 5′ ATGACGGAATATAAGCTGGT and 3′ CGCCAGGCTCACCTCTATA; H-ras 61: 5′ AGGTGGTCATTGATGGGGAG and 3′ AGGAAGCCCTCCCCGGTGCG; K-ras 12/13: 5′ ATGACTGAATATAAACTTGT and 3′ CTCTATTGTTGGATCATATT; K-ras 61: 5′ AAGTAGTAATTGATGGAGAA and 3′ AGAAAGCCCTCCCCAGTCCT; N-ras 12/13: 5′ ATGACTGAGTACAAACTGGT and 3′ CTCTATGGTGGGATCATATT; N-ras 61: 5′ CAAGTGGTTATAGATGGTGA and 3′ AGGAAGCCTTCGCCTGTCCT. The PCR mix contained 10 mM Tris (pH 8.3), 50 mM KCl, 1.5 mM Mg Cl_2, 0.1% gelatin and 10 mM each of dATP, dCTP, dTTP, and dGTP. The first cycle was performed as follows: denaturation was for 5 min at 95 C, annealing at 55 C for 2 min, and extension at 70 C for 2 min. In subsequent cycles, denaturation was for 2 min at 94 C, annealing for 2 min at 55 C, and extension for 2 min at 70 C. After completion, 10 μl PCR product was electrophoresed on 4% NuSieve agarose (FMC, Rockland, ME) gels and the size of the amplified DNA compared to that of appropriate molecular size markers by ethidium bromide staining.

Since there are 57 possible point mutations in the two codons of the three ras genes, positive controls (*i.e.* tumor DNA containing such ras point mutations) for many of these are not easily obtained. To overcome this problem, we used oligonucleotides containing the desired base substitutions as one of the flanking primers in an amplification reaction. Since the amplification proceeds to include the sequence of the primers, the nucleotide substitutions are incorporated into the product of the PCR. A total of 10 different rat mutant positive controls were thus generated and served to control the specificity of the hybridization and washing conditions.

Oligonucleotide Probe Hybridization

Twenty microliters of the PCR mixture were denatured by heating (95 C, 2 min) in 100 μl 0.4 *N* NaOH, 25 mM EDTA, and then cooled rapidly to 4 C. One hundred microliters of 2 M Tris HCl (pH 7.4) were then added and the mixture applied to nylon transfer membrane filters (Micron Separations Inc., Westboro, MA) under vacuum using a slot blot apparatus. DNA was fixed by UV light illumination. Oligonucleotides specific for the wild type and possible point mutants of codons 12, 13, and 61 of H-ras, K-ras and N-ras was purchased from Clontech Laboratories, or the UCLA Molecular Biology Institute (Table 2). Oligonucleotide probes were prepared by end-labeling with γ-^{32}P ATP (6000 Ci/mmol, Dupont, Claremont, CA) (25). Briefly, 200 ng oligonucleotide were incubated with 10 μl γ-^{32}P ATP and 2 μl T4 polynucleotide kinase (BRL, Bethesda, MD) in 50 mM Tris HCl (pH 7.5), 10 mM $MgCl_2$, 5 mM dithiothreitol, 0.1 mM spermidine, and 0.1 mM EDTA at 37 C for 60 min. After incubation, the probes were isolated with Sep-Pak 18 columns (Water Co., Milford, MA). Membranes were hybridized with 5 × 10^6 cpm/ml end-labeled probe in 10 ml hybridization mix: 5× SSPE, 5× Denhardt's, 0.5% SDS for 12 h at 50 C. The membranes were washed twice with 250 ml 6 × SSC, 0.1% SDS at Td-2 C (26) for 30 min, and were then autoradiographed at −70 C for 6–18 h.

Sequencing

PCR amplified DNA from the multinodular goiter tumor sample was separated on an agarose gel, transferred to a solid support and hybridized with an H-ras probe to confirm the appropriateness of the reaction. The PCR amplified product was restricted with *Eco*RI and ligated into a plasmid vector (pBluescript II KS$^-$, Stratagene, La Jolla, CA), which was used to transform a suitable bacterial host (XL-1 Blue, Stratagene). Minipreps were prepared by alkaline lysis and the DNA was further purified for sequencing with polyethylene glycol (27).

Double stranded template was prepared for sequencing by the alkaline denaturation technique (28). Sequencing was performed by the method of dideoxy chain termination (29) with an oligonucleotide primer synthesized specifically for the flanking region of the polylinker of the vector and a modified T7 polymerase (Sequenase 2.0, U.S. Biochemicals, Cleveland, OH). Dual aliquots of template were simultaneously sequenced with dGTP and the analog 7-deaza-dGTP (Boehringer Mannheim, Indianapolis, IN) and ^{35}S dATP label (Amersham, Arlington Heights, IL). The dual sequences were electrophoresed on a 6% polyacrylamide, 7 M urea gel. After removing the urea, the gel was dried and autoradiographed on blue film (RP, Kodak, Rochester, NY). The sequence was manually read.

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