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(54) Title: DETECTION OF POINT MUTATIONS IN GENES ENCODING GTP BINDING PROTEINS

(57) Abstract

The present invention provides a method for detecting whether a point mutation is present in a nucleic acid encoding a GTP bindingprotein or protein subunit. Methods are also provided for characterizing point mutations, if present. In a preferred embodiment, the method involves amplification of a nucleic acid segment followed by sequence-specific probe hybridization. The method is preferred for nucleic acids which encode a G-protein α subunit or p21 *ras* protein.

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DETECTION OF POINT MUTATIONS IN GENES ENCODING GTP BINDING PROTEINS

This application is a continuation-in-part of copending Serial No. 477,260, filed Feburary 7, 1990, which is incorporated herein by reference.

The present invention relates to the identification of point mutations within nucleic acids encoding GTP binding proteins in human samples. Point mutations within GTP binding proteins are associated with malignancies. The invention provides specific primers and probes for the detection and classification of these point mutations and potential oncogenes. The identification of oncogenes is important in the study of cell growth and carcinogenesis. The invention provides methods which relate specific point mutations to specific tumor types. In a preferred embodiment point mutations are described within nucleic acids encoding G-proteins.

G-proteins function as intermediates in transmembrane signalling pathways (Gilman , 1987, Ann. Rev. Biochem. 56:615). These pathways consist of receptors, G-proteins and effector molecules and are regulated by the cyclic association of GTP and GDP with G-proteins. Each G-protein consists of three subunits: α, β, and γ. The specificity of the interaction with the effector molecule is dictated by the α subunit.

Several G-proteins have been purified and characterized; Gs and Gi are involved in stimulation and inhibition, respectively, of adenylate cyclase activity. Three Giα subunits Giα1, Giα2, and Giα3 have been identified and cloned (Itoh et al., 1988, J. Biol. Chem. 263:6656-6664). Gt activates cGMP phosphodiesterase in response to photosignal transduction (Mattera et al., 1986, FEBS Lett. 206:36-42, and Didsbury et al., 1987, FEBS Lett 211:160-164). Other G-proteins have been sequenced including Go and Gz (Jones and Reed, 1987, J. Biol. Chem. 262:14241-14249, and Strathmann et al., 1989, Proc. Natl. Acad. Sci. USA: 86:7407-7409). Additionally, Gk (Yatani et al., 1987, Science 235:207) and Golf (Jones and Reed, 1989, Science 244:790) have been identified as G-proteins.

Gs activity raises the level of cAMP in cells by stimulating adenylyl cyclase. In pituitary somatotrophs, cAMP stimulates secretion of human growth hormone and causes cellular proliferation. Recently, a subset of human pituitary tumors were described having elevated levels of growth hormone and cAMP (Vallar et al., 1987, Nature 330:566-568). Landis et al., 1989, Nature 340:692-696, proposed that the abnormal cell proliferation observed by Vallar et al. was the result of a defect in the G-protein responsible for controlling cAMP levels, resulting in an accumulation of cAMP. Landis et al. identified patients harboring tumors secreting excessive amounts of growth hormone and determined that four tumors had constutively elevated levels of Gs activity. RNA was purified from fresh tissue, reverse transcribed, and cloned. The

entire Gsα coding region of the cDNA was sequenced and point mutations within codons 201 and 227 were identified. These mutations are Gsp mutations. Gsp mutations are a class of mutations that activate Gs, which normally mediates stimulation by thyrotropin (TSH) of thyrocyte proliferation and production of thyroid hormones. (Lyons et al., 1990, Science, 249:655-659)

Arginine 201 is a major site of ADP-ribosylation of Gsα by cholera toxin. This modification allows constitutive adenylyl cyclase activation (Lo and Hughes, 1987, FEBS Lett 224:1-3). Glutamine 227 is predicted to be a Gsα equivalent of glutamine 61 in ras p21 proteins (Landis et al. supra.). Mutational replacement of Gln-61 in ras p21 produces a protein that promotes malignant transformation (Der et al., 1986, Cell 44:167-176).

The ras genes encode highly related proteins approximately 21,000 daltons in molecular weight (p21s). While the exact functions of these proteins in cellular signalling pathways remains elusive, the p21s have GTPase enzymatic activities and interact with a GTPase activating protein (GAP) (Bishop, 1983, Ann. Rev. Biochem. 52:301-354, and McCormick, 1989, Cell 56:5-8).

The human ras gene family, which includes the closely related Ha-, Ki-, and N-ras genes, is one of the potential targets for mutational changes that have been implicated in the development of many human malignancies (Bos, 1988, Mutation Research 195:255-271). These alterations are either point mutations in codon 12, 13, or 61, or alternatively a 5- to 50- fold amplification of the wild-type gene. These changes convert the ras proto-oncogenes into oncogenes.

Polymerase chain reaction (PCR) methods have been used to detect known point mutations in ras oncogenes in genomic DNA isolated from tumors (Verlaan-de Vries et al., 1986, Gene 50:313-320, and Almoguera et al., 1988, Cell 5:549-554). Farr et al., 1988, Proc. Natl. Acad. Sci. USA 85:1629-1633, have combined PCR with oligonucleotide dot blot methods to examine specific ras gene point mutations in DNA isolated from patients afflicted with acute myeloid leukemia (AML).

The present invention provides methods for screening nucleic acids encoding G-proteins. Methods for screening ras genes are also provided. The nucleic acids may be RNA or DNA. Primers and probes are provided which aid in the identification of potential oncogenes and characterization of point mutations within an oncogene or potential oncogene. The invention provides primers and probes which are particularly suitable for detection of point mutations in nucleic acids encoding Gz, Gs, Go, Ga, and Gi proteins in endocrine tumors.

The present invention provides a method for detecting whether a point mutation is present in a nucleic acid encoding a G-protein α subunit, in a sample, that comprises:

(a) hybridizing a G-protein α subunit probe to said sample and (b) determining whether hybridization has occurred.

In another embodiment, the method comprises a method for detecting point mutation, if present, in a nucleic acid encoding a G-protein α subunit in a sample, comprising:

(a) treating the sample with a G-protein α subunit primer pair, an agent for polymerization, and deoxynucleoside 5' triphosphates under conditions such that an extension product of each primer can be synthesized, wherein said primers are sufficiently complementary to separate strands of a nucleic acid encoding a segment of a G-protein α subunit to hybridize thereto so that the extension product synthesized from one member of said pair, when separated from its complementary strand, can serve as a template for synthesis of the extension product of the other member of said pair;

(b) separating the primer extension products from the templates on which the extension products were synthesized to form single-stranded molecules;

(c) treating the single-stranded molecules generated in step (b) with the primers of step (a) under conditions such that a primer extension product is synthesized using each of the single-stranded molecules produced in step (b) as a template;

(d) repeating steps (b) and (c) at least once to provide amplified DNA;

(e) hybridizing a G-protein α subunit probe to said amplified DNA, wherein said probe contains a nucleic acid sequence that will hybridize to a sequence, selected from a wild type and mutant nucleic acid sequence, within said amplified DNA; and

(f) determining if hybridization has occurred.

The present invention provides novel primers and probes useful for detecting potentially oncogenic point mutations within a nucleic acid encoding a G-protein α subunit.

The present invention also provides kits for amplifying and detecting point mutations, if present, within a nucleic acid encoding a G-protein α subunit.

Figure 1 shows the identification of point mutations in Gsα and Giα2 genes. A region containing the indicated codons of the Gsα or Giα2 gene was amplified by PCR from genomic DNA isolated from either fresh frozen tissue or paraffin-embedded tissue. Point mutations were detected with high-stringency hybridization of sequence-specific oligonucleotides to the amplified product. Each panel represents hybridization with a different oligonucleotide.

Analysis of Gsα genes is shown in Figure 1A and described in Example 2. This analysis identified mutations in Arg 201 and Gln 227 codons of Gsα in 18 biochemically-characterized human growth hormone secreting pituitary tumors.

Analysis of Giα2 genes is shown in Figure 1B and described in Example 3. This analysis identified mutations in codon 179 of Giα2 in three human adrenocortical tumors and one ovarian granulosa cell tumor.

Figure 2 provides the GVA (gel visualization assay) analysis of ras RNA/PCR products from a normal human spleen and the K562 cell line as described in Example 5.

Figure 3 shows the results of a southern blot analysis of ras RNA/PCR products using ASO probes specific for activating point mutations in characterized cell lines. The experiment is described in Example 6.

Figure 4 provides the GVA analysis of ras RNA/PCR from alcohol-fixed, paraffin-embedded samples as described in Example 7.

Figure 5 provides the GVA analysis of ras RNA/PCR from stained and unstained microscope slides of human bone marrow as described in Example 8.

Figure 6 is schematic diagram of ras format II filters showing the positions of wild type and mutant ras oligonucleotide probes as described in Example 9.

The present invention provides a method for detecting and characterizing point mutations, if present, in a nucleic acid encoding a G-protein α subunit. The point mutations detected by these methods are believed to be involved in oncogenesis. The nucleic acid is a G-protein subunit gene, RNA transcription product, cDNA product, or a subsequence thereof. The method involves amplifying by a polymerase chain reaction, a segment of nucleic acid encoding at least one G-protein amino acid of interest. For each nucleic acid segment suspected of comprising a potentially oncogenic point mutation, a pair of oligonucleotide primers are provided for amplification in a polymerase chain reaction. The primers will amplify wild type or mutant nucleic acid segments. Genes that encode G-proteins are proto-oncogenes in the normal somatotrophic state and are referred to as wild type. If a point mutation is present, the gene is a putative oncogene. Thus, the present methods, primers, and probes allow one skilled in the art to distinguish between these two types of G-protein genes.

In the embodiment of the invention illustrated below, point mutations are detected by oligonucleotide probes. To detect an oncogenic point mutation in a nucleic acid encoding a G-protein, the probes contain either the wild type nucleic acid sequence or single nucleotide changes within codons which correspond to amino acids 49, 201, and 227 in the sequence of Gsα. The amino acid at 226 may be of interest as well. These single nucleotide changes affect the translation product of the gene. Of course, in some samples, a mutated oncogene may not be present. In such a sample only the wild type probe will hybridize to the sample. Thus, where a point mutation is not

detected, the wild type probe serves to verify the presence of the amplified product in the sample.

G-protein α subunits share a high degree of homology. Matsuoka et al., 1988, Proc. Natl. Acad. Sci. USA 85:5384-5388, provide a comparison of the amino acid sequences of several Gα subunits. Although the Gα subunits vary in length, the degree of homology between the published amino acid sequences is sufficiently high such that sequence alignment is possible. In this way, the amino acid corresponding to Gsα Gly 49, Gsα Arg 201, Gsα Gly 226, and Gsα Gln 227 can be determined for all Gα subunits. For example, in Giα2, Arg 179, and Gln 205 correspond to Arg 201 and Gln 227 in Gsα.

The present invention also provides probes which detect point mutations in nucleic acid encoding ras proteins. Such probes contain single base changes as well as the wild type sequences for codons encoding amino acids at p21 positions 12, 13, and 61.

The examples below describe methods to detect the presence of a point mutation within a nucleic acid sequence contained in a sample. For example, the sequence to be examined can be assayed by hybridization using a wild type probe and a pool of probes, each probe containing a point mutation at a specific position. Alternatively, the methods can be used to detect and classify a point mutation. In this case, the probes, one comprising the wild type sequence and the others comprising point mutations which affect the translation product at a specific position, are used individually. According to the method, only the probe comprising the exact nucleic acid sequence contained within the sample nucleic acid will hybridize to the nucleic acid in the sample.

Those skilled in the art will recognize that the disclosed methods and probes enable new oncogenes to be detected and classified. These methods led to the discovery that, for example, the gene encoding Giα2 is a proto-oncogene. The present invention has led to the discovery of new point mutations that may give rise to cancer. In addition, the invention provides primers and probes that can be used to identify oncogenic point mutations in genes encoding Giα1, Giα3, Goα, and Gzα if such mutations exist. Similarly, these methods may be used to design primers and probes to identify new oncogenic point mutations in other G-protein α subunits such as Gtα, Gkα, and Golfα. These point mutations may well be related to oncogenesis and their identification lays a critical foundation for experimental research in oncogenesis. Regardless of oncogenicity, these point mutations are useful to discriminate between individuals. Methods which discriminate between individuals based on nucleic acid sequence differences are used in, for example, forensic medicine.

The disclosed examples of the invention, relating to G-proteins, provide methods, primers, and probes to detect and classify point mutations in nucleic acids encoding Gsα and Gi2α. These methods are also suitable for detecting point mutations, if present, and classifying those mutations in any nucleic acid encoding a G-protein α subunit. Tables 1-4 provide primers and probes suitable for detecting and classifying point mutations in nucleic acids encoding Gsα, Giα1, Giα2, Giα3, Goα, and Gzα. These methods are directly applicable to the detection of point mutations in other Gα subunits, for example, Gtα, Gk, or Golf. Table 4 provides probes which correspond to codons 49, 201, and 227 in the Gsα subunit.

In a preferred method for detecting and classifying point mutations within oncogenes or proto-oncogenes, the nucleic acid containing the region of interest is amplified by a polymerase chain reaction prior to detection. Primers useful in these methods are suitable for amplification of proto-oncogenes as well as activated proto-oncogenes. Those skilled in the art will recognize that with the disclosed methods, primers, and probes, new oncogenes can be readily detected. For example, the primers and probes of the invention led to the discovery of novel point mutations and, thus, a novel putative oncogene, Giα2. Novel oncogenic genes encoding Giα1, Giα3, Goα, and Gzα may also be discovered by the methods and compositions provided. Primers of use in the present invention hybridize to genomic DNA at sites such that, in a PCR reaction, the primers amplify a specific region of the G-protein DNA. The specific region amplified comprises at least one codon which corresponds to Gly 49, Arg 201, or Gln 227 in Gsα.

In one embodiment of the present invention, primers are selected such that the resultant amplified fragment comprises both codons 201 and 227 (or correspondingly 179 and 205 in Giα2). However, this is not an essential aspect of the invention. If, for example, a large intron or more than one intron exists between these codons in the genomic DNA, or if the DNA is degraded as in some paraffin embedded tissue, amplification primers are designed for the region comprising codon 201 and separately for the region comprising codon 227. Such amplification reactions are run separately or simultaneously in one reaction vessel.

Amplification requires the use of primer pairs that will amplify a discrete region of DNA present in a sample. These primer pairs are oligonucleotides. The PCR products generated from these primers are then analyzed by hybridization with sequence specific probes. Sequence specific probes may also be allele-specific probes. An allele-specific probe (ASO) will hybridize to an allele-specific sequence in a nucleic acid within a sample. An allele-specific sequence is a component of an individual's genotype. A sequence-specific probe comprises a specific sequence which may or may

not exist as an allele. Thus, until a sequence is identified in at least one individual, probes which are sequence-specific are not necessarily allele-specific. However, as the term allele-specific probe is used by those of skill in the art, these terms are used interchangeably herein.

Amplification of DNA by PCR is disclosed in U.S. Patent Nos. 4,683,195 and 4,683,202 (both of which are incorporated herein by reference). Methods for amplifying and detecting nucleic acids by PCR using a thermostable enzyme are disclosed in U.S. Patent No. 4,965,188, which is incorporated herein by reference. PCR amplification of DNA involves repeated cycles of heat-denaturing the DNA, annealing two oligonucleotide primers to sequences that flank the DNA segment to be amplified, and extending the annealed primers with DNA polymerase. The primers hybridize to opposite strands of the target sequence and are oriented so that DNA synthesis by the polymerase proceeds across the region between the primers, effectively doubling the amount of the DNA segment. Moreover, because the extension products are also complementary to and capable of binding primers, each successive cycle essentially doubles the amount of DNA synthesized in the previous cycle. This results in the exponential accumulation of the specific target fragment, at a rate of approximately 2n per cycle, where n is the number of cycles.

In the disclosed embodiment, Taq DNA polymerase is preferred although this is not an essential aspect of the invention. Taq polymerase, a thermostable polymerase, is active at high temperatures. Methods for the preparation of Taq are disclosed in U.S. Patent No. 4,889,818 and incorporated herein by reference.

The choice of primers for use in PCR determines the specificity of the amplification reaction. In the present invention, primers are used that will amplify G-protein or ras p21 sequences present in a sample. The primers of the invention can include degenerate primers. These are mixtures of oligonucleotides synthesized to have any one of several nucleotides incorporated at a selected position during synthesis. For example, the primers may be sufficiently complementary to all types of ras genes to amplify a DNA sequence of any ras DNA present in the sample. Illustrative primers of this type are referred to, for example, as "pan" ras primers. The primers are designed to amplify a region of DNA, cDNA, or RNA that contains sequences specific to any ras p21 gene: c-N-ras, c-Ha-ras, or c-Ki-ras. Because the "pan" ras primers span large intron sequences, cDNA and RNA templates are preferred. The amplified DNA can therefore be used to classify the ras gene present in the sample.

Alternatively, it may be desirable to use primer pairs which are specific for each gene to be detected. Such a primer pair will amplify a specific DNA or RNA segment encoding, for example, c-Ki-ras, c-N-ras, c-Ha-ras, Giα1, Giα2, Giα3, Gsα, Goα,

or Gzα. In one embodiment, three separate pairs of ras primers are included in one PCR reaction such that each pair will specifically amplify either c-N-ras, c-Ki-ras, or c-Ha-ras. The primers are designed so that each PCR product has a discrete size. Thus, three primer pairs are used to simultaneously amplify several DNA or RNA segments. The identity of the segment(s) amplified can then be determined by, for example, gel electrophoresis and size determination. The presence of point mutations and the classification of such mutations can be subsequently determined by the methods provided.

When more than one nucleic acid segment is characterized, it is not essential that primers are designed such that the resulting amplification products are of different sizes. Amplification reactions using different primer pairs can be run independently of one another and analyzed simultaneously, for example, using individual lanes on an acrylamide gel. Alternatively, several primer pairs can be used simultaneously in one reaction, and the amplification products divided and analyzed to characterize the sample by, for example, separate probe hybridizations.

In another embodiment of the present invention, nested primers are used (Mullis et al., 1986, Cold Spring Harbor Symposium on Quantitative Biology 51:263, incorporated herein by reference). This method may be preferred when the amount of nucleic acid in a sample is extremely limited, for example, where archival, paraffin embedded samples are used. When nested primers are used, the nucleic acid is first amplified with an outer set of primers. This amplification reaction is followed by a second round of amplification cycles using an inner set of primers.

Once a sample has been treated with a primer pair under conditions suitable for PCR, the method of the invention requires in a preferred embodiment that the amplified product is characterized. It may be preferred, but is not essential in the practice of the invention, to determine whether amplification has occurred. The use of an internal amplification control to assure the competency of a sample for PCR is within the scope of the invention and reduces the likelihood of false negative results. There are a variety of ways to determine whether amplification has occurred. A portion of the reaction mixture can be subjected to gel electrophoresis and the resulting gel stained with ethidium bromide and exposed to ultraviolet light to observe whether a product of the expected size is present. Labeled primers or deoxyribonucleotide 5'-triphosphates can be added to the PCR reaction mixture, and incorporation of the label into the amplified DNA measured to determine if amplification occurred. Another method for determining if amplification has occurred is to test a portion of PCR reaction mixture for ability to hybridize to a labeled oligonucleotide probe or mixture of probes designed to hybridize to only the amplified DNA. Alternatively, the determination of amplification and

characterization of a point mutation can be carried out in one step by testing a portion of the PCR reaction mixture for its ability to hybridize to one or more specific probes.

Due to the enormous amplification possible with the PCR process, small levels of DNA carryover from samples with high DNA levels, positive control templates or from previous amplifications can result in PCR product, even in the absence of purposefully added template DNA. If possible, all reaction mixes are set up in an area separate from PCR product analysis and sample preparation. The use of dedicated or disposable vessels, solutions, and pipettes (preferably positive displacement pipettes) for RNA/DNA preparation, reaction mixing, and sample analysis will minimize cross contamination. See also Higuchi and Kwok, 1989, Nature, 339:237-238 and Kwok, and Orrego, in: Innis et al. eds., 1990 PCR Protocols: A Guide to Methods and Applications, Academic Press, Inc., San Diego, CA, which are incorporated herein by reference.

One particular method for minimizing the effects of cross contamination of nucleic acid amplification is described in U.S. Serial No. 609,157, filed November 2, 1990, which is incorporated herein by reference. The method involves the introduction of unconventional nucleotide bases, such as dUTP, into the amplified product and exposing carryover product to enzymatic and/or physical-chemical treatment to render the product DNA incapable of serving as a template for subsequent amplifications. For example, uracil-DNA glycosylase will remove uracil residues from PCR product containing that base. The enzyme treatment results in degradation of the contaminating carryover PCR product and serves to "sterilize" the amplification reaction.

The present invention has led and will continue to lead to the discovery of many previously unknown or uncharacterized oncogenes. For example, using the methods, primers, and probes of the invention, four clinical samples examined were discovered to contain two different Giα2 oncogenes. These new oncogenes are an important aspect of the present invention, as are the point mutations that distinguish these from wild type, and the probes that hybridize to these gene sequences in specific fashion.

In one embodiment, the present invention provides a number of probes for use in detecting and characterizing potential oncogenes. These probes are set forth in Table 2, below. Those skilled in the art will recognize that although the specific primers and probes of the invention exemplified herein have a defined number of nucleotide residues, one or more nucleotide residues may be added or deleted from a given primer or probe typically without great impact on the suitability of that primer or probe in the present methods. The essential aspect of these probes is their ability to discriminate between wild type and mutant sequences. When a portion of the PCR

reaction mixture contains DNA that hybridizes to a probe, the sample contains DNA comprising the wild type or mutant sequence according to the specific sequence of the probe.

An important aspect of the present invention relates to detecting the novel probes provided for use in the present methods. There are a number of ways to determine whether a probe has hybridized to a DNA sequence contained in a sample. Typically, the probe is labeled in a detectable manner, the target DNA (i.e., the amplified DNA in the PCR reaction buffer) is bound to a solid support, and determination of whether hybridization has occurred simply involves determining whether the label is present on the solid support. This procedure can be varied, however, and works just as well when the target is labeled and the probe is bound to the solid support.

The hybridization probes disclosed herein are sequence-specific oligonucleotide probes for each G-protein α subunit codon to be characterized. As described above, sequence-specific probes are similar to allele-specific probes in use and utility. Methods for utilizing ASOs are described in Saiki et al., 1986, Nature 324:163-166, incorporated herein by reference. The probes may be used individually for detecting, for example, a wild type sequence. Alternatively, the probes may be used in a panel format for characterizing a tumor genotype. The tumor genotype may be compared to, for example, somatic tissue or other tumor types.

The probes can be used in a variety of different hybridization formats. Although solution hybridization of a nucleic acid probe to a complementary target sequence is clearly within the scope of the present invention, commercialization of the invention will likely result in the use of immobilized probes and thus a quasi "solid-phase" hybridization. In this format, the probe is covalently attached to a solid support and target sequences are hybridized with the probe. A preferred method for immobilizing probes on solid supports is disclosed in U.S. patent application S.N. 347,495, filed May 4, 1989, incorporated herein by reference. According to this method, sequence-specific probes are attached to a solid support by virtue of long stretches of T residues which are added during probe synthesis on an automated synthesizer after the hybridizing sequence is synthesized.

For example, in a fixed probe format, the following probes are useful for detecting point mutations in a gene encoding Gsα at amino acid positions 201 and 227.

Table 1

Probe Name	Sequence	Amino Acid Position
JFL316	5'TCGCTGCCGTGTCCTGGAC	201
JFL317	5'TCGCTGCAGTGTCCTGGACT	201
JFL318	5'TCGCTGCGGTGTCCTGGAC	201
JFL319	5'TCGCTGCTGTGTCCTGGACT	201
JFL320	5'TCGCTGCCATGTCCTGGACT	201
JFL321	5'TCGCTGCCTTGTCTTGGACT	201
JFL322	5'TGGGTGGCCAGCGGCGATGA	227
JFL323	5'TGGGTGGCCTGCGCGATGA	227
JFL324	5'TGGGTGGCCCGCGCGATG	227
JFL325	5'TGGGTGGCCGGCGCGATG	227
JFL326	5'TGGGTGGCGAGCGCGATGA	227
JFL327	5'TGGGTGGCTAGCGCGATGA	227
JFL328	5'TGGGTGGCCATCGCGATGA	227
JFL329	5'TGGGTGGCAAGCGCGATGA	227
JLF330	5'TGGGTGGCCACCGCGATG	227

A fixed probe format is suitable for detecting other gsp mutations, as well, and is demostrated in Example 5 for detecting point mutations in ras gene PCR products.

Many methods for labeling nucleic acids, whether probe or target, are known in the art and are suitable for purposes of the present invention. In one embodiment illustrated below the probes were labeled with radioactive phosphorous ^{32}P, by treating the probes with polynucleotide kinase in the presence of radiolabelled ATP. However, other non-radioactive labeling systems may be preferred, i.e., horseradish peroxidase-avidin-biotin systems. Horse-radish peroxidase (HRP) can be detected by its ability to covert diaminobenzidine to a blue pigment. A preferred method for HRP-based detection uses tetramethyl-benzidine (TMB) as described in Clin. Chem. 33:1368 (1987). An alternative detection system is the enhanced chemiluminescent (ECL) detection kit commercially available from Amersham. The kit is used in accordance with manufacturer's instructions. A variety of alternative dyes and chromogens and corresponding labels are available for nucleic acid detection systems (see, e.g., U.S. patent application S.N. 136,166, filed December 18, 1987).

Another non-radioactive alternative detection method uses terminal transferase (Tdt) and biotinylated dUTP to add homopolymer tails to the oligonucleotide probes. Biotin serves as the detectable moiety. Following probe hybridization, the filters are

washed as usual. Hybridized biotin is detected with strep-avidin conjugated HRP (Se-eqence® available from Cetus) according to manufacturer's instructions. The ECL system is then used to visualize the biotin-HRP product.

Probes are typically labeled with radioactive phosphorous ^{32}P, by treating the probes with polynucleotide kinase in the presence of radiolabeled ATP. However, for commercial purposes non-radioactive labeling systems may be preferred, such as, horseradish peroxidase-avidin-biotin or alkaline phosphatase detection systems. HRP can be used in a number of ways. For example, if the primer or one or more of the dNTPs utilized in a PCR amplification is labeled (for instance, the biotinylated dUTP derivatives described by Lo et al., 1988, Nuc. Acids Res. 16:8719) instead of the probe, then hybridization can be detected by assay for the presence of labeled PCR product. In a preferred embodiment, probes are biotinylated and detected with the ECL system described above. For example, biotinylated probes are prepared by direct biotinylation of the oligonucleotide rather than incorporation of biotin-dUTP during PCR. For 5' biotinylation of oligonucleotides direct solid phrase synthesis using biotin containing phosphoramidites is done according to Alves et al., 1989, Tetra. Let 30:3098; Cocuzza, 1989, Tetra Let. 30:6287; and Barabino et al., 1989, EMBO J. 8:4171. Solid phase synthesis of biotinylated oligonucleotides at any internal or terminal (5' or 3') position is also suitable for preparing biotinylated primers and probes (Pieles et al., 1989, NAR 18:4355, and Misiura et al., 1989, NAR 18:4345). Alternatively, probes and primers are conjugated to HRP, for example, by the method disclosed in WO89/2932, and Beaucage et al., 1981, Tetra. Lett. 22:1859-1862. These references are incorporated herein by reference.

Those skilled in the art will recognize that with the above description, primers, and probes for amplifying, detecting, and characterizing new G-protein point mutations can be readily obtained. It will also be readily apparent to those skilled in the art that the specific primers and probes provided in the examples are merely illustrative of the invention. Primers and probes of the invention can also be prepared to amplify and detect sequence variations within areas G-protein sequences other than those specifically exemplified herein, for example, codons corresponding to Gsα 49.

The method of the present invention is applicable for detecting a point mutation within a gene encoding a GTP binding protein or the expression product of that gene. Thus, the method can detect specific point mutations in a sample containing RNA, or DNA, or both. If the sample contains RNA, the nucleic acid will be reverse transcribed, providing a double-stranded DNA template prior to amplification. Procedures for reverse transcribing RNA are known (see Maniatis et al., 1982,

Molecular Cloning: A Laboratory Manual, Cold Spring Harbor, NY). In one embodiment, the methods are used to detect point mutations in RNA.

Alternatively, if RNA is abundant, the probes (or the complement of a probe sequence) are suitable for direct detection and characterization of an oncogene or proto-oncogene mRNA suspected of being present in the sample or in the reverse transcribed cDNA product. Similarly, if DNA is abundant, for example, as in a fresh tissue sample, the probes are useful for direct detection of gene sequences. Thus, amplification by PCR is not an essential component of the present invention.

Samples suitable for analysis by the methods described may be fresh or archival. Fresh samples may be, for example, biopsied tumor, tissue samples, or blood. Archival samples may be, for example, frozen or paraffin embedded. In one embodiment of the invention, paraffin-embedded samples are analyzed using G-protein primers and probes. In another embodiment, methods are provided for ras oncogene detection in RNA purified from surgical biopsy samples or cultured cells. Methods are also provided for extracting RNA from air-dried bone marrow sides and alcohol-fixed paraffin embedded tissues. For paraffin embedded tissues containing intact DNA Table 2 provides primers and conditions for amplifying potential oncogenic sites in Gi_3, Gi_2, $Gs\alpha$, Gi_1, Gz, and Go.

Table 2

Primers and PCR Conditions for G-Protein Analysis

Gene Product	Amino Acid Position	Primer Pair	Cycling Parameters 1	2	3	Number of Cycles	Product Size (bp)
$Gi_3\alpha$	201 (178)	JFL 110, sense GAT CTG GAT AGA ATA TCC CAG JFL 112, antisense GGT GAA ATG TGT TTC TAC AAT	95°C 30 sec	55°C 30 sec	72°C 30 sec	40	99
$Gi_3\alpha$	227 (204)	JFL 113, sense TTC CCC TTG CGC AGG ATG TTT JFL 115, antisense CAG AAC AAG GTC ATA ATC ACT	95°C 30 sec	60°C 30 sec	72°C 30 sec	40	120
$Gi_2\alpha$ nested primer set	201 + 227 (179 + 205) outers	JL 54, sense CCC CCC ATC CCC AGC TAC CT JL 57 outer antisense TCT CAC CAT CTC CTC GTC CTC	95°C 60 sec	50°C 120 sec	72°C 120 sec	30	~1,000
	201 + 227 (179 + 205) inners	JL 55, sense ATT GCA CAG AGT GAC TAC ATC CCC JL 56, antisense GGC GCT CAA GGC TAC GCA GAA	95°C 60 sec	56°C 60 sec	72°C 60 sec	30	504
$Gs\alpha$	49	JFL 226, sense AAC AGC AGA CCT CCC TGC CCA AAG JFL 227, antisense CCC CCC TGC ACA GAT TTG ACA CTT	95°C 60 sec	60°C 30 sec	72°C 30 sec	50	171
$Gs\alpha$ nested primer set	201 + 227 outers	JFL 69, sense GCG CTG TGA ACA CCC CAC GTG TCT JFL 70, antisense CGC AGG GGG TGG GCG GTC ACT CCA	95°C 60 sec	50°C 120 sec	72°C 120 sec	30	1,200

Table 2 - Continued

Primers and PCR Conditions for G-Protein Analysis

Gene Product	Amino Acid Position	Primer Pair	Cycling Parameters 1	2	3	Number of Cycles	Product Size (bp)
Gsα nested primer set	201 + 227 inners	JFL 135, sense GTG ATC AAG CAG GCT GAC TAT GTG JFL 136 antisense GCT GCT GGC CAC CAC GAA GAT GAT	95°C 30 sec	57°C 40 sec	72°C 45 sec	30	526
$Gi_1\alpha$	201 (173)	JFL 223, sense TTG GAC AGA ATA GCT CAA CCA AAT SP 33 antisense GAA AGT AAA ATG GGT TTC AAC	95°C	53°C	72°C	40	99
$Gi_1\alpha$	227 (199)	JFL 224, antisense TAG AAC CAG GTC GTA GTC ACT SP 34, sense AAA TGT TTG ATG TGG GAG GT	95°C	53°C	72°C	40	114
$Gi_2\alpha$	49 (42)	JFL 235, sense GCC CTC TGT TCC AGG TGC JFL 237, antisense ACT TAC TTC ATC TGC TTG ACG	95°C 30 sec	55°C 30 sec	72°C 30 sec	45	63
$Gi_2\alpha$	201 (179)	JFL 55, sense ATT GCA CAG AGT GAC TAC ATC CCC JFL 212, antisense GCT CAC TTG AAG TGT AGG	95°C 60 sec	57°C 30 sec	72°C 30 sec	50	118
$Gi_2\alpha$	227 (205)	JFL 215, sense CTG CAG GAT GTT TGA TGT GG JFL 56, antisense GGC GCT CAA GGC TAC GCA GAA	95°C 60 sec	57°C 30 sec	72°C 30 sec	50	~123

Table 2 - Continued

Primers and PCR Conditions for G-Protein Analysis

Gene Product	Amino Acid Position	Primer Pair	Cycling Parameters 1	2	3	Number of Cycles	Product Size (bp)
Goα	201	SP 9, sense CTG GAC AGC CTG GAT CGG ATT GGG SP 10, antisense GAG GTT CTT GAA TGT GAA GTG GGT	95°C 60 sec	50°C 60 sec	72°C 60 sec	35	87
Goα	227	JFL 139, sense AAC CTC CAC TTC AGG CTG TTT SP11, antisense GTG GAG CAC CTG GTC ATA GCC GCT	95°C 60 sec	48°C 90 sec	72°C 60 sec	50	129
Gzα	201 + 227	SP 15, sense AAC GAC CTG GAG CGC ATC GCC JFL 201, antisense TGT GAC GCC CTC GAA GCA GT	95°C 60 sec	55°C 60 sec	72°C 60 sec	40	~200
Gsα	201	JFL 135, sense GTG ATC AAG CAG GCT GAC TAT GTG JFL 286, antisense TA ACA GTT GGC TTA CTG GAA	95°C 30 sec	55°C 30 sec	72°C 30 sec	40	222
Gsα	227	JFL 229, sense CCC CAG TCC CTC TGG AAT AAC CAG JFL 136, antisense GCT GCT GGC CAC CAC GAA GAT GAT	95°C 30 sec	55°C 30 sec	72°C 30 sec	50	165

* Numbers in parentheses indicate the exact amino acid position within the named G protein subunit. Gi_3 amino acid position 178 corresponds to position 201 in Gsα, and position 204 corresponds to position 227 in Gsα. For $Gi_1\alpha$ the amino acid positions 173 and 199 correspond to positions 201 and 227, respectively in Gsα. For Gi2α amino acid positions 42, 179, and 205 correspond to Gsα 49, 201, and 227, respectively.

In some instances DNA samples are less well preserved and, consequently, amplification of long segments is problematic. Example 1 describes primers for amplifying short segments of DNA that include oncogenic regions of gsp.

Thus, the present invention provides methods for detecting activated oncogenes at the genomic (DNA) level. The methods are also suitable for monitoring the expression of proto-oncogenes and oncogenes. The level of oncogene expression can be monitored during and following, for example, chemotherapeutic treatment. Remission and recurrence of tumors can be monitored as well. Comparing the activation of proto-oncogenes into oncogenes at the genomic level, with the expression of activated oncogenes at the mRNA level, provides valuable information for analysis of carcinogenesis and therapies. These methods may also provide information regarding a predipostion to specific malignancies.

The invention provides needed tools useful in analysis of carcinogenesis events. Probes are provided for determining allelic dominance for a specific oncogene. For example, probes specific for Giα2 alleles can be used to analyze somatic cells as well as tumor cells. These probes can also distinguish, in the case of a tumor comprising one mutant and one wild type allele, the relative abundance of the mRNA products of these alleles. One skilled in the art will recognize the utility of such analyses following study of and studying mutagenic events.

In another aspect, the primers and probes described provide a method for phenotyping a cell. The cell may be a tumor cell or a somatic cell. Analysis using the methods of the invention provides information relating to the proto-oncogene and oncogene profile of a cell. In this way, events related to the presence of, for example, more than one G-protein point mutation, may be discerned which were previously undetectable.

These methods provide a means for associating a specific malignancy with a specific oncogene or point mutation. For example, of 306 samples for 15 tumor types analyzed for Gsα, 18 point mutations were detected; all 18 Gsα oncogenes were detected in pituitary adenomas and thyroid tumors. In another example using Giα2 primers and probes, 254 samples representing 14 different tumor types were analyzed. Four tumors of two types had Giα2 oncogenes; one ovarian granulosa cell tumor and three adrenal cortical tumors. Analyses using the methods and probes provided has led to the discovery that the proto-oncogenes encoding G-protein α subunits are activated in endocrine tumors. These studies suggest that distribution of Gsα and Giα2 oncogenes is restricted among specific endocrine target cells.

An analysis of G-protein oncogenes and tumor specificity provides information of useful in determining the role of G-proteins, where that role is as yet undefined. For

example, the data suggests that corticotropin (ACTH), which stimulates cortisol secretion via Gs, adenylyl cyclase, and cAMP, does not utilize Gs and cAMP to stimulate proliferation of ACTH target cells. Tumors derived from adrenal cortical cells (the target cell of ACTH) do not harbor the Gsα oncogene. Those skilled in the art will recognize that the methods of the present invention provide essential tools for identifying new oncogenes, such as Giα2 (alternatively referred to herein as gip2) and exploring the diverse mix of signalling pathways that mediate regulation of proliferation in endocrine target cells.

The present invention also provides a number of previously unknown point mutations. These sequences encode corresponding mutant proteins and can be used to synthesize novel, mutant, proteins. Such proteins, or protein subunits or subsequences, can be used to generate antibodies useful in the detection of mutant G-proteins. These antibodies would provide important tools for screening, for example, biopsied tissue to detect mutant G-proteins, and thus provide important information regarding the genetic make-up of an individual or the carcinogenic state of the sampled tissue.

A mutant G-protein may be dominated in vivo by a normal G-protein unless carcinogenesis is triggered by other events. Thus, antibodies enabled by the present invention would also find use in, for example, screening transplantation tissue as an indicator of potential oncogenic complications.

It will be apparent to those skilled in the art that the method of the present invention is amenable to commercialization as a kit for the quantitation of one or more nucleic acids in a sample. For example, in its simplest embodiment, such a kit would provide an oligonucleotide primer pair for amplification of a G-protein α subunit segment and corresponding wild type oligonucleotide probe. In another embodiment, a kit may contain an array of G protein probes fixed onto a solid support and a corresponding primer pair for amplifying and detecting oncogenic point mutations in genes encoding G protein subunits. In another embodiment, a kit may contain an oligonucleotide primer pair, corresponding G-protein α subunit wild type and mutant probes, a DNA polymerase, a RNA polymerase, a reverse transcriptase, nucleotide triphosphates, restriction enzymes, and buffers for carrying out cDNA synthesis, restriction enzyme digests, and amplification by PCR. Further, the kits may contain a thermostable DNA polymerase; for example, the thermostable DNA polymerase Taq isolated from Thermus aquaticus as an agent of polymerization.

To facilitate the understanding of the invention, brief definitions are provided below.

"Proto-oncogene" refers to the wild type form of gene encoding a protein in which a point mutation affecting the amino acid sequence encodes a protein which may have a carcinogenic or tumorigenic effect.

"Oncogene" refers to a proto-oncogene containing a point mutation and encoding protein which may have a carcinogenic or tumorigenic effect. Oncogenes may alternatively be referred to herein as "activated proto-oncogenes."

"G-protein α subunit primers" refer to primer pairs which hybridize to complementary strands of a nucleic acid encoding a G-protein α subunit and will function in a PCR reaction to amplify a nucleic acid segment comprising one or more codons suspected of harboring a point mutation. In the disclosed embodiment, these codons encode amino acids corresponding to Gsα amino acids 49, 201, and 227. However, those skilled in the art will recognize that potentially oncogenic point mutations may exist at other positions as well. G-protein α subunit primers can readily be designed to amplify such regions according to the methods described herein.

"G-protein α subunit probe," "G-protein probe," or "probe" as used herein refers to an oligonucleotide probe designed to characterize the nucleic acid sequence encoding an amino acid at a position suspected of containing a point mutation. In the preferred embodiment of the present invention, individual probes comprise the wild type nucleic acid sequence, or a nucleic acid containing a point mutation, within a specific codon. Specific codons include any codon within a nucleic acid encoding a G-protein, which when replaced with a non-wild type sequence results in a G-protein oncogene. In the present examples, the specific codons include for each G-protein α subunit, codons encoding the amino acids corresponding to Goα amino acid positions 49, 102, and 227.

For any specific codon and any specific G-protein, one wild type probe and nine point mutation probes may be designed and used in the present methods. However, it is not an essential aspect of the invention that probes be included for practice of these methods. In fact, the use of only one probe may be sufficient to discriminate between two individuals or between the presence and absence of a point mutation.

The following examples provide an illustration of the present invention. They are not a limitation to the scope of the invention. Those skilled in the art will recognize that the primers and probes disclosed can be modified, for example, by altering the length of an oligonucleotide without altering the purpose and effectiveness of the described invention.

Example 1

Materials and Methods

A. Source of Tumors

Human pituitary tumors specimens were supplied by Charles Wilson (University of California, San Francisco) and Anna Spada (Milan, Italy) provided biochemically characterized pituitary samples. Additional samples were provided by Hans Feichtinger and Kurt Grünewald at the University of Innsbruch, Austria, and Claudia Landis, Griffith Harsh, Quan-Yang, Duh and Orlo Clark at the University of California, San Francisco.

B. Sample Preparation

For isolation of genomic DNA from paraffin-embedded tissue, 3-5 adjacent 5 mm sections were cut from paraffin blocks and mounted on glass slides (Wright et al. PCR Protocols: A Guide to Methods and Applications eds. M. Innis, D. Gelfand, J. Sninsky, and T. White, Academic Press, San Diego, pp. 153-158, incorporated herein by reference). One slide was stained with hematoxylin and eosin and used as a guide to select a region composed entirely of tumor on the other slides. With a razor blade, excess paraffin and unwanted tissue were removed from the unstained slides, and the remaining tumor tissue was scraped into a sterile 1.5 ml microcentrifuge tube. To remove contaminating paraffin, the sample was incubated with 5 mls of octane (anhydrous, Aldrich) or Hemo-De (Fischer) at room temperature for 30 minutes with shaking. The tissue sample was pelleted by centrifugation (5 minutes, 1000X g) and the supernatant was discarded. The tissue sample was extracted 2X with 500 mls absolute ethanol to remove traces of octane and then vacuum dried. To digest the tissue and release the genomic DNA, the sample was treated with 0.2 mg/ml proteinase K in 100 mls digestion buffer (50 mM Tris, pH 8.5; 1 mM EDTA; 0.5% Tween 20) at 37°C overnight. The sample was centrifuged to remove undigested debris and the DNA-containing supernatant was incubated at 95°C for 8 minutes to denature proteolytic enzymes and nucleases.

Fresh frozen samples were prepared according to Verlaan-de Vries et al., 1986, Gene 50:313.

C. Amplification Procedure

Nested amplification primers were used in the PCR amplification procedure to improve specificity and yield (Mullis et al., supra). Genomic DNA (100-500 ng DNA from fresh tissue or 10 mls of the DNA solution from paraffin-embedded tissue) was first amplified with 30 pmols of the outer primers (see Table 1) in 100 mls of 0.1 mM

dNTPs; 50 mM KCl; 20 mM Tris, pH 8.3; 2.5 mM MgCl2; 100 g/ml BSA; and 1.5 units Taq polymerase (Perkin-Elmer Cetus). A thermocycler (Perkin-Elmer Cetus) was used for amplification. The amplification program was: 5 minutes at 95°C followed by 30 cycles of 1 minute at 95°C, 2 minutes at 50°C, and 2 minutes at 72°C. A second amplification reaction with 30 pmols of the inner primers was done using 2 µls of the initial amplification mixture in the same dNTP and buffer conditions as above and 0.5 units Taq polymerase. The program for the 2nd amplification reaction was: 30 seconds at 94°C, 40 seconds at 57°C, and 45 seconds at 72°C. Five µls of the final product was sized on a 2% Nusieve, 1% Seakem agarose gel and visualized by ethidium bromide staining. A 526 base pair fragment was obtained for Gsα and a 504 bp fragment for Giα2.

D. Dot Blot Procedure

Nylon filters (Pall Biodyne-B, 0.45 mm) were briefly rinsed in water and mounted on a Bio-RAD dot-blot apparatus. Four mls of each final amplification product were denatured in 0.4 N Na OH and 25 mM EDTA for 5 minutes and spotted on the filter. The DNA was crosslinked to the filter using a Stratalinker (Stratagene) set at auto crosslink. The filters were prehybridized in 5X SSPE and 0.5% SDS at 50°C for 30 minutes. One ng of a ^{32}P end-labeled oligonucleotide was added to the prehybridization solution and incubated at 50°C for 45-60 minutes. The hybridized filters were washed briefly in 2X SSPE and 0.1% SDS at room temperature, followed by a 10 minute incubation in 3 M tetramethyl ammonium chloride, 0.2% SDS, and 50 mM Tris, pH 8.0 (TMACl) at the following temperatures: for Gsα codon 201, 64.5°C; Gsα codon 227, 67°C; Giα2 codon 179, 61.5°C; Giα2 codon 205, 67.5°C. When other probes are employed, hybridization is carried out as described above. The filters are then washed in TMACl at 58°C for 10 minutes. The wash temperature is adjusted in 1°C increments until only the wild type and mutant signals can be detected. In this way, the appropriate wash temperature is determined. Under these conditions, the chosen temperature allows only fully complementary hybrids to stay formed, resulting in a positive dot on a filter. The filters were exposed to Kodak X-AR film for 2-6 hours at -70°C with intensifying screens. For subsequent hybridization with different oligonucleotides, the nylon filters were stripped by boiling the filters for five minutes in 2X SSPE, 0.1% SDS and then processed as described above.

E. Sequencing

For double-stranded sequencing of PCR product, a single band of appropriate size was excised from an ethidium bromide-stained agarose gel under UV light. The excised band was placed into a Costar spin-X 0.22 µm cellulose acetate filter unit,

frozen at -70°C for 15 minutes and spun in an Eppendorf microcentrifuge for 15 minutes at full speed. The DNA-containing eluate was transferred to a microcentrifuge tube, 20 µg glycogen was added, and DNA was precipitated with 0.2 volumes of 3 M sodium acetate and 0.3 volumes ISO propanol. DNA was pelleted in a microcentrifuge, washed with 70% ethanol, vacuum dried, and resuspended in 20 µl double distilled water. The sample was sequenced according to the Sequenase (United States Biochemical) protocol using 7.75 µl DNA solution and 2.5 pmol sequencing primer.

F. Oligonucleotides

Table 3 provides primer pairs for amplification of nucleic acid segments possibly containing oncogenic point mutations. If primer sequence is within the coding region (exon) of the α subunit, the primer pair is suitable for amplification of either a DNA or cDNA template. The nucleic acid sequence of the G-protein α subunits are published for Giα (see Bray et al., 1987, Proc. Natl. Acad. Sci. USA 84:5115-5119), Gsα (see Kozasa et al., 1988, Proc. Natl. Acad. Sci. USA 85:2081-2085), Goα (see Lavu et al., 1988, Biochem. Biophys. Res. Comm. 150:811-815), and Gzα (see Fong et al., 1988, Proc. Natl. Acad. Sci. USA 85:3066-3070). For each primer pair, the size of the amplification product from a genomic DNA template is shown. Note that for Giα1, Giα2, Giα3, Goα, and Gzα the designation 201 and 227 refers to the codon encoding the amino acid corresponding to position 201 or 227 in amino acid sequence of Gsα.

Table 3

Amplification Primers for Detection of G-Protein Point Mutations

		Primer Position
	Gsα 201/227 Outer Primers	
JFL69	5' GCG CTG TGA ACA CCC CAC GTG TCT	intron
JFL70	5' CGC AGG GGG TGG GCG GTC ACT CCA	intron
	Gsα 201/227 Inner Primers (526 bp)	
JFL135	5' GTG ATC AAG CAG GCT GAC TAT GTG	exon
JFL136	5' GCT GCT GGC CAC CAC GAA GAT GAT	exon

			Primer Position
		Gsα - 201 (289 bp)	
	JFL228	5' AAG AAA CCA TGA TCT CTG TTA TAT	intron
	JFL135	5' GTG ATC AAG CAG GCT GAC TAT GTG	exon
		Gsα - 227 (263 bp)	
	JFL229	5' CCC CAG TCC CTC TGG AAT AAC CAG	intron
	JFL136	5' GCT GCT GGC CAC CAC GAA GAT GAT	exon
		Gsα - 49 (171 bp)	
	JFL226	5' AAC AGC AGA CCT CCC TGC CCA AAG	intron
	JFL227	5' CCC CCC TGC ACA GAT TTG ACA CTT	intron
		Giα1 - 201/227	
	JFL223	5' TTG GAC AGA ATA GCT CAA CCA AAT	exon
	JFL224	5' TAG AAC CAG GTC GTA GTC ACT	exon
		Giα2 - 201/227 Outer Primers	
	JL54	5' CCC CCC ATC CCC AGC TAC CT	exon/intron
	JL57	5' TCT CAC CAT CTC CTC GTC CTC	exon/intron
		Giα2 - 201/227 Inner Primers (504 bp)	
	JL55	5' ATT GCA CAG AGT GAC TAC ATC CCC	exon
	JL56	5' GGC GCT CAA GGC TAC GCA GAA	exon
		Giα3 - 201 Outer Primers	
	JFL109	5' TGT CTT TTA TTT AGT ATC AA	exon/intron
	JFL110	5' GAT CTG GAT AGA ATA TCC CAG	exon/intron
		Giα3 - 201 Inner Primers (99 bp)	
	JFL110	5' GAT CTG GAT AGA ATA TCC CAG	exon
	JFL112	5' GGT GAA ATG TGT TTC TAC AAT	exon
		Giα3 - 227 Outer Primers	
	JFL113	5' TTC CCC TTG CGC AGG ATG TTT	exon/intron
	JFL114	5' ACA TAC CAT CTC CTC GTC CTC	exon/intron
		Giα3 - 207 Inner Primers (120 bp)	
	JFL115	5' CAG AAC AAG GTC ATA ATC ACT	exon
	JFL113	5' TTC CCC TTG CGC AGG ATG TTT	
		Goα - 201 (87 bp)	
	SP9	5' CTG GAC AGC CTG GAT CGG ATT GGG	exon
	SP10	5' GAG GTT CTT GAA TGT GAA GTG GGT	exon
		Goα - 227 (129 bp)	
	JFL139	5' AAC CTC CAC TTC AGG CTG TTT	exon
	SP11	5' GTG GAG CAC CTG GTC ATA GCC GCT	exon
		Gzα - 201/227 (~200bp)	
	JFL201	5' TGT GAC GCC CTC GAA GCA GT	exon
	SP15	5' AAC GAC CTG GAG CGC ATC GCC	exon

Table 4

G-Protein Probes for the Detection of Point Mutations and Wild Type Alleles

Gsα - 49									
5'	TA	GGT	GCT	GGA	GAA	TCT	GGT	3'	Gly
				AGA					Arg
				CGA					Arg
				GAA					Glu
				GCA					Ala
				GTA					Val
Gsα - 201									
5'	TT	CGC	TGC	CGT	GTC	CTG	ACT	3'	Arg
				TGT					Cys
				GGT					Gly
				AGT					Ser
				CAT					His
				CCT					Pro
				CTT					Leu
Gsα - 227									
5'	GTG	GGT	GGC	CAG	CGC	GAT	GA	3'	Gln
				TAG					Thr
				GAG					Glu
				AAG					Lys
				CGT					Arg
				CCG					Leu
				CTG					Pro
				CAC					His
				CAT					His
Giα1 - 201									
5'	TC	AGA	ACT	AGA	GTG	AAA	ACT	3'	Arg
				AGC					Ser
				AGT					Ser
				GGA					Gly
				ACA					Tar
				AAA					Lys
				ATA					Ile
Giα1 - 227									
5'	TG	GGA	GGT	CAG	AGA	TCT	GAG	3'	Gln
				GAG					Glu
				AAG					Lys
				CTG					Leu
				CCG					Pro
				CGG					Arg
				CAT					His
				CAC					His

Giα2 - 201									
5'	TA	CGG	ACC	CGC	GTA	AAG	ACC	3'	Arg
				AGC					Ser
				GGC					Gly
				TGC					Cys
				CAC					His
				CCC					Pro
				CTC					Leu
Giα2 - 227									
5'	GTG	GGT	GGT	CAG	CGG	TCT	GA	3'	Gln
				CTG					Leu
				CCG					Pro
				CGG					Arg
				AAG					Lys
				GAG					Glu
				CAT					His
				CAC					His
Giα3 - 201									
5'	TT	CGG	ACG	AGA	GTG	AAG	ACC	3'	Arg
				AGC					Ser
				AGT					Ser
				GGA					Gly
				ATA					Ile
				ACA					Thr
				AAA					Lys
Giα3 - 227									
5'	GTA	GGT	GGC	CAA	AGA	TCA	GA	3'	Gln
				CAT					His
				CAC					His
				CTA					Leu
				CCA					Pro
				CGA					Arg
				AAA					Lys
				GAA					Glu
Goα - 201									
5'	CTC	CGA	ACC	AGG	GTC	AAA	AC	3'	Arg
				GGG					Gly
				TGG					Trp
				AAG					Lys
				ACG					Thr
				ATG					Met
				AGC					Ser
				AGT					Ser

Goα - 227								
5' GTC	GGA	GGC	CAG	CGA	TCT	GA	3'	Gln
			AAG					Lys
			GAG					Glu
			CCG					Pro
			CGG					Arg
			CTG					Leu
			CAT					His
			CAC					His
Gzα - 201								
5' TG	CGC	TCC	CGG	GAC	ATG	ACC	3'	Arg
			GGG					Gly
			TGG					Trp
			CAG					Gln
			CCG					Pro
			CTG					Leu
Gzα - 227								
5' TG	GGG	GGG	CAG	AGG	TCA	GAG	3'	Gln
			AAG					Lys
			GAG					Glu
			CCG					Pro
			CTG					Leu
			CAC					His
			CAT					His
			CGG					Arg

For each G-protein codon to be characterized, oligonucleotide probes are shown in Table 4 as follows. For each position, i.e., Gsα 201, the full probe sequence is shown for the wild type allele. The wild type codon at the potentially oncogenic site is underlined, and the translated amino acid is shown to the right. A set of probes is provided where the sequence is identical to the wild type except at the codon to be characterized. Thus, for non-wild type probes only that codon and the translated amino acid product is shown in the table. Only those point mutations encoding amino acids different from the wild type amino acid are shown.

Table 5 shows stretches of Gsα sequence surrounding the arginine-201 and glutamine-227 codons which are highly conserved in G-protein α chains of vertebrates, yeast, and slime mold. Published sequences include rat Gsα, Giα2, Giα3, and Goα, human Gzα (Katada and Vi, 1982, Proc. Natl. Acad. Sci. USA 79:3129), Gtα of bovine retinal rod cells (Chambard et al., 1987, Nature 326:800), the α chain of the G-protein (called GPA1/SCG1) that mediates pheromone signalling in Saccharomyces cerevisiae (Corven et al., 1989, Cell 59, and Itoh et al., 1988, J. Biol. Chem. 263:6656), and an α chain (Gα1) from Dictyostelium discoideum (Zarbl et al., 1985, Nature 315:382). The number is parenthesis following each sequence is the actual

amino acid position of the last amino acid in the sequence shown. In the table, a one letter amino acid code is used where:

D = Asp	K = Lys
L = Leu	G = Glu
R = Arg	M = Met
C = Cys	A = Ala
V = Val	Q - Gln
T - Thr	F = Phe
S = Ser	E = Glu
I = Ile	

Table 5
Conservation of Sequences Among G-Protein α Chains

	201		227	
sα	D L L R C R V L T S	(205)	F D V G G Q R D E R R	(232)
iα2	- V - - T - - K - T	(183)	- - - - - - - - S - - K	(210)
iα3	- V - - T - - K - T	(182)	- - - - - - - - S - - K	(210)
oα	- I - - T - - K - T	(183)	- - - - - - - - S - - K	(209)
zα	- I - - S - D M - T	(183)	V - - - - - - - S - - K	(210)
tα	- V - - S - - K - T	(177)	- - - - - - - - S - - K	(205)
GPA1/SCG1	- I - K G - I K - T	(301)	L - A - - - - - S - - K	(328)
Gα1	- V - - S - T K - T	(184)	V - - - - - - - S - - K	(211)

Example 2

Identification of G-Protein Point Mutations in Gsα Genes

To determine the frequency of mutations in codons 201 and 227 of Gsα genes in pituitary and other tumors, the polymerase chain reaction (PCR) was used to amplify a specific region of genomic DNA and high-stringency hybridization of sequence-specific oligonucleotides to detect point mutations in the amplified product. To detect mutations in the Gsα gene, a single region including both codons 201 and 227 and an intervening intron was amplified from tumor genomic DNA prepared from fresh frozen or paraffin embedded samples as described in Example 1. The amplification primers shown in Table 3, designated "Gsα 201/227 Outer Primers" and "Gsα 201/227 Inner Primers," were used according to the method described above. Oligonucleotides specific for wild type or single-base mutations at codon 201 (6 possible missense mutations) or codon 227 (7 possible missense mutations, 1 nonsense mutation) were hybridized to the amplified product (Table 4). Genomic DNA from more than 300 tumors was analyzed either in the form of high molecular weight DNA prepared from fresh tissue or as obtained from paraffin-embedded tissue. Group 1 tumors had low basal adenylyl cyclase activity that responded normally to stimulatory agents, group 2 tumors had marked elevation of basal adenylyl cyclase activity that responded poorly to stimulatory agents.

The hybridization results are shown in Figure 1A. The hybridization probes shown in Table 4 were used as follows: R201 indicates the wild type probe for Gsα Arg 201; R201C indicates that the probe used contained a point mutation (CGT to TGT) encoding cysteine; and R201H indicates that the probe used contained a point mutation (CGT to CAT) encoding histidine. The fourth panel was probed with the probe corresponding to Gln 227 containing a point mutation (CAG to CGG) encoding arginine.

Of the many tumor types analyzed, mutations were detected only in GH-secreting tumors of the pituitary gland. Among 42 GH-secreting pituitary tumors, 18 (43%) contained Gsα mutations. Of these, 16 mutations were in codon 201 (14 arginine to cysteine, 2 arginine to histidine) and two were in codon 227 (both glutamine to arginine). These results are summarized in Table 6. For two patients in whose tumors Gsα point mutations were detected, samples of white blood cells were available; neither sample contained a mutant Gsα gene, whether assessed by sequencing cDNA clones (2) or by allele-specific oligonucleotide analysis. This result indicates that the mutations are somatic and thus likely to have played a direct causal role in the development of the tumors. Furthermore, a normal Gsα allele was present

in all tumors where a mutant allele was detected, suggesting that mutations that activate Gs are dominant.

Of 42 GH-secreting pituitary tumors, 16 were biochemically characterized in terms of adenylyl cyclase activity. Eight tumors showing elevated adenylyl cyclase were predicted to harbor an activated Gsα; each of these tumors contained a mutation in codon 201 or codon 227 (Figure 1A). No mutations were detected in eight tumors that showed normal adenylyl cyclase activity. Although Gsα mutations in other codons may also inhibit GTPase, the strong concordance between elevated adenylyl cyclase activity and a mutation in codon 201 or codon 227 indicates that activating mutations at other sites are relatively infrequent.

Table 6 provides a summary of human tumors screened for mutations in codons 201 and 227 for Gsα and codons 179 and 205 for Giα2. DNA for PCR amplification Mullis et al., supra., was isolated from either fresh tissue, Verlaan-de Vries et al., 1986, Gene 50:313, or paraffin-embedded tissue Kozasa et al., 1988, Proc. Natl. Acad. Sci. USA 85:2081. Eighteen growth hormone (GH) secreting pituitary adenomas contained a mutation in either Gsα codons 201 or 227. One ovarian granulosa cell tumor and three adrenal cortical tumors (two adenomas, one carcinoma) contained a mutation in Giα2 codon 179.

Table 6

sα and iα2 Mutations in Human Tumors

Tumor Type	α2		iα2	
	Tested	Mutation	Tested	Mutation
Pituitary adenoma				
GH	42	18 (43%)	4	0
Prolactin	12	0	7	0
TSH	2	0	1	0
ACTH	7	0	2	0
Nonsecretor	3	0	2	0
Ovarian granulosa cell tumor	6	0	6	1 (17%)
Adrenal cortical tumor[1]	9	0	11	3 (27%)
Thyroid tumors[2]	24	0	24	0
Melanoma	16	0	16	0
Glioblastoma	29	0	29	0
Ovarian adenocarcinoma	8	0	10	0
Gastric adenocarcinoma	13	0	14	0
Renal cell carcinoma	17	0	17	0
Breast adenocarcinoma	30	0	25	0
Bladder transitional cell carcinoma	8	0	9	0
Pancreatic adenocarcinoma	12	0	-	0
Acute myelogenous leukemia	8	0	-	0
Squamous cell carcinoma	33	0	33	0
Colonic adenocarcinoma	-	0	20	0
Hepatoma	15	0	12	0
Prostatic tumor[3]	12	0	12	0
	306		254	

1. Adenoma (5 for sα, 4 for iα2); carcinoma (6 for sα, 5 for iα2)

2. Papillary carcinoma 6; follicular carcinoma 3; follicular adenoma 3; Hürthle cell carcinoma 1; multinodular goiter 3; medullary carcinoma 8

3. Benign hyperplasia 6, adenocarcinoma 6

Table 7 provides a list of wild type codons for the conserved arginine and conserved glutamine in Gsα and Giα2 genes. The table also shows single-nucleotide base changes (in bold) and the resulting amino acid changes. Oligonucleotides specific for wild type and each missense or nonsense single-base change listed were used to screen human tumors, with the exception of base changes that would be silent (marked with asterisks). Mutations detected in the tumors listed in Table 6 are underlined. "Term" indicates termination or stop signal.

Table 7

Possible Mutations in Two Key Codons of Gsα and Giα2

	Arginine			Glutamine		
sα	WT=CGT (Arg 201)			WT=CAG (Gln 227)		
	TGT Cys	CAT His	CGC Arg*	TAG Term	CGG Arg	CAA Gln*
	GGT Gly	CCT Pro	CGG Arg*	CAG Glu	CCG Leu	CAC His
	AGT Ser	CTT Leu	CGA Arg*	AAG Lys	CTG Pro	CAT His
iα2	WT=CGC (Arg 179)			WT=CAG (Gln 205)		
	TGC Cys	CAC His	CGT Arg*	TAG Term	CGG Arg	CAA Gln*
	GGC Gly	CCC Pro	CGG Arg*	GAG Glu	CCG Leu	CAC His
	AGC Ser	CTC Leu	CGA Arg*	AAG Lys	CTG Pro	CAT His

Example 3

Identification of G-Protein Point Mutations in Giα2 Genes

In order to investigate the possibility that mutational activation of signaling pathways mediated by other G-proteins might lead to abnormal proliferation and tumor formation, a large panel of human tumors was screened for mutations in a Giα gene. In Giα2 the coding sequence and intron between the two codons to be tested, arginine-179 (corresponding to Gsα Arg 201) and glutamine-205 (corresponding to Gsα Gln 227), is short enough to allow PCR amplification of a single genomic DNA fragment containing both codons (Itoh et al., 1988, J. Biol. Chem. 263:6656).

The primers and probes used for detection and characterization of Giα2 are shown in Tables 3 and 4. Samples were prepared and analyzed as described in Example 1. The hybridization results are shown in Figure 1B. In the figure, the first two rows of each panel were probed with the wild type probe for codon Arg 179 (R179). The third and fourth rows were hybridized to the Giα2/201 probe of Table 2 containing a point mutation (CGT or TGT) encoding cysteine (R179C). The last two rows of each panel were hybridized to the Giα2/227 probe shown in Table 4 comprising a point mutation (CGT or CAT) encoding histidine (R179H). The amplification methods are described in Example 1.

Table 6 summarizes the hybridization results. Mutations in codon 179 of Giα2 were detected in two different endocrine tumor types 3 of 11 tumors of the adrenal cortex and one of 6 ovarian granulosa cell tumors. The adrenal tumor lacking a wild type allele was an adenocarcinoma; the other 2 adrenal tumors were adenomas. No mutations were found in codon 205. The high frequency of codon 179 mutations in tumors of two related cell types suggests that these mutations converted the Giα2 gene into an oncogene, referred to herein as gip2 (for Gi protein-2).

Strikingly, the amino acids that replaced arginine-179 in Giα2, cysteine and histidine, were the same as those that replaced the cognate arginine at position 201 in Gsα oncogene products found in pituitary tumors (Landis et al.). It is possible that, of the six possible missense mutations that can result from single-base changes in these codons of Gsα and Giα2, only these mutant proteins are biologically active. Also, all mutations found so far, in either Gsα or Giα2, are transition mutations (Table 7); consequently, these mutations may reflect a common mutagenic mechanism.

In one tumor of the adrenal cortex, a normal allele of Giα2 was not detected (Figure 1B). Sequence analysis of PCR products revealed a single sequence, corresponding to the codon 179 mutation. This result makes it likely that the normal allele was missing, although the possibility that both alleles contain the same mutation cannot be excluded. Loss of the normal allele suggests that its protein product

interferes with the oncogenic effect of the mutant protein, so that failure to express normal Giα2 confers an additional selective advantage on cells carrying an activating Giα2 mutation in the other allele.

Example 4

Detection of *gsp* Mutations in Paraffin-Embedded Samples by Microdissection

Changes in the two Gsα codons affected by gsp mutations were detected in formalin-fixed, paraffin-embedded tissue blocks of human tumors by isolating genomic DNA, amplifying appropriate Gsα sequences with the polymerase chain reaction (PCR), and screening the amplified products for their ability to hybridize with allele-specific oligonucleotides. In Examples 1-3 DNA from all cells in a 5 mm tissue section was analyzed. DNA from one thyroid tumor had two different gsp mutations, in addition to the wild type allele. To ask whether the two mutations were located in different regions of the 5 m tissue slice, an adjacent 5 mm section from the same tumor was divided into several smaller fragments; genomic DNA was isolated from each fragment separately and subjected to PCR amplification and screening with allele-specific oligonucleotides. The two gsp mutations were detected in different fragments; in addition, several fragments contained only the wild type codons at positions 201 and 227 of Gsα.

The latter observation suggested that the heterogeneous distribution of mutations in 5 mm sections may go undetected due to dilution by wild type DNA sequences from cells in parts of the sections that contained no mutations. Accordingly, this modified microdissection approach was applied to additional tumors. Identification of a high prevalence of gsp mutations some in tumors earlier tested as negative indicated that this approach is more sensitive at detecting gsp mutations.

A. Malignant Thyroid Tumors

To explore the roles of gsp mutations in pathogenesis of malignant thyroid tumors, the microdissection technique to tissue blocks of primary tumor or lymph node metastases from 37 patients with differentiated thyroid carcinoma. These tissue fragments were also tested for mutations in specific codons of the three human *ras* genes.

Stained sections from these surgical specimens were examined and each microdissected fragment was classified as malignant or benign thyroid tissue; in every case, the pathologic diagnosis applied to at least 90% of the cells in the fragment. Both the pathologic examination and the DNA analysis were performed in a blinded fashion: The pathologist diagnosed histology of tissue fragments without knowledge of the

presence or absence of mutations, and the DNA analyses were performed on coded samples.

B. Sample Preparation

Multiple 5 mm sections were cut from each tissue block and one was stained with hematoxylin and eosin. Regions of the stained slide, 30-100 mm^2 in area, were demarcated with a pen and designated by number; when possible, demarcations separated histologically distinct regions. Using the stained slide as a template, an adjacent unstained 5 mm section was divided into corresponding fragments with a razor blade. These fragments were transferred into separate tubes and each was processed for PCR and hybridization screening, exactly as described in Example 1.

C. PCR Amplification

Using 20-25 base pair (bp) oligonucleotide primers upstream and downstream of codons 201 and 227 in the human Gsα gene, templates ranging from 165 to 1,200 bp in length. Primers chosen for PCR amplification of regions around codons 12, 13 and 61 of the human H-ras, Ki-ras and N-ras genes yielded products of 112-117 bp. Amplification of formalin-preserved, paraffin-embedded tissues was most effective with relatively short PCR products. Amplification with nested primers was required when the first PCR of larger DNA fragments produced an unsatisfactory amount of amplified DNA, as judged by agarose gel electrophoresis and ethidium bromide staining.

PCR conditions were as generally described in Example 1, however, the oligonucleotides and cycling temperatures used were as were as follows.

For nested primer amplification of Gsα codons 201-227: Outer sense, (JFL69) 5'GCG CTG TGA ACA CCC CAC GTG TCT; outer antisense (JFL70), 5'CGC AGG GGG TGG GCG GTC ACT CCA; product 1,200 bp; optimal PCR condition, 30 cycles of 1, 2, and 2 min at 95°/50°/72°; inner sense (JFL135), 5'GTG ATC AAG CAG GCT GAC TAT GTG; inner antisense (JFL136), 5'GCT GCT GGC CAC CAC GAA GAT GAT; product 526 bp; optimal PCR condition, 30 cycles 30, 40, and 45 sec at 95°/57°/72°.

For Gsα codon 201: sense (JFL135), 5'GTG ATC AAG CAG GCT GAC TAT GTG; antisense (JFL286),5' TA ACA GTT GGC TTA CTG GAA; product 222 bp; optimal PCR conditions: 40 cycles of 30, 30, and 30 sec at 95°/55°/72°. For Gsα codon 227: sense (JFL229),5' CCC CAG TCC CTC TGG AAT AAC CAG; antisense (JFL136), 5'GCT GCT GGC CAC CAC GAA GAT GAT; product 165 bp; optimal PCR condition, 50 cycles of 60, 30, and 30 sec at 95°/55°/72°.

For ras gene amplications the following standard PCR conditions of 50 cycles of 60, 30, and 30 sec 95°/55°/72° were used. Ras primers were as follows.

Ha-ras codons 12 and 13: sense (JFL243), 5'AGA CCC TGT AGG AGG ACC CCG GGC C; antisense (JFL244), 5'ATA GTG GGG TCG TAT TCG TCC ACA A; product 150 bp.

For Ha-ras codon 61: sense (JFL252), 5'GTC ATT GAT GGG GAG ACG TG; antisense (JFL253), 5'ACA CAC ACA GGA AGC CCT CC; product 112 bp;

for Ki-ras codons 12 and 13: sense (EK371), 5'CCT GCT GAA AAT GAC TGA ATA TAA A; antisense (EK372), 5'T ATT GTT GGA TCA TAT TCG TCC ACA; product 118 bp;

for Ki-ras codon 61: sense (JFL248), 5'GTA ATT GAT GGA GAA ACC TG; antisense (JFL249), 5'ATA CAC AAA GAA AGC CCT CC; product 112 bp.

For N-ras codons 12 and13: sense (JFL216), 5'CTT GCT GGT GTG AAA TGA CT; antisense (JFL257), 5'GGT GGG ATC ATA TTC ATC TA; product 150 bp.

For N-*ras* codon 61: sense (JFL218), 5'GTT ATA GAT GGT GAA ACC TG; antisense (JFL242), 5'GGC AAA TAC ACA GAG GAA GCC TTC; product 112 bp.

Hybridization dot blots like that shown were scored by counting in an AMBIS radioanalytic imaging system as described above. Replicate PCR amplifications and analyses of adjacent 5 mm sections produced similar results, indicating that the procedure was accurate and reproducible as well as sensitive.

To avoid false positives by nonspecific hybridization of mutant probes to the PCR product in the absence of mutations, a level of hybridization was set, below which a sample would be considered negative for a particular mutation. This level was set at 20% of the signal detected by hybridization of the wild type probe to unmutated DNA in the sample. Hybridization signals were determined to be reproducible at this level, but not below it. Consequently, a positive result (20% or more of the amplified DNA contains a mutation) indicates that at least 40% of the cells in the assayed tissue fragment contain the mutation, if — as expected for dominant somatic mutations — each cell has one wild type and one mutant allele.

D. Hybridization and Detection of Point Mutations

For mutation-specific olinucleotide hybridization, the PCR product was spotted (dot-blot apparatus, Bio-Rad) and covalently bound to a nylon filter (Pall Biodyne-B, 0.45 um) using UV light at the auto-crosslink setting (Stratalinker, Stratagene). Hybridization with [^{32}P]-radiolabeled mutation-specific oligonucleotides 20 bp long were performed exactly as described in Example 1.

E. Criterion for Presence of a Mutation

To verify the presence of point mutations we scanned all a_s hybridization reactions with an AMBIS radioanalytic imaging system (Ambis Co., San Diego, CA), which measures the emission of radioactivity from each mutant (CPM_m) or wild type (CPM_{wt}) dot on the filter. The [^{32}P] b radioactivity of mutant oligonucleotides nonspecifically bound to the same filters after high stringency washing was termed background activity (CPM_b), and ranged from 5 to 10 % of the wild type hybridization signal. A dot was considered to represent a *gsp* mutation if (CPM_m - CPM_b) divided by (CPM_{wt} - CPM_b) was greater than or equal to 0.2. This criterion thus required that amplified DNA samples judged as positive for a mutation must exhibit a mutant signal 20% of that observed with wild type. Applying this criterion also minimized the chance that a spuriously positive result could result from contamination of a gsp-negative sample by DNA from a gsp-positive sample.

F. Controls

The microdissection procedure gave negative results for Gsα and ras mutations in sections of normal connective tissue and human thyroid removed during parathyroidectomy. To confirm positive results, PCR products from six fragments for which hybridization results indicated R201C mutations at levels near the demonstrated cutoff point (i.e., 22-35%) were sequenced. Genomic DNA was amplified using one biotinylated and one non-biotinylated primer, to generate unilateral biotinylated PCR products. After binding the biotinylated PCR product to streptavidin coated beads (Dynabeads, Dynal) the complementary strand was denatured and aspirated, leaving single stranded DNA. Sequencing was performed using the Sequenase kit (Sequenase, USB). In all six cases the sequencing gels showed both the wild type and the mutant 201 codon, confirming the results of dot blot hybridization.

G. Number and Distribution of Mutations

Gsp mutations were found in surgical specimens from 24 of 37 patients (65 %) with differentiated thyroid cancer. Gsp mutations in these patients were heterogeneously distributed among microdissected fragments; overall, 81 of 266 thyroid tissue fragments (30.5 %) contained detectable gsp mutations. Among the 24 surgical specimens with at least one gsp positive fragment, a gsp mutation was found in 60 of 120 histologically malignant fragments (50 %). The mutations were not confined to fragments with obviously malignant tissue, however; indeed, in the same tumors 21 of 60 histologically benign fragments (36.2 %) also contained gsp mutations.

Of the 24 patients in whom the microdissection technique revealed gsp mutations, only five were originally suspected of harboring gsp on the basis of the previous testing procedure, which used the entire 5 m section as a single sample. In each of these five previously positive cases, more than half of the fragments tested with the new procedure were positive for gsp mutations, a frequency found in only one other case. These observations indicate that without microdissection the dilution of samples by DNA from regions without mutations can cause a substantial proportion — perhaps more than 75% — of thyroid gsp mutations to be missed.

Specimens from six of the 37 patients harbored two different gsp mutations, and the tumor of one patient (number 22, Table 8) contained three different gsp mutations. These multiple mutations were found in separate fragments of these specimens: Of the 31 gsp-positive fragments tested in patients with more than one gsp mutation, only one fragment contained two mutations. Multiple gsp mutations in a single tumor did not correlate with clinical aggressiveness or other characteristics of the tumor (Table 8).

Of the 37 specimens examined, 15 were obtained from tumor metastases to areas physically separate both from the original primary tumor and from normal thyroid tissue — i.e., spread beyond the strap muscles to lymph nodes in 13 cases and to lung in two cases. Of these 15 specimens, 11 harbored gsp mutations. In three cases (patients 12, 19 and 24), these metastases revealed two different gsp mutations.

Occasional reports have documented more than one ras mutation in a tumor (e.g., in human cutaneous melanoma, see Van't Veer et al., 1989, Mol. Cell Biol., 9:3114-3116). The present study is the first to find a high incidence of multiple mutations of a single oncogene in individual tumors. The microdissection technique will likely find a similar multiplicity of oncogenes in other tumors as well.

H. *Ras* Mutations

Mutational substitutions of amino acid residues in three key positions of $p21^{ras}$ — glycine-12, glycine-13, and glutamine-61 — inhibit GTP hydrolysis and promote its ability to trigger neoplastic transformation. Other investigators have reported prevalences of ras mutations in thyroid cancer ranging from 17 to 60 % (LeMoine et al., 1989, Oncogene, 4:159-164 and Wright et al., 1989, Brit. J. Cancer, 60:576-577). Consequently, the 37 surgical specimens from patients with thyroid cancer were tested for oncogenic mutations affecting the three key codons of the three human ras genes. Microdissected fragments from 12 of 37 patients (32 %) contained N-ras mutations. These included 12 fragments with codon-61 mutations (Q61R), 18 codon-13 mutations (nine G13C, nine G13D), and a single fragment with a codon-12 mutation (G12C).

N-*ras* mutations resembled *gsp* mutations in several respects: Both were heterogeneously distributed, sometimes multiple in a single patient, and present in benign as well as malignant thyroid tissue (Table 8). Of 117 microdissected fragments tested in the 12 *ras*-positive patients, 31 (26 %) contained an N-*ras* mutation. Two patients had more than one different *ras* mutation.

Detection of ras and gsp mutations in the same microdissected fragment does not mean that both mutations are present in the same cell; indeed, further microdissection would probably segregate different cell populations containing each mutation.

I. New *Gsp* Mutations

The thyroid tumors exhibited four previously unreported mutations, including substitutions of proline or serine for arginine-201 and substitutions of histidine or proline for glutamine-227.

J. Conclusions

The microdissection technique for finding point mutations greatly extends the practical resolution for detecting certain oncogenes, to the point that it can detect a mutation present in 40% or more of the few thousand cells in a 5 m x 30-100 mm^2 fragment of tissue. Microdissection may serve to uncover heterogeneously distributed oncogenes in non-thyroid tumors also. The increased sensitivity of this technique also shows that a conclusion that a particular oncogene mutation is not present in a tumor can be wrong. If based upon PCR amplification of large fragments of tumor, such a negative conclusion must be qualified; in fact, a negative result only indicates that the mutation is not present in a substantial proportion of the cells in the tumor fragment. The results of this analysis are presented in Table 8.

Table 8
Gsp and ras Mutations in Differentiated Thyroid Cancer

Patient Characteristics				Gsp mutations				Ras mutations			
				Carcinoma		Benign		Carcinoma		Benign	
No.	Age/Sex	Histol./TNM	R	n/N	Codon	n/N	Codon	n/N	Codon	n/N	Codon
1	25F	P100		ND		5/15	R201C	ND		0/15	
2	35F	P100		1/4 1/4	Q227H Q227R	1/4 1/4	Q227H Q227R	0/4		2/4	Q61R
3	43M	P100		ND		2/6	Q227H	ND		0/6	
4	53F	P100		1/2	Q227H	1/2	Q227H	0/2		0/2	
5	63M	P100		ND		1/3	Q227H	ND		0/3	
6	52F	P200		1/5	Q227H	ND		0/5		ND	
7	53F	P200		ND		2/2	R201C	ND		0/2	
8	58F	P200		1/3	Q227H	0/3		2/3	G13D	3/3	G13D
9	24F	P120	+	ND		2/10	Q227H	ND		0/1	
10	47M	P210		0/1		4/4	Q227H	0/1		1/4	G12C
11	41M	P210	+	4/8	R201C	ND		0/8		ND	
12	45F	P220	+	2/4 2/4	Q227H Q227R	ND		0/4		ND	

Table 8 - Continued
Gsp and ras Mutations in Differentiated Thyroid Cancer

Patient Characteristics				Gsp mutations				Ras mutations			
				Carcinoma		Benign		Carcinoma		Benign	
No.	Age/Sex	Histol./TNM	R	n/N	Codon	n/N	Codon	n/N	Codon	n/N	Codon
13	31F	P220	+	0/1		ND		0/1		ND	
14	44F	P220		1/7	R201C	ND		0/7		ND	
15	70F	P130	+	0/2		ND		0/2		ND	
16	66F	Px2x	+	3/9	R201C	ND		0/9		ND	
17	56F	P211		1/3	R201C	ND		0/3		ND	
18	52M	Px21	+	8/14 1/14	Q227H R201P	ND		0/14		ND	
19	68M	Px31	+	1/9 2/9	Q227H Q227R	ND		3/9	G13D	ND	
20	76F	P221		1/8	Q227P	2/9	Q227H	0/8		1/9 1/9	G13C G13D
21	22F	P431	+	0/4		ND		0/4		ND	
22	68F	F300	+	3/12 1/12 3/12	Q227H Q227H R201C	ND		0/12		ND	
23	44F	Fx20	+	0/3		0/5		0/3		0/5	

Table 8 - Continued
Gsp and ras Mutations in Differentiated Thyroid Cancer

Patient Characteristics				Gsp mutations				Ras mutations			
				Carcinoma		Benign		Carcinoma		Benign	
No.	Age/Sex	Histol./TNM	R	n/N	Codon	n/N	Codon	n/N	Codon	n/N	Codon
24	70F	Fx20	+	3/4 1/4	Q227H R201C	ND		1/4	G13C	ND	
25	73F	Fx30	+	1/4	Q227R	ND		0/4		ND	
26	42M	Fx31	+	0/32		ND		4/32	G13C	ND	
27	49M	F411	+	13/13 3	R201C	ND		5/13	Q61R	ND	
28	24F	P200		0/3		ND		0/3		ND	
29	34F	P200		0/3		ND		0/3		ND	
30	42M	P300		0/6		ND		0/6		ND	
31	56M	P300		0/4		0/1		1/4	Q61R	1/1	Q61R
32	62M	P310		2/6	R201S	ND		0/6		ND	
33	62M	P321		0/2		0/4		1/2 1/2	G13C Q61R	1/4	Q61R
34	74M	Px31	+	2/5	R201C	0/2		1/5	Q61R	0/2	
35	35F	F200		0/5		0/2		0/5		0/2	

Table 8 - Continued
Gsp and ras Mutations in Differentiated Thyroid Cancer

Patient Characteristics				Gsp mutations				Ras mutations			
				Carcinoma		Benign		Carcinoma		Benign	
No.	Age/Sex	Histol./TNM	R	n/N	Codon	n/N	Codon	n/N	Codon	n/N	Codon
36	63M	Fx00		0/3		ND		0/3		ND	
37	56M	F410	+	0/5				2/5	G13C	ND	
TOTAL				60/1		21/7		21/1		10/7	
				94		2		94		2	

K. Table 8

Patients numbered 1-27 and 28-37 (left column) underwent surgery in Düsseldorf and San Francisco, respectively. Gap and ras mutations are listed as present or absent in microdissected fragments of thyroid tissue; fragments containing no thyroid tissue are not included. Each fragment tested was classified as "carcinoma" or "benign"; for each, 90% or more of the cells seen in the corresponding part of the stained section were histologically malignant or benign, as indicated. Mutations are enumerated as a fraction of n mutations detected in N fragments tested; ND indicates that no fragment of the particular classification (carcinoma or benign thyroid) were present in the material available for analysis. Although Harvey- and Kirsten-*ras* mutations were also sought, only N-*ras* were found.

Clinical characteristics of patients: The "Histo./TNM" column (third from the left) indicates whether the tumor was diagnosed as a papillary (P) or follicular (F) thyroid carcinoma, and provides the numerical clinical classification (TNM) of thyroid cancer devised by the World Health Organization[21], in which T, N, and M are numbers referring to different characteristics: T (varying from 1 to 4) indicates increasing size and extent of the tumor mass; N (varying from 1 to 3) indicates increasing numbers of lymph node metastases; M (0 or 1) indicates the absence or presents of distant metastases; "x" indicates that the information was not known to us. Fifteen patients (nos. 11, 12, 13, 15, 16, 17, 19, 21, 24, 25, 26, 27, 32, 34, and 37) had lymph node metastases outside the bed of the thyroid gland, and two (nos. 19 and 26) also had lung metastases. The fifth column (R) indicates whether (+) or not (-) the patient had received therapeutic radiation therapy (^{131}I or external) before the operation.

Table 9 provides a summary of pituitary *gsp* mutations identified in Examples 1-4.

Table 9
Transition vs. Transversion Mutations in Human Endocrine Tumors

		Arginine-201 (CGT)				Glutamine-227 (CAG)					
		TGT Cys	CAT His	CCT Pro	AGT Ser	CAT His	CGG Arg	CCG Pro	CTG Leu	Total	Transversion (%)
Pituitary*	Transitions	14	2	0	0	0	2	0	0	18	0
	Transversion	0	0	0	0	0	0	0	0	0	
MNG	Transitions	24	0	0	0	0	0	0	0	0	
	Transversion	0	0	0	0	0	0	0	0	0	
Thyroid ca. (Malignant fragments)	Transitions	8	0	0	0	0	5	0	0	13	48
	Transversion	0	0	1	1	9	0	1	0	12	
Thyroid ca. (Benign fragments)	Transitions	2	0	0	0	0	1	0	0	3	70
	Transversions	0	0	0	0	7	0	0	0	7	

Example 5

Materials and Methods

A. Human Tissues

Human tissues were obtained from surgical biopsy and either RNA immediately extracted or rapidly frozen to -70°C until use. All human tissues were obtained by informed consent in approval with the University of California, Davis Human Subjects Review Committee. Tissues were also supplied by the Tissue Bank of the Department of Pathology University of California, Davis) under direction of Dr. Robert D. Cardiff.

Microscope slides with bone marrow smears on them were analyzed. One was hematoxylin and eosin (H+E) stained slide, while the other was merely air dried bone marrow. Both slides were stored at room temperature for several months prior to RNA extraction.

Other archival samples were either methanol- or ethanol-fixed prior to being embedded in paraffin.

B. Cell Lines

Human tumor cell lines previously characterized to have known activating point mutations in various ras alleles were used. Cell lines EJT24 (bladder transitional cell carcinoma) (Tabin et al., 1982, Nature 300:143-149, and Reddy et al., 1982, Nature 300:149-152) and SK-N-SH (neuroblastoma) (Taparowsky et al., 1983, Cell 344:581-586) were a gift of Dr. R. Cardiff. Calu-1 (lung carcinoma), SW 480 (colon carcinoma, and PA-1 (teratocarcinoma) were obtained from the American Type Culture Collection (ATCC) (Capoh et al., 1983, Nature 304:507-513, and Tainsky et al., 1984, Science 225:643-645). HL-60 (promyelocytic leukemia) was a gift of Dr. J. Lawrence (Murray et al., 1983, Cell 33:749-757).

Other cell lines were used as negative controls for ras mutations because they are not known to contain activated alleles. K562 (erythroleukemia) was supplied by the Cetus Tissue Culture Collection (CTCC) (Lozzio and Lozzio, 1975, Blood 45:321-334). Cell line G-2101 (renal clear cell carcinoma) was originated in our lab (Gumerlock et al., 1988, In Vitro Cell Devel. Biol. 24:429-434). The cell strain T-3891 (fetal lung) is a normal, nonimmortalized fibroblastic culture (Rossitto et al., 1988, J. Virol. 140:431-435). All of the above cell lines were maintained according to the instructions of the supplier.

C. RNA Extractions from Fresh or Frozen Tissues and Cell Lines

Total cellular RNA was extracted from tissues and cell lines using modifications of the previously described guanidinium-isothiocyanate-phenol-chloroform methods

(Maniatis et al., 1982, In. Molecular Cloning, New York, Cold Spring Harbor Page 190, and Chirgwin et al., 1979, Biochem. 18:5294-5299). Guanidinium isothiocyanate solution (5 M guanidinium isothiocyanate, 25 mM sodium citrate, 0.5% sarcosyl, pH 7.0) (GITC) was prepared to 5% β-mercaptoethanol (GITC-ME) just prior to use. Tissue bits were powdered in liquid nitrogen in a mortar, further ground in the mortar upon additional of GITC-ME and 1.5 ml of the slurry was layered onto a cesium chloride (CSCL) density gradient in 13 x 51 mm polyallomer tubes (Beckman Laboratories). The CsCl density gradient was prepared by layering 1.5 ml of a 40% CsCl density gradient was prepared by layering 1.5 ml of a 40% CsCl solution in 20 mM Tris-HCl, 2 mM EDTA, pH 7.5 (TE) onto 2.0 ml of 5.7 M CsCl in TE. RNA was pelleted through this density gradient by ultracentrifugation at 40,000 rpm in an SW-50.1 rotor at room temperature for 16 to 19 hours.

The RNA pellet was suspended in 50 µl TE-SDS (10 mM Tris-HCl, 1 mM EDTA, pH 7.4 wit 0.5% SDS) in a microcentrifuge tube for phenol-chloroform extraction. TE saturated phenol was mixed 1:1 (v/v) with a chloroform:isoamyl alcohol (24:1) solution. An equal volume of this phenol:chloroform solution was added to the RNA solution, vortexed vigorously for 10 seconds and phase separated in a microfuge for two minutes. This extraction was repeated, and the aqueous phase containing the RNA was placed in a 2 ml microcentrifuge tube for precipitation. The RNA was precipitated by addition of 5 M NaCl to create a final concentration of 0.3 M NaCl followed by addition of two volumes of ice-cold 100% ethanol. This solution was placed at -70°C for a minimum of one hour. The tube was then warmed to room temperature to melt the ice and spun in a microfuge at 4°C for 15 minutes to pellet the RNA precipitate. The supernant was decanted and residual liquid was removed by vacuum desiccation. When nearly dry, the RNA pellet was redissolved in TE (without SDS) and precipitated a second time. This time the RNA pellet was redissolved in 50-100 µl of 0.2X TE. RNA concentrations were determined by reading optical density at 260 mm in a spectrophotometer and calculated by setting 1.0 O.D. equivalent to 40 µg per ml RNA.

D. RNA Extraction from Air-dried Bone Marrow Slides

Microscope slides of human bone marrow were extracted for RNA. One was stained with H+E while the other was merely air-dried and left unstained. The cells on these slides were scraped with razor blades into microcentrifuge tubes. To the tubes was added 1 ml of GITC-ME buffer, and the tubes were shaken vigorously on a rotary shaker for 60 minutes to dissolve the cells. The solution was then put in a 2 ml microcentrifuge tube. To precipitate DNA away from the RNA in solution, 0.1 ml of 2

M sodium acetate (pH 4.8) was added to each tube. The DNA precipitation and an extraction were performed by adding 1 ml of phenol:chloroform to the tube, inverting the tubes multiple times, and placing the tubes on wet ice for 15 minutes. This method of quick RNA extraction including the precipitation of DNA away from the RNA is modification of that described by Chomczynski and Sacchi, 1987, Anal. Biochem. 162:156-159. Following the incubation of ice, DNA remains at the interface between the organic and aqueous phases. The tubes were spun in a microfuge at 4°C for 20 minutes, and the aqueous phase containing the RNA was removed and transferred to a new 2 ml tube for precipitation of the RNA. to each tube was added 750 µl of isopropanol, and the tubes were inverted several times before placing at -20°C for one hour. Precipitated RNA was pelleted by spinning in a microfuge at °C for 20 minutes. The RNA was redissolved in 300 µl of GITC-ME and precipitated a second time by addition of 300 µl of isopropanol and placing at -20°C for one hour. RNA was pelleted again as above, supernatant was discarded, and the RNA pellets washed with 1 ml of 70% ethanol. Once again, the RNA was pelleted, supernatant discarded, and the pellet dried under vacuum desiccation. The RNA was finally redissolved in 50 µl of 0.2 X TE and quantitated. Both slides each yielded over 15 µg of RNA.

E. RNA Extraction from Alcohol-fixed Paraffin-Embedded Tissues

Fifty micron sections of paraffin blocks were cut and deparaffinized in 1 ml of xylenes by vigorous shaking in a microfuge tube for 30 minutes. Tissue bits were pelleted by microfuging for five minutes and the xylenes decanted. Residual xylenes were removed by washing with 100% ethanol and repelleting the tissue bits. To the tissue 1 ml of the GITC-Me solution described above was added. Tubes were vigorously shaken on a rotary shaker for one hour to dissolve the tissues. All subsequent steps in the RNA isolation were the same as those described above for the bone marrow slides where DNA was precipitated away from RNA. Each fifty micron section yielded approximately 25 µg of RNA.

F. RNA/PCR Procedure

The strategy for amplification of mRNA sequences in the polymerase chain reaction (RNA/PCR) is based on that previously described (Kawasaki et al., 1988, Proc. Natl. Acad Sci. USA 85:5698-5702). Total cellular RNA was converted to a pool of cDNAs by reverse transcription. This cDNA was then subjected to PCR using gene-specific primer pairs. The primer pairs were designed to be homologous to exon sequences separated by one or more introns. Primer A, the upstream primer of the pair, comprised same sequence (5'--->3') as that determined for the non-template

strand used in RNA transcription of the gene (the same sequence as the RNA itself with thymine substituted for uracil). Primer B, the downstream primer, was designed to be complementary to and anti-parallel to the non-template strand. Primers were generally 21 to 24 bases in length (21- to 24-mers) with a GC content of 40-60%.

With the primer pair spanning an intron, PCR amplification of spliced mRNA results in amplified products of a predicted length containing only the exon sequences of the gene. Amplification of unspliced RNA or any contaminating genomic DNA in the RNA preparation yields products of a larger size including the intron sequences. The smaller product predicted from the spliced mRNA sequence will only be produced if spliced mRNA transcribed from the gene of interest is present. Therefore, the present of the predicted band on an ethidium bromide stained gel is an unequivocal assay for that gene's transcription or expression. This assay is referred as the Gel Visualization Assay (GVA) for gene expression.

The predicted amplified products were confirmed by use of internal probe hybridization to the PCR products. For this reason, oligonucleotides were prepared in sets of three: two as the amplimer pair and a third internal to both amplimers to be used as a probe for the resulting products of PCR. However, with confirmation of the predicted band, GVA can be used to screen for gene expression in a extremely rapid and sensitive fashion.

G. Ras Primers and Probes

Seven sets of ras-specific RNA/PCR primers were designed and are listed in Table 6. An upstream primer specific for exon 1 in each of the three human ras genes, c-N-ras (primer EK 221), c-Ha-ras-1 (EK 222), and c-Ki-ras-2 (EK 223) was prepared. these were each used separately in combination with a generic exon 2 downstream primer (EK 225) targeted to a conserved region. In addition, a generic upstream primer (EK 224) was used, paired with EK 225 to detect "pan"-ras expression. The products of each of these primer sets were predicted to be a size of 200 bp.

Primer pairs were also designed for each of the three genes to yield different-sized RNA/PCR products. Using GVA to score gene expression, the production of different-sized products allowed all three gene expression assays to be run simultaneously in the same reaction mixture and then viewed in a single lane of a gel. The result saved in enzyme expenses and allowed scale-up of the number of samples to be screened by a factor of three.

RNA/PCR products from c-N-ras (primers EK 365 and EK 366), c-Ha-ras-1 (EK 367 and EK 368), and c-Ki-ras-2 (EK 369 and EK 370) messages of 299 bp, 259

bp, and 234 bp, respectively. Each upstream primer was designed to have a greater than six base mismatch with the other two upstream primers to prevent cross amplification of the other ras messages. These three sets of primers were able to be used simultaneously in the same reaction mixture.

In these experiments, allele-specific oligonucleotide (ASO) probes hybridization was used to detect point mutations at the 12th and 61st codons to screen for activating point mutations in ras alleles. The 20-mer probes used are listed in Table 7. These probes were used according to the procedure described in Farr et al., 1988, Proc. Natl. Acad. Sci. USA 85:9268-9272.

H. Synthesis of cDNA from RNA

Complementary DNA (cDNA) was synthesized from the extracted total cellular RNA essentially as previously described (Kawasaki et al., 1988, Proc. Natl. Acad. Sci. USA 85:5698-5702). One microgram of total RNA was reverse transcribed with Moloney Murine Leukemia Virus (Mo-MuLV) reverse transcriptase (Bethesda Research Laboratories) in a 20 µl reaction in 1 X PCR buffer (50 mM KCl, 20 mM Tris-HCl pH 8.3, 2.5 mM $MgCl_2$, and 0.01% BSA) containing 20 U RNAsin (Promega), 1 µl of a 10 mM each stock of nucleotide triphosphates (dATP, dCTP, dGTP, and dTTP), and 100 pmoles of random hexamer primers. The change to random hexamer primers rather than oligo-dT primers was based on a recent report showing better yield of RNA/PCR products (Noonan and Roninson, 1988, Nucl. Acid Res. 16:10366). Reverse transcription reactions were incubated at room temperature for 15 minutes, 42°C for 30 minutes, and 95°C for five minutes. The 95°C incubation was done to heat denature the Mo-MuLV reverse transcriptase enzyme. These cDNAs were stored at 4°C until use in the PCR reaction.

I. PCR Reactions Using cDNA

The PCR method was used according to Saiki et al., as described for DNA using recombinant thermostable DNA polymerase originating from Thermus aquaticus (rTaq) (Perkin Elmer-Cetus). Slight modifications included reducing the amount of primers to 10 pmoles each, reducing the dNTPs to 1 µl of the 10 mM each stock described above, and using the rTaq enzyme at 2 U per reaction in a total 100 µl reaction. The substrate for RNA/PCR was 2 µl of the 20 µl cDNA described above. This amount corresponds to 100 ng of the initial 1 µg of total cellular RNA used in the reverse transcription reaction. Where three gene reactions were run simultaneously, this corresponds to a minimum of 30 gene expression studies from 1 µg of total cellular RNA.

PCR thermal profiles of 95°C for one minute (denaturing of double strands), 55°C for 30 seconds (annealing of amplimers), and 72°C for 30 seconds (synthesis of DNA) were performed in a programmable heat block (Perkin Elmer-Cetus) for 30-50 cycles. A 30 second synthesis step at 72°C was sufficient to produce RNA/PCR products of at least 935 bp.

J. Gel Visualization Assay (GVA) for mRNA Expression

Discrete gene expression was scored by the gel visualization assay (GVA) following RNA/PCR. RNA/PCR products were screened by running 9 µl of the reaction mixture in 2% NuSieve (FMC, Rockland, MD), 1% agarose gels in Tris-borate EDTA buffer (TBE). For size markers, the 123 bp DNA ladder (Bethesda Research Laboratories) was used. Gels of 75 ml were run in wide mini-sub cells (Bio-Rad Laboratories) in TBE at a constant 100 volts for approximately 90 minutes. Gels were stained in an ethidium bromide solution (0.5 µg per ml) for 30 minutes, detained for 30 minutes, and photographed under ultraviolet light with a Polaroid Land camera.

K. ASO Probing of RNA/PCR Products

RNA/ PCR products to be probed were run in 2% agarose gels in a Tris-borate EDTA electrophoresis buffer (TBE) in a mini-gel system. alkaline transfer to Zeta-Probe nylon filters (Bio-Rad Laboratories) in a wick-action transfer was done with a 0.4 N NaOH solution in water. Transfers were allowed to proceed for 90 minutes. Following transfer, blots were neutralized in 2X SSC for 5 minutes. Blots were prehybridized in a solution of 3 M tetra-methyl ammonium chloride (TMAC), 50 mM Tris-HCl pH 7.5, 2 mM EDTA, 5X Denhardt's solution, and 0.3% SDS at 55°C for one hour with circular agitation. Hybridization with ASO probes that were kinase-labeled with gamma-^{32}P-ATP was done in 5 ml of the TMAC buffer listed above with 2 x 10^6 cpm per ml of probe added. Hybridization continued at 55°C for one hour. The hybridization buffer and the first wash of 2X SSC with 0.1% SDS (50 ml) at room temperature were discarded as radioactive waste. A second wash was done at room temperature with 2X SCC with 0.1% SDS. Blots were quickly rinsed in the TMAC buffer minus the Denhardt's solution and extensively washed in the same buffer at 61°C for one hour. This wash was the critical wash for allowing the discriminatory ability of the ASO probes to distinguish point mutations. Blots were then blotted dry with Whatmann 3 MM paper and exposed to Kodak XAR film for at -70°C for one hour. Film was then developed in an automatic processor and data interpreted.

Table 10

Oligonucleotide primers for ras RNA/PCR. All sequences are listed in a 5'--->3' direction (in EK 224, K=T or G in EK 225, R=A or G).

EK 221: N-ras exon 1 (upstream)	GACTGAGTACAAAGTGGTGGTGG
EK 222: Ha-ras-1 exon 1 (upstream)	GACGGAATATAAGCTGGTGGTGG
EK 223: Ki-ras-2 exon 1 (upstream)	GACTGAATATAAACTTGTGGTAG
EK 224: generic ras exon 1 (upstream)	ATGACTGAATATAAACTGGTGGTGGTKGG
EK 225: generic ras exon 2 (downstream)	CATGTACTGGTCCCTCATGGCRCTG
EK 365: N-ras exon 1 (upstream)	CGGGGTCTCCAACATTTTTCC
EK 366: N-ras exon 2 (downstream)	GTATTGGTCTCTCATGGCACT
EK 367: Ha-ras-1 exon 1 (upstream)	AGGAGACCCTGTAGGAGGACC
EK 368: Ha-ras-1 exon 2 (downstream)	GTACTGGTCCCGCATGGCGCT
EK 369: Ki-ras-2 exon 1 (upstream)	CGGGAGAGAGGCCTGCTGAAA
EK 370: Ki-ras-2 exon 2 (downstream)	GTACTGGTCCCTCATTGCACT

Table 11

Oligonucleotides for ras ASO probing. All sequences are listed in a 5'--->3' direction (R=A or G; H=C, A, or T; V= A, C, or G; S=G, A, or T).

JN 01: Ha-ras-1 12 (w.t.)	GTGGGCGCCGGCGGTGTGGG
JN 02: Ha-ras-1 12-1	GTGGGCGCCHGCGGTGTGGG
JN 03: Ha-ras-1 12-2	GTGGGCGCCGHCGGTGTGGG
JN 04: Ha-ras-1 61 (w.t.)	TACTCCTCCTGGCCGGCGGT
JN 05: Ha-ras-1 61-1	TACTCCTCCTHGCCGGCGGT
JN 06: Ha-ras-1 61-2	TACTCCTCCVGGCCGGCGGT
JN 07: Ha-ras-1 61-3	TACTCCTCRTGGCCGGCGGT
JN 08: Ki-ras-2 12 (w.t.)	CCTACGCCACCAGCTCCAAC
JN 09: Ki-ras-2 12-1	CCTACGCCACSAGCTCCAAC
JN 10: Ki-ras-2 12-2	CCTACGCCASCAGCTCCAAC
JN 11: Ki-ras-2 61 (w.t.)	TACTCCTCTTGACCTGCTGT
JN 12: Ki-ras-2 61-1	TACTCCTCTTHACCTGCTGT
JN 13: Ki-ras-2 61-2	TACTCCTCTVGACCTGCTGT
JN 14: Ki-ras-2 61-3	TACTCCTCRTGACCTGCTGT
JN 15: N-ras 12/13 (w.t.)	GGAGCAGGTGGTGTTGGGAA
JN 16: N-ras 12-1	GGAGCAHGTGGTGTTGGGAA
JN 17: N-ras 12-2	GGAGCAGHTGGTGTTGGGAA
JN 18: N-ras 13-1	GGAGCAGGTHGTGTTGGGAA

JN 19: N-ras 13-2	GGAGCAGGTGHTGTTGGGAA
JN 20: N-ras 61 (w.t.)	TACTCTTCTTGTCCAGCTGT
JN 21: N-ras 61-1	TACTCTTCTTHTCCAGCTGT
JN 22: N-ras 61-2	TACTCTTCTVGTCCAGCTGT
JN 23: N-ras 61-3	TACTCTTCRTGTCCAGCTGT

Example 6

Results

The results of GVA of RNA/PCR amplification of human ras family mRNAs are shown in Figure 2. The samples used were a normal spleen and the cell line K562. Lanes 1 and 14 contain the 123 bp DNA ladder. Negative controls (no RNA) for each reaction are shown in lanes 4, 7, 10, and 13. Lanes 2-4 display the RNA/PCR products utilizing the "pan" ras primers EK 224 and EK 225 on the RNA from the normal human spleen, the K562 cell line, and the negative control of no RNA, respectively. As predicted, the 200 bp amplified product is present. Lanes 5-7 display the results using the c-N-ras-specific primers EK 365 and EK 336. The samples are displayed in the same sequence and as predicted, a 299 bp product is present indicating expression a the c-N-ras gene is both samples of human cells. c-Ha-ras-1 expression is shown in lanes 8-10. The primers EK 367 and EK 368 produce a 259 bp product and that is clearly seen in lane 9 (K562 cells). No product is seen from RNA isolated from the normal human spleen (lane 8). The lack of a product is interpreted to reflect lack of c-Ha-ras-2 mRNA or levels of the message below that detectable after 30 cycles of RNA/PCR in the normal human spleen. This result shows the utility of the GVA for the detection of mRNA after RNA/PCR.

Lanes 11-13 contain the RNA/PCR products using the c-Ki-ras-2 primers EK 369 and EK 370. The predicted product of 234 bp is present in both lanes 11 (normal human spleen RNA) and 12 (K-562 RNA) indicating expression of the gene in both samples; however, the abundance of message is less in the normal human spleen from that in the K562 cell line.

Example 7

Utilization of the ras RNA/PCR Products in the Screening for Activating Point Mutations

ASO probe hybridization with a different point mutation specific probe was conducted as described in Example 5. The results are shown in Figure 3.

The cell line samples on each blot are the same: lane 1, EJ/T24 RNA amplified with primers EK 222 and EK 225 (c-Ha-ras-1); lane 2, EJ/T24 RNA amplified with

primers EK 224 and EK 225 ("pan" ras); lane 3, PA-1 RNA amplified with primers EK 221 and EK 225 (C-N-ras); lane 4, SW-48- RNA amplified with primers EK 223 and EK 225 (c-Ki-ras-2); lane 5, SW-480 RNA amplified with primers EK 224 and EK 225 ("pan" ras); lane 6, HL-60 RNA amplified with primers EK 221 and EK 225 (c-N-ras); lane 7, Calu-1 RNA amplified with primers EK 223 and EK 225 (c-Ki-ras-2); lane 8, Calu-1 RNA amplified with primers EK 224 and EK 225 ("pan" ras); lane 9, G2101 RNA amplified with primers EK 224 and EK 225 ("pan" ras); lane 20, 123 bp DNA ladder. The blot in Panel A has been probed with a pool of oligonucleotides specific for activating point mutations at the second nucleotide of the 12th codon of c-Ha-ras-1 (JN 03).

As predicted, lanes 1 and 2 containing the EJ/T24 RNA amplified both the c-Ha-ras-1 primers and the "pan" ras primers are positive for the characterized EJ/T24 mutation. Panel B blot has been probed with a pool of oligonucleotides specific for activating point mutations at the second nucleotide of the 12th codon of c-N-ras (JN 17). The PA-2 cell line is known to contain a mutation at this position, and lane 3 is positive as expected. Panel C blot has been probed with oligonucleotide pool JN 09 targeted to mutations at the first nucleotide of codon 12 in c-Ki-ras-2.

The SW-480 cell line contains one of those mutations and lanes 4 and 5 containing RNA/PCR products for that cell line are positive. Because the signal in lane 5 is quite weak, it may indicate that the mutant allele's message is in low abundance with respect to all other ras messages in the cell as the "pan" ras primers were used for that lane or that the "pan" ras primers are less efficient at amplifying c-Ki-ras-2 messages with respect to the other two ras genes.

Panel D blot has been probed with pool JN 22 specific for mutations at the second position of the 61st codon of c-N-ras. Cell line HL-60 has a mutation at the position and is positive in lane 6. This panel, in combination with Panel B, illustrates that the RNA/PCR products amplified by the primers EK 221 and EK 225 (c-N-ras) contain sequences of both the 12th, 13th, and 61st codons of that gene. This is a significant advance for ras point mutation screening in that a single PCR reaction allows one to screen both activating hotspots in a single product as opposed to the need for two reactions when screening genomic DNA due to those two spots being present in exons 1 and 2 separated by a large intron. There is also the additional value of screening for expressed mutations in mRNA as opposed to the possibility of detecting mutations in non-expressed alleles.

Example 8

RNA/PCR products from alcohol-fixed paraffin-embedded samples were analyzed by GVA (Figure 4). Lanes 1, 5, and 12 contain the 123 bp DNA ladder. Samples in lanes 2, 6, and 9 have been amplified with primers EK 365 and 366 (c-N-ras: 299 bp), those in lanes 3, 7, and 10 with primers EK 367 and EK 368 (c-Ha-ras-1: 259 bp), and those in lanes 4, 8, and 11 with primers EK 369 and EK 370 (c-Ki-ras-2: 234 bp). Lanes 2-4 are the negative controls with no RNA added to the reverse transcriptase reaction of RNA/PCR. No products are seen in those lanes other than the primer dimers mentioned above. Lanes 6-8 contain RNA/PCR products from the Calu-1 cell line and the products corresponding to ras messages from all three genes are present. Lanes 9-11 contain the RNA/PCR products from the cell line G-2101. In this case, there is a lack of any signal from c-Ha-ras-1 messages indicating lack of expression.

These reactions were run for 50 cycles of RNA/PCR which increases the presence of background bands, but also the technique for precipitating DNA away from RNA which was used in the preparation of these RNAs is not totally efficient and can result in contaminating genomic DNA in the RNA preparations. The contaminating genomic DNA can result in specific amplification of the ras genes containing the intron between exons 1 and 2 but it will be a much larger size than that predicted for the spliced mRNA.

Example 9

The "pan" ras primers were used to amplify reverse transcribed RNA products. Sample preparation and the amplification procedure were as described in Example 4.

The results of "pan" ras amplification of RNA isolated from the air-dried stained microscope slide preparations of human bone marrow are shown in Figure 5. The predicted 200 bp RNA/PCR products using primers EK 224 and EK 225 are shown for the unstained slide (lane 3) and the stained slide (lane 4) adjacent to the negative control (no RNA) in lane 2 and the 123 bp DNA ladder in lane 1. These are the results of a 50 cycle RNA/PCR run.

Example 10

Detection of ras Mutations by Format II

The synthesis of biotinylated primers, poly T tailing of the probes and preparation of the format II filters are as described in commonly assigned, copending U.S. Serial No. 197,000 incorporated herein by reference (also see Chiang et al., 1989, BioTechniques 7(4):360).

Three sets of filters representing 21 mutation specific oligo probes for N-, H-, and K-ras, respectively, are shown in Figure 6. The sequences of the ras probes are listed in Table 12. All probes were designed to have approximately the same melting temperature (~50 -52°C) so that hybridization conditions could be standardized for all the oligonucleotides. Five pmoles of each tailed probe were spotted onto Biodyne Nylon membranes (Pall Biosupport, NY) and UV immobilized.

The ras oligonucleotide bound filters were hybridized in 5X SSPE, 0.5% SDS with alkali denatured PCR products for 60 minutes at 42°C. Washing was done in 3M tetramethylammonium chloride to minimize the influence of base composition among the various nucleotides. The filters were briefly rinsed with 2X SSPE, 0.1% SDS, then incubated in the same buffer with 2 µg/ml streptavidin-horse radish peroxidase conjugate ("Sequence," Cetus Corporation) for 30 minutes at room temperature. The filters were then washed for five minutes with the same buffer without the conjugate. Reagents of the ECL gene detection system (Amerrsham) were added and incubated for one minute at room temperature. Filters were then wrapped in Saran wrap and the light signal produced was detected by exposing Kodak XRP film to the filters for 20 seconds to one minute.

Table 12

Individual ras Probes for Format II Detection

N-ras codon 12	YZ1	GAGCAGGTGGTGTTGG
	YZ21	GAGCAAGTGGTGTTGG
	YZ22	GAGCATGTGGTGTTGG
	YZ23	GAGCACGTGGTGTTGG
	YZ2	GAGCAGATGGTGTTGG
	YZ24	GAGCAGCTGGTGTTGG
	YZ25	GAGCAGTTGGTGTTGG
N-ras codon 13	YZ26	GCAGGTAGTGTTGGGA
	YZ50	GCAGGTTGTGTTGGGA
	YZ27	GCAGGTCGTGTTGGGA
	YZ28	GCAGGTGATGTTGGGA
	YZ29	GCAGGTGCTGTTGGGA
	YZ3	GCAGGTGGTGTTGGGA

N-ras codon 16	YZ4	AGCTGGACAAGAAGAGT
	YZ30	AGCTGGAGAAGAAGAGT
	YZ5	CAGCTGGAAAAGAAGAG
	YZ31	AGCTGGACGAGAAGAG
	YZ6	AGCTGGACTAGAAGAGT
	YZ32	AGCTGGACCAGAAGAG
	YZ51	AGCAGGACTGAAGAGT
	YZ33	AGCAGGACACGAAGAG
H-ras codon 12	YZ7	GGAGCCGGCGGTG
	YZ34	GGCGCCAGCGGTGT
	YZ35	GGCGCCTGCGGTGT
	YZ36	GGCGCCCGCGGTG
	YZ37	GGCGCCGACGGTGT
	YZ8	GGCGCCGTCGGTGT
	YZ38	GGCGCCGCCGGTG
H-ras codon 13	YZ39	GCCGGCAGTGTGGG
	YZ9	GCCGGCTGTGTGGG
	YZ40	GCCGGCCGTGTGGG
	YZ41	GCCGGCGATGTGGG
	YZ42	GCCGGCGCTGTGGG
	YZ43	GCCGGCGTTGTGGG
H-ras codon 61	YZ10	GCCGGCCAGGAGGA
	YZ11	GCCGGCGAGGAGGA
	YZ44	CGCCGGCAAGGAGG
	YZ45	GCCGGCCGGGAGG
	YZ46	GCCGGCCTGGAGGA
	YZ47	GCCGGCCCGGAGG
	YZ48	CGCCGGCCATGAGG
	YZ49	GCCGGCCACGAGGA
K-ras codon 12	YZ52	GGAGCTGGTGGCGTA
	YZ53	GGAGCTAGTGGCGTAG
	YZ54	GGAGCTTGTGGCGTAG
	YZ55	GGAGCTCGTGGCGTA
	YZ56	GGAGCTGATGGCGTAG
	YZ57	GGAGCTGCTGGCGTA
	YZ58	GGAGCTGTTGGCGTAG
K-ras codon 13	YZ59	GCTGGTAGCGTAGGC
	YZ60	GCTGGTTGCGTAGGC
	YZ61	GCTGGTCGCGTAGGC
	YZ62	GCTGGTGACGTAGGC
	YZ63	GCTGGTGTCGTAGGC
	YZ64	GCTGGTGCCGTAGGC

K-ras codon 61	YZ65	AGCAGGTCAAGAGGAG
	YZ66	AGCAGGTGAAGAGGAG
	YZ67	AGCAGGTAAAGAGGAGT
	YZ68	GCAGGTCGAGAGGAG
	YZ69	AGCAGGTCTAGAGGAG
	YZ70	GCAGGTCCAGAGGAG
	YZ71	AGCAGGTCATGAGGAG
	YZ72	GCAGGTCACGAGGAG

In the Claims

1. A method for detecting a G-protein α subunit point mutation in a nucleic acid segment present in a biological sample, said method comprising:

(a) treating the sample with a G-protein α subunit primer pair, an agent for polymerization, and deoxynucleoside 5' triphosphates under conditions such that an extension product of each primer can be synthesized, wherein said primers are sufficiently complementary to separate strands of a nucleic acid encoding a segment of a G-protein α subunit to hybridize thereto so that the extension product synthesized from one member of said pair, when separated from its complementary strand, can serve as a template for synthesis of the extension product of the other member of said pair;

(b) separating the primer extension products from the templates on which the extension products were synthesized to form single-stranded molecules;

(c) treating the single-stranded molecules generated in step (b) with the primers of step (a) under conditions such that a primer extension product is synthesized using each of the single-stranded molecules produced in step (b) as a template;

(d) repeating steps (b) and (c) at least once to provide amplified DNA;

(e) hybridizing a G-protein α subunit probe to said amplified DNA, wherein said probe contains a nucleic acid sequence that will hybridize to a sequence selected from a wild type and a mutant nucleic acid sequence within said amplified DNA; and

(f) determining if hybridization has occurred.

2. The method of Claim 1, wherein steps (b) and (c) are repeated at least five times and said agent of polymerization is a thermostable DNA polymerase.

3. The method of Claim 2, wherein said thermostable DNA polymerase is Taq polymerase.

4. The method of Claim 1, wherein the sample is removed from a human tumor.

5. The method of Claim 4, wherein said human tumor is an endocrine tumor.

6. The method of Claim 1, wherein said primer pair will amplify a nucleic acid segment encoding a subsequence of a G-protein α subunit selected from the group consisting of Giα1, Giα2, Giα3, Gzα, Goα, and Gsα.

7. The method of Claim 6, wherein said nucleic acid segment encodes at least one amino acid selected from the group consisting of the amino acids corresponding to Gsα 49, 201, and 227.

8. The method of Claim 7, wherein said primer pair is selected from the group consisting of: JFL69 and JFL70; JFL135 and JFL136; JFL228 and JFL135; JFL229 and JFL136; JFL226 and JFL227; JFL223 and JFL224; JL54 and JL57; JFL109 and JLF110; JFL110 and JFL112; JFL113 and JFL114; JFL115 and JFL113; SP9 and SP10; JFL139 and SP11; JFL201 and SP15; JL55 and JL56; JFL223 and SP33; and JFL224 and SP34; JFL235 and JFL237; JL55 and JFL212; JFL215 and JL56; and JFL135 and JFL286.

9. The method of Claim 1, wherein said probe comprises a sequence which hybridizes to DNA encoding a subsequence of a G-protein subunit selected from the group consisting of Giα1, Giα2, Giα3, Goα, Gsα, and Gzα.

10. The method according to Claim 1, wherein said probe hybridizes to a G-protein subsequence encoding the amino acid corresponding to the Gsα amino acid at position 49, 201, or 227.

11. The method of Claim 10, wherein said probe is selected from the group consisting of

5' TA GGT GCT GGA GAA TCT GGT 3';
5' TA GGT GCT AGA GAA TCT GGT 3';
5' TA GGT GCT CGA GAA TCT GGT 3';
5' TA GGT GCT GAA GAA TCT GGT 3';
5' TA GGT GCT GCA GAA TCT GGT 3';
5' TA GGT GCT GTA GAA TCT GGT 3';

5' TT CGC TGC CGT GTC CTG ACT 3';
5' TT CGC TGC TGT GTC CTG ACT 3';
5' TT CGC TGC GGT GTC CTG ACT 3';
5' TT CGC TGC AGT GTC CTG ACT 3';
5' TT CGC TGC CAT GTC CTG ACT 3';
5' TT CGC TGC CCT GTC CTG ACT 3';
5' TT CGC TGC CTT GTC CTG ACT 3';

5' GTG GGT GGC CAG CGC GAT GA 3';
5' GTG GGT GGC TAG CGC GAT GA 3';
5' GTG GGT GGC GAG CGC GAT GA 3';
5' GTG GGT GGC AAG CGC GAT GA 3';
5' GTG GGT GGC CGT CGC GAT GA 3';
5' GTG GGT GGC CCG CGC GAT GA 3';
5' GTG GGT GGC CTG CGC GAT GA 3';
5' GTG GGT GGC CAC CGC GAT GA 3';
5' GTG GGT GGC CAT CGC GAT GA 3';

5' TC AGA ACT AGA GTG AAA ACT 3';
5' TC AGA ACT AGC GTG AAA ACT 3';
5' TC AGA ACT AGT GTG AAA ACT 3';
5' TC AGA ACT GGA GTG AAA ACT 3';
5' TC AGA ACT ACA GTG AAA ACT 3';
5' TC AGA ACT AAA GTG AAA ACT 3';
5' TC AGA ACT ATA GTG AAA ACT 3';

5' TG GGA GGT CAG AGA TCT GAG 3';
5 TG GGA GGT GAG AGA TCT GAG 3';
5' TG GGA GGT AAG AGA TCT GAG 3';
5' TG GGA GGT CTG AGA TCT GAG 3';
5' TG GGA GGT CCG AGA TCT GAG 3';
5' TG GGA GGT CGG AGA TCT GAG 3';
5' TG GGA GGT CAT AGA TCT GAG 3';
5' TG GGA GGT CAC AGA TCT GAG 3';

5' TA CGG ACC CGC GTA AAG ACC 3';
5' TA CGG ACC AGC GTA AAG ACC 3';
5' TA CGG ACC GGC GTA AAG ACC 3';
5' TA CGG ACC TGC GTA AAG ACC 3';
5' TA CGG ACC CAC GTA AAG ACC 3';
5' TA CGG ACC CCC GTA AAG ACC 3';
5' TA CGG ACC CTC GTA AAG ACC 3';

5' GTG GGT GGT CAG CGG TCT GA 3';
5' GTG GGT GGT CTG CGG TCT GA 3';
5' GTG GGT GGT CCG CGG TCT GA 3';
5' GTG GGT GGT CGG CGG TCT GA 3';
5' GTG GGT GGT AAG CGG TCT GA 3';
5' GTG GGT GGT GAG CGG TCT GA 3';
5' GTG GGT GGT CAT CGG TCT GA 3';
5' GTG GGT GGT CAC CGG TCT GA 3';

5' TT CGG ACG AGA GTG AAG ACC 3';
5' TT CGG ACG AGC GTG AAG ACC 3';
5' TT CGG ACG AGT GTG AAG ACC 3';
5' TT CGG ACG GGA GTG AAG ACC 3';
5' TT CGG ACG ATA GTG AAG ACC 3';
5' TT CGG ACG ACA GTG AAG ACC 3';
5' TT CGG ACG AAA GTG AAG ACC 3';

5' GTA GGT GGC CAA AGA TCA GA 3';
5' GTA GGT GGC CAT AGA TCA GA 3';
5' GTA GGT GGC CAC AGA TCA GA 3';
5' GTA GGT GGC CTA AGA TCA GA 3';
5' GTA GGT GGC CCA AGA TCA GA 3';
5' GTA GGT GGC CGA AGA TCA GA 3';
5' GTA GGT GGC AAA AGA TCA GA 3';
5' GTA GGT GGC GAA AGA TCA GA 3';

5' CTC CGA ACC AGG GTC AAA AC 3';
5' CTC CGA ACC GGG GTC AAA AC 3';
5' CTC CGA ACC TGG GTC AAA AC 3';
5' CTC CGA ACC AAG GTC AAA AC 3';
5' CTC CGA ACC ACG GTC AAA AC 3';
5' CTC CGA ACC ATG GTC AAA AC 3';
5' CTC CGA ACC AGC GTC AAA AC 3';
5' CTC CGA ACC AGT GTC AAA AC 3';

5' GTC GGA GGC CAG CGA TCT GA 3';
5' GTC GGA GGC AAG CGA TCT GA 3';
5' GTC GGA GGC GAG CGA TCT GA 3';
5' GTC GGA GGC CCG CGA TCT GA 3';
5' GTC GGA GGC CGG CGA TCT GA 3';
5' GTC GGA GGC CTG CGA TCT GA 3';
5' GTC GGA GGC CAT CGA TCT GA 3';
5' GTC GGA GGC CAT CAC TCT GA 3';

5' TG CGC TCC CGG GAC ATG ACC 3';
5' TG CGC TCC GGG GAC ATG ACC 3';
5' TG CGC TCC TGG GAC ATG ACC 3';
5' TG CGC TCC CAG GAC ATG ACC 3';
5' TG CGC TCC CCG GAC ATG ACC 3';
5' TG CGC TCC CTG GAC ATG ACC 3';

5' TG GGG GGG CAG AGG TCA GAG 3';
5' TG GGG GGG AAG AGG TCA GAG 3';
5' TG GGG GGG GAG AGG TCA GAG 3';
5' TG GGG GGG CCG AGG TCA GAG 3';
5' TG GGG GGG CTG AGG TCA GAG 3';
5' TG GGG GGG CAC AGG TCA GAG 3';
5' TG GGG GGG CAT AGG TCA GAG 3';
5' TG GGG GGG CGG AGG TCA GAG 3';

5' TCG CTG CCG TGT CCT GGA C 3';
5' TCG CTG CAG TGT CCT GGA CT 3';
5' TCG CTG CGG TGT CCT GGA C 3';
5' TCG CTG CTG TGT CCT GGA CT 3';
5' TCG CTG CCA TGT CCT GGA CT 3';
5' TCG CTG CCT TGT CTT GGA CT 3';
5' TGG GTG GCC AGC GGC GAT GA 3';
5' TGG GTG GCC TGC GCG ATG A 3';
5' TGG GTG GCC CGC GCG ATG 3';
5' TGG GTG GCC GGC GCG ATG 3';
5' TGG GTG GCG AGC GCG ATG A 3';

5'	TGG	GTG	GCT	AGC	GCG	ATG	A	3';
5'	TGG	GTG	GCC	ATC	GCG	ATG	A	3';
5'	TGG	GTG	GCA	AGC	GCG	ATG	A	3'; and
5'	TGG	GTG	GCC	ACC	GCG	ATG		3'.

12. The method of Claim 1, wherein said nucleic acid is RNA and is reverse transcribed to provide a double-stranded cDNA copy prior to step (a).

13. The method of Claim 1, wherein said sample is paraffin embedded tissue.

14. An oligonucleotide primer pair for the amplification of a subsequence of a nucleic acid encoding a G-protein α subunit wherein said subsequence comprises a nucleic acid sequence encoding an amino acid corresponding to the Gsα amino acid at a position selected from the group consisting of 49, 201, and 227.

15. An oligonucleotide primer pair according to Claim 14, wherein said G-protein α subunit is selected from the group consisting of Gsα, Goα, Giα1, Giα2, Giα3, and Gzα.

16. An oligonucleotide probe for distinguishing between oncogenes and proto-oncogenes encoding G-protein α subunits, wherein said probe hybridizes to a region of nucleic acid encoding an amino acid selected from the group consisting amino acids corresponding to the Gsα amino acid at positions 49, 201, and 227.

17. An oligonucleotide primer pair for the amplification of a subsequence of a nucleic acid encoding a G-protein α subunit wherein the primer pair is selected from the group consisting of: JFL69 and JFL70; JFL135 and JFL136; JFL228 and JFL135; JFL229 and JFL136; JFL226 and JFL227; JFL223 and JFL224; JL54 and JL57; JFL109 and JLF110; JFL110 and JFL112; JFL113 and JFL114; JFL115 and JFL113; SP9 and SP10; JFL139 and SP11; JFL201 and SP15; JL55 and JL56; JFL223 and SP33; and JFL224 and SP34; JFL235 and JFL237; JL55 and JFL212; JFL215 and JL56; and JFL135 and JFL286.

18. A probe for the detection of a point mutation in a nucleic acid encoding a segment of a G-protein α subunit wherein the probe is selected from the group consisting of:

5' TA GGT GCT GGA GAA TCT GGT 3';
5' TA GGT GCT AGA GAA TCT GGT 3';
5' TA GGT GCT CGA GAA TCT GGT 3';
5' TA GGT GCT GAA GAA TCT GGT 3';
5' TA GGT GCT GCA GAA TCT GGT 3';
5' TA GGT GCT GTA GAA TCT GGT 3';

5' TT CGC TGC CGT GTC CTG ACT 3';
5' TT CGC TGC TGT GTC CTG ACT 3';
5' TT CGC TGC GGT GTC CTG ACT 3';
5' TT CGC TGC AGT GTC CTG ACT 3';
5' TT CGC TGC CAT GTC CTG ACT 3';
5' TT CGC TGC CCT GTC CTG ACT 3';
5' TT CGC TGC CTT GTC CTG ACT 3';

5' GTG GGT GGC CAG CGC GAT GA 3';
5' GTG GGT GGC TAG CGC GAT GA 3';
5' GTG GGT GGC GAG CGC GAT GA 3';
5' GTG GGT GGC AAG CGC GAT GA 3';
5' GTG GGT GGC CGT CGC GAT GA 3';
5' GTG GGT GGC CCG CGC GAT GA 3';
5' GTG GGT GGC CTG CGC GAT GA 3';
5' GTG GGT GGC CAC CGC GAT GA 3';
5' GTG GGT GGC CAT CGC GAT GA 3';

5' TC AGA ACT AGA GTG AAA ACT 3';
5' TC AGA ACT AGC GTG AAA ACT 3';
5' TC AGA ACT AGT GTG AAA ACT 3';
5' TC AGA ACT GGA GTG AAA ACT 3';
5' TC AGA ACT ACA GTG AAA ACT 3';
5' TC AGA ACT AAA GTG AAA ACT 3';
5' TC AGA ACT ATA GTG AAA ACT 3';

5' TG GGA GGT CAG AGA TCT GAG 3';
5 TG GGA GGT GAG AGA TCT GAG 3';
5' TG GGA GGT AAG AGA TCT GAG 3';
5' TG GGA GGT CTG AGA TCT GAG 3';
5' TG GGA GGT CCG AGA TCT GAG 3';
5' TG GGA GGT CGG AGA TCT GAG 3';
5' TG GGA GGT CAT AGA TCT GAG 3';
5' TG GGA GGT CAC AGA TCT GAG 3';

5' TA CGG ACC CGC GTA AAG ACC 3';
5' TA CGG ACC AGC GTA AAG ACC 3';
5' TA CGG ACC GGC GTA AAG ACC 3';
5' TA CGG ACC TGC GTA AAG ACC 3';
5' TA CGG ACC CAC GTA AAG ACC 3';
5' TA CGG ACC CCC GTA AAG ACC 3';
5' TA CGG ACC CTC GTA AAG ACC 3';

5' GTG GGT GGT CAG CGG TCT GA 3';
5' GTG GGT GGT CTG CGG TCT GA 3';
5' GTG GGT GGT CCG CGG TCT GA 3';
5' GTG GGT GGT CGG CGG TCT GA 3';
5' GTG GGT GGT AAG CGG TCT GA 3';
5' GTG GGT GGT GAG CGG TCT GA 3';
5' GTG GGT GGT CAT CGG TCT GA 3';
5' GTG GGT GGT CAC CGG TCT GA 3';

5' TT CGG ACG AGA GTG AAG ACC 3';
5' TT CGG ACG AGC GTG AAG ACC 3';
5' TT CGG ACG AGT GTG AAG ACC 3';
5' TT CGG ACG GGA GTG AAG ACC 3';
5' TT CGG ACG ATA GTG AAG ACC 3';
5' TT CGG ACG ACA GTG AAG ACC 3';
5' TT CGG ACG AAA GTG AAG ACC 3';

5' GTA GGT GGC CAA AGA TCA GA 3';
5' GTA GGT GGC CAT AGA TCA GA 3';
5' GTA GGT GGC CAC AGA TCA GA 3';
5' GTA GGT GGC CTA AGA TCA GA 3';
5' GTA GGT GGC CCA AGA TCA GA 3';
5' GTA GGT GGC CGA AGA TCA GA 3';
5' GTA GGT GGC AAA AGA TCA GA 3';
5' GTA GGT GGC GAA AGA TCA GA 3';

5' CTC CGA ACC AGG GTC AAA AC 3';
5' CTC CGA ACC GGG GTC AAA AC 3';
5' CTC CGA ACC TGG GTC AAA AC 3';
5' CTC CGA ACC AAG GTC AAA AC 3';
5' CTC CGA ACC ACG GTC AAA AC 3';
5' CTC CGA ACC ATG GTC AAA AC 3';
5' CTC CGA ACC AGC GTC AAA AC 3';
5' CTC CGA ACC AGT GTC AAA AC 3';

5' GTC GGA GGC CAG CGA TCT GA 3';
5' GTC GGA GGC AAG CGA TCT GA 3';
5' GTC GGA GGC GAG CGA TCT GA 3';
5' GTC GGA GGC CCG CGA TCT GA 3';
5' GTC GGA GGC CGG CGA TCT GA 3';
5' GTC GGA GGC CTG CGA TCT GA 3';
5' GTC GGA GGC CAT CGA TCT GA 3';
5' GTC GGA GGC CAT CAC TCT GA 3';

5' TG CGC TCC CGG GAC ATG ACC 3';
5' TG CGC TCC GGG GAC ATG ACC 3';
5' TG CGC TCC TGG GAC ATG ACC 3';
5' TG CGC TCC CAG GAC ATG ACC 3';
5' TG CGC TCC CCG GAC ATG ACC 3';
5' TG CGC TCC CTG GAC ATG ACC 3';

5' TG GGG GGG CAG AGG TCA GAG 3';
5' TG GGG GGG AAG AGG TCA GAG 3';

5' TG GGG GGG GAG AGG TCA GAG 3';
5' TG GGG GGG CCG AGG TCA GAG 3';
5' TG GGG GGG CTG AGG TCA GAG 3';
5' TG GGG GGG CAC AGG TCA GAG 3';
5' TG GGG GGG CAT AGG TCA GAG 3';
5' TG GGG GGG CGG AGG TCA GAG 3';

5' TCG CTG CCG TGT CCT GGA C 3';
5' TCG CTG CAG TGT CCT GGA CT 3';
5' TCG CTG CGG TGT CCT GGA C 3';
5' TCG CTG CTG TGT CCT GGA CT 3';
5' TCG CTG CCA TGT CCT GGA CT 3';
5' TCG CTG CCT TGT CTT GGA CT 3';
5' TGG GTG GCC AGC GGC GAT GA 3';
5' TGG GTG GCC TGC GCG ATG A 3';
5' TGG GTG GCC CGC GCG ATG 3';
5' TGG GTG GCC GGC GCG ATG 3';
5' TGG GTG GCG AGC GCG ATG A 3';
5' TGG GTG GCT AGC GCG ATG A 3';
5' TGG GTG GCC ATC GCG ATG A 3';
5' TGG GTG GCA AGC GCG ATG A 3'; and
5' TGG GTG GCC ACC GCG ATG 3'.

19. A method for detecting a point mutation, if present, in a nucleic acid encoding a G-protein α subunit in a sample comprising:

(a) hybridizing a G-protein α subunit probe to said sample, and

(b) determining whether hybridization has occurred.

20. A kit for detecting point mutations in a nucleic acid encoding a G-protein α subunit, comprising, in a separate container:

(a) a G-protein primer pair suitable for providing an amplified G-protein DNA segment in a PCR reaction;

(b) a G-protein probe comprising a wild type sequence which will hybridized to said DNA segment if it is a wild type; and

(c) a G-protein probe comprising a sequence containing a point mutation for detecting a point mutation if present in said DNA segment.