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Most Human Carcinomas of the Exocrine Pancreas Contain Mutant c-K-*ras* Genes

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Summary

Using in vitro gene amplification by the polymerase chain reaction (PCR) and mutation detection by the RNAase A mismatch cleavage method, we have examined c-K-*ras* genes in human pancreatic carcinomas. We used frozen tumor specimens and single 5 μm sections from formalin-fixed, paraffin-embedded tumor tissue surgically removed or obtained at autopsy. Twenty-one out of 22 carcinomas of the exocrine pancreas contained c-K-*ras* genes with mutations at codon 12. In seven cases tested, the mutation was present in both primary tumors and their corresponding metastases. No mutations were detected in normal tissue from the same cancer patients or in five gall bladder carcinomas. We conclude from these results that c-K-*ras* somatic mutational activation is a critical event in the oncogenesis of most, if not all, human cancers of the exocrine pancreas.

Introduction

The product of the mammalian *ras* gene family belongs to the group of proto-oncogene products involved in cell growth and differentiation. The membrane bound *ras* proteins possess guanine nucleotide binding and low intrinsic hydrolytic activities and appear to be involved in the process of signal transduction across the cellular membrane in mammals (reviewed in Barbacid, 1987). Single amino acid substitutions at several positions in the protein lead to the activation of the *ras* oncogenic potential in cultured rodent fibroblasts (reviewed in Varmus, 1984).

The role that somatic mutational activation of *ras* genes plays in mammalian tumorigenesis has been well documented in several carcinogen-induced animal tumor model systems (reviewed in Barbacid, 1987). The high incidence of *ras* oncogenes and the carcinogen-specific nature of the activating mutations observed in some of these systems provide strong support for the notion that *ras* activation is playing a reproducible and predictable role in the initiation and/or progression of chemical carcinogenesis.

ras oncogenes activated by single nucleotide substitutions have been associated with a significant percentage of human tumors of diverse histological origins (for a recent review, see Bos, 1987). However, information on the incidence of activated *ras* oncogenes in human cancer is still very incomplete. Recent advances in methods able to detect single point mutations in higher eukaryotic genes are beginning to provide accurate information on the frequency of *ras* oncogene activation in several types of human tumors. For instance, activated c-K-*ras* oncogenes have been found in approximately 40% of human colorectal tumors (Bos et al., 1987, Forrester et al., 1987a). However, in contrast with some carcinogen-induced animal tumor model systems, no examples of activated *ras* oncogenes present in the majority of a well-defined class of spontaneous human tumor have been reported.

Using a combination of techniques, including specific in vitro gene amplification by the polymerase chain reaction or PCR (Mullis and Faloona, 1987; Saiki et al., 1985), the application of PCR to 5–10 μm sections of formalin-fixed, paraffin-embedded tissue (Shibata et al., 1988), and mutation detection by cleavage at single base mismatches by RNAase A in DNA:RNA and RNA:RNA heteoduplexes (Myers et al., 1985; Winter et al., 1985), we report here the presence of c-K-*ras* genes with mutations at codon 12 in 21 out of 22 human carcinomas of the exocrine pancreas.

Results

Detection of Mutant c-K-*ras* Genes in Human Pancreatic Carcinomas by RNAase A Cleavage of RNA: Amplified DNA Hybrids

As part of our screening of primary human tumors for the presence of mutant c-K-*ras* genes using the RNAase A mismatch cleavage method (Myers et al., 1985; Winter et al., 1985), we analyzed pancreatic carcinomas. Total cellular RNA was prepared from frozen tumors and hybridized to an antisense RNA probe corresponding to the first coding exon of the c-K-*ras* gene. The RNA hybrids were then digested with RNAase A, and the resistant products were analyzed by denaturing polyacrylamide gel electrophoresis. Mutant oncogenes were detected by the presence of RNA subbands generated by RNAase A cleavage at the mismatches present in the RNA:RNA heteroduplexes (Forrester et al., 1987a). Four human carcinomas of the exocrine pancreas that we tested contained mutant c-K-*ras* genes at codon 12 (data not shown and Table 1), suggesting that the frequency of mutant c-K-*ras* genes could be very high in this type of human carcinoma.

Fresh specimens of pancreatic carcinomas are very scanty because of the poor prognosis of this type of human cancer, which often precludes surgery. Therefore, we adapted the RNAase A mismatch cleavage method to the analysis of single-point mutations in c-K-*ras* sequences present in formalin-fixed paraffin-embedded tumor specimens, which are readily available from biopsies, surgical resections, or autopsies. The method is based on the use of in vitro gene amplification by PCR (Mullis and Faloona, 1987; Saiki et al., 1985), and on the observation that a sin-

Table 1. Incidence of c-K-*ras* Genes Mutant at Codon 12 in Human Pancreatic Carcinomas

Case	Donor[a]	Tissue[b] N	P	M	Comments[c]
1	67 M	NA	+	NA	(A)
2	64 F	NA	NA	+	(B) (C)
3	65 M	–	+	NP	(A) (C)
4	69 F	NA	+	NA	(B) (C)
5	38 M	–	+	NA	(C)
6	54 M	–	+	+	
7	32 M	–	+	+	
8	63 F	–	+	+	
9	35 M	–	+	+	
10	58 M	ND	ND	+	
11	51 F	–	+	NA	(C)
12	59 F	–	+	NP	(C)
13	62 F	–	+	NP	
14	54 M	–	+	ND	
15	68 M	–	+	NA	
16	54 M	–	+	+	(C)
17	83 F	–	–	–	(D)
18	80 F	–	+	NP	
19	68 M	–	+	+	
20	81 M	–	+	NP	
21	60 F	ND	+	+	
22	65 M	–	ND	+	

[a] The age and sex of the cancer patients is indicated.

[b] Presence (+) or absence (–) of mutant c-K-*ras* genes in normal (N), primary (P), or metastatic (M) carcinoma pancreatic tissue of the same patient. NA: tissue not available. ND: not determined because of inadequate DNA amplification. NP: metastases not present.

[c] Unless otherwise indicated, the samples were analyzed using 5 μm formalin-fixed paraffin-embedded tissue sections obtained from autopsy cases by PCR amplification of c-K-*ras* sequences, followed by RNAase A mismatch cleavage of the DNA:RNA hybrids.

(A) Cases analyzed by RNAase A cleavage of both DNA:RNA and RNA:RNA hybrids. PCR was performed with DNA purified from nonfixed tumor specimens.

(B) Cases analyzed by RNAase A mismatch cleavage of RNA:RNA duplexes using total RNA from nonfixed frozen tumors. The experimental conditions for RNA preparation, hybridization, and RNAase A digestion were as described previously (Winter et al., 1985; Forrester et al., 1987a).

(C) Specimens surgically removed.

(D) This patient survived 9 months after diagnosis while the average survival of ten patients with the activated c-K-*ras* gene was only 1.9 months (range 1–4 months).

gle 5–10 μm thick formalin-fixed paraffin section is a suitable substrate for PCR (Shibata et al., 1988). A 5 μm section of the fixed tissue is stained with hematoxylin and eosin for histological identification. The adjacent 5 μm section of the specimen is treated to remove the paraffin by extraction with organic solvents, and the tissue is then subjected to PCR. Primers complementary to the coding and noncoding DNA strands flanking the first coding exon of the c-K-*ras* gene are extended by E. coli DNA polymerase I (Klenow fragment). The amplification reaction is performed by successive cycles of denaturation, annealing, and primer-mediated elongation (Saiki et al., 1985). The amplified DNA product is hybridized to a RNA probe corresponding to the first coding exon of the c-K-*ras* gene (Figure 1), and the RNA:DNA hybrids are digested with RNAase A and analyzed as described above. The result of a typical experiment is shown in Figure 2. When DNA

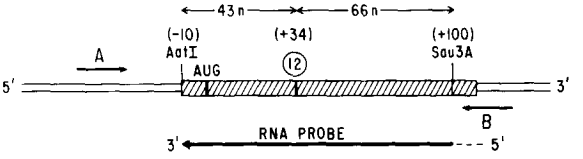

Figure 1. Schematic Representation of PCR-Mediated Amplification of c-K-*ras* Gene Sequences

The region flanking the c-K-*ras* first coding exon is indicated with the position of the oligomers (A and B) used for in vitro amplification. The numbers indicate the distances (in nucleotides) and relative positions of the region covered by the RNA probe as well as codon 12. The open bars indicate intron sequences, the stippled bar exon sequences. For details see Experimental Procedures.

amplified from surgically removed normal pancreatic tissue was hybridized to the RNA probe and digested with RNAase A, only a major protected band of about 110 nucleotides was observed (lane 3N, corresponding to case 3 of Table 1). In contrast, RNAase A treatment of the DNA:RNA hybrids corresponding to amplified DNA biochemically purified from the SK-CO-1 tumor cell line (lane S), which contains a c-K-*ras* gene with a valine mutation resulting from a G to T substitution at the second position of codon 12 (Forrester et al., 1987b), generated two codon 12 mismatch-specific subbands of 66 and 43 nucleotides (see Figure 1). These subbands were also present after hybridization and RNAase A treatment of DNA amplified from 5 μm sections of three distinct pancreatic carci-

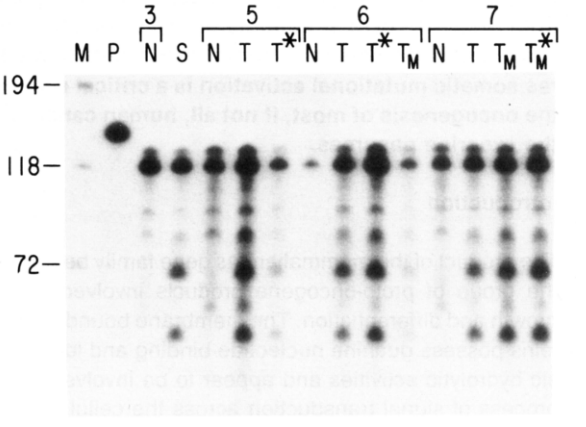

Figure 2. RNAase A Mismatch Cleavage Analysis of PCR-Amplified c-K-*ras* DNA Sequences from Human Carcinomas of the Exocrine Pancreas

DNA sequences amplified from 5 μm sections of formalin-fixed pancreatic tissues (5 μl of the 200 μl PCR reaction) were hybridized to an uniformly labeled RNA probe directed by pAKINgly. The hybrids were digested with RNAase A for 30 min and analyzed in a denaturing urea-polyacrylamide gel. N: sections corresponding to normal pancreatic tissue. T and Tm: primary and metastatic tumor tissue, respectively; T*: smaller areas of the sections (see Figure 3). The numbers at the top indicate the cases corresponding to Table 1. 3N and S correspond to amplified c-K-*ras* sequences (1 μl of the final PCR reactions) using purified DNA (1 μg) from normal pancreas of case 3 and SK-CO-1, respectively. Numbers at left indicate the position and size (in nucleotides) of HaeIII restriction fragments of φX174 DNA used as molecular weight markers (lane M). Lane P: undigested RNA probe (100 cpm). The picture corresponds to a 20 hr exposure of the X-ray film.

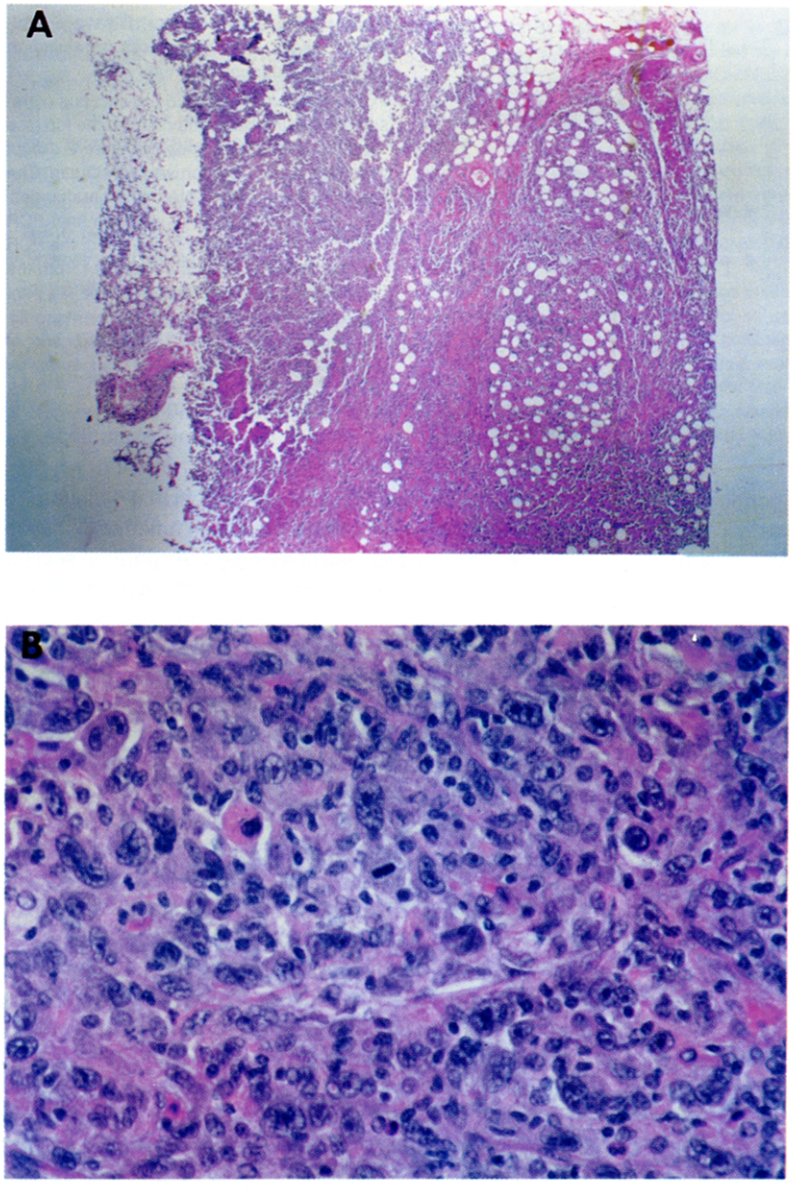

Figure 3. Histology of Paraffin Sections of Pancreatic Carcinomas Stained with Hematoxylin and Eosin

(A) Case 6T* : The section (0.4 × 0.4 cm) shows a poorly differentiated primary pancreatic ductal adenocarcinoma (20×).
(B) Case 5T* : The section (0.5 × 0.5 cm) shows a poorly differentiated primary pancreatic ductal adenocarcinoma (400×).

nomas (cases 5, 6, and 7). Moreover, similar RNAase A cleavage patterns were obtained with amplified DNA from two different regions of the tumor specimens, a large area (T) and a smaller area (T*) and from either primary (T) or metastatic (Tm) tumors from cases 6 and 7. However, the mismatch-specific bands were absent when DNA was amplified from 5 μm formalin-fixed paraffin sections of normal pancreatic tissue from these cancer patients (lanes N). Other fainter bands were also present above and below the 110 nucleotide protected band. However, these bands were present in all samples, and probably represent incomplete digestion products and nonspecific RNAase A cleavage at AT-rich regions in the DNA:RNA heteroduplexes. The differences in the relative intensities of the bands between lanes result from differences in the extent of DNA amplification by PCR.

These results indicate that these three distinct pancreatic carcinomas contained c-K-*ras* oncogenes with mutations at codon 12, and that in the two cases analyzed, the mutant oncogene was present in both the primary tumors and in their corresponding metastases. The histology immediately adjacent to some of the sections used for the experiment of Figure 2 is shown in Figure 3.

Mutant c-K-*ras* Genes Are Present in Most Human Pancreatic Carcinomas

Similar analysis of nine other distinct tumors (cases 8 through 16, see Table 1) is shown in Figure 4. In all cases,

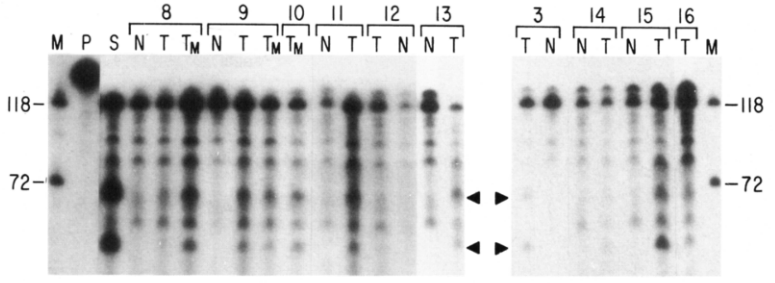

Figure 4. RNAase A Mismatch Cleavage Analysis of c-K-*ras* DNA Sequences from Human Pancreatic Adenocarcinomas

PCR-amplified DNA from 5 μm sections corresponding to the cases indicated at the top (see Table 1) were analyzed as in Figure 2 except that RNAase A digestion was for 60 min. The amounts of amplified DNA using paraffin sections ranged from 3 to 10 μl of the total PCR reaction. Lanes 3 and S: 1 μl each of the PCR reaction, using 1 μg of purified DNA from the corresponding normal (N) or tumor (T) pancreatic tissue of case 3 or SK-CO-1 (lane S). The picture corresponds to a 4 day exposure of the X-ray films (36 and 42 hr exposures for lanes 3, 13, and 16 and for lanes 14 and 15, respectively). The symbols are as in Figure 2.

DNA amplified from paraffin sections corresponding to tumor tissue either primary (lanes T) or metastatic (lanes Tm) yielded the diagnostic codon 12 mismatch bands although their relative intensities varied between the different samples. In lanes 8T and 12T, the mismatch-specific bands were very faint. However, the presence of these bands was confirmed in a different experiment (data not shown).

The mismatch diagnostic bands were not present when DNA:RNA hybrids corresponding to amplified DNA from 5 μm sections of normal tissue (pancreas or kidney) from the same cancer patients were digested with RNAase A (lanes N), although in some cases the nonspecific background bands were quite prominent (for instance, the band located between the 66 and the 43 nucleotide subbands). A diffuse band migrating slightly faster than the 66 nucleotide mismatch-specific subband was also present in some of the normal samples. However, no background band was present at the position of the smaller 43 nucleotide mismatch-specific subband in any of the lanes corresponding to amplified DNA from normal tissues. Therefore, this band represents the clearest diagnostic feature of the presence of a mutant c-K-*ras* gene at codon 12 in all of these tumor samples. Five additional pancreatic carcinomas (cases 18–22, Table 1) were found positive in the assay (data not shown).

The bands of 66 and 43 nucleotides are not due to an artifact dependent on the nature of the tumor tissue in the formalin fixed sections because they were absent when 5 μm paraffin sections from a carcinoma of the exocrine pancreas (case 17, Table 1) and from five gall bladder carcinomas were similarly analyzed (data not shown).

The results of these experiments are summarized in Table 1. Twenty-one out of 22 carcinomas of the exocrine pancreas tested positive for the presence of c-K-*ras* genes mutant at codon 12. In all seven cases analyzed, the mutation was present in both primary tumors and in their corresponding metastases.

Discussion

In taking advantage of the PCR in vitro gene amplification technique to increase the sensitivity of the RNAase A mismatch cleavage method, we have shown that mutations in the c-K-*ras* gene are readily detected in amounts of tissue as small as that contained in a single 5 μm paraffin section of tumor specimens. Therefore, our approach allows unambiguous identification of the tissue analyzed by conventional staining of adjacent sections and represents an improvement over the method described by Bos et al. (1987) because it is simpler and faster and does not involve cryostat sectioning of fresh tissue.

The possibility of using formalin-fixed paraffin-embedded tissue specimens that can be stored at room temperature for many years, including those obtained from autopsies, greatly increases the scope of the method. Using PCR on formalin-fixed paraffin-embedded sections, hypotheses linking the presence of specific DNA sequences or genomic mutations to biological phenomena can be rapidly tested (Shibata et al., 1988). Thus analysis of the frequency of mutant oncogenes in many types of tumors, including those of rare incidence, as well as retrospective correlative studies of oncogene mutational activation and the clinical evolution of the cancer patients (survival and recurrence rates), should be greatly facilitated.

Pancreatic carcinoma is the fourth leading cause of death in males of cancer in the United States and Europe. This reflects the aggressive nature and poor prognosis of the disease, with metastases present in a high proportion of cases at the time of diagnosis. Our findings on the incidence of mutant c-K-*ras* genes in pancreatic carcinomas imply that most, if not all, human carcinomas of the exocrine pancreas contain activated c-K-*ras* oncogenes, and provide a molecular genetic diagnostic marker for this cancer. Of 22 total cases that we analyzed, only one was found to be negative in our assay. This tumor could contain a c-K-*ras* gene activated by a point mutation at another position. For instance, mutant c-K-*ras* genes at codon 61 have been found in human tumors of lung (Yamamoto and Perucho, 1984) and colon (Bos et al., 1987) and in a pancreatic carcinoma cell line (Hirai et al., 1985). We also have detected c-K-*ras* genes mutant at codon 12 in seven out of nine pancreatic carcinoma cell lines (unpublished data).

The reason for the high incidence of mutant c-K-*ras* in cancer of the exocrine pancreas is not clear. However, it

is noteworthy that a mutant c-H-*ras* gene induces acinar carcinomas in the pancreas of transgenic mice when placed in front of the elastase I gene promoter (Quaife et al., 1987). This is the only reported situation where a single activated *ras* oncogene seems capable of inducing tumorigenesis in vivo, because neoplastic growth of the pancreatic cells is detectable immediately after elastase gene expression commences after 14 days of gestation. Therefore, the reproducible activation of the c-K-*ras* gene in pancreatic carcinomas (in contrast with other types of carcinomas) could be due to a selection phenomenon favored by the apparent sensitivity of cells from the exocrine pancreas to *ras* activation rather than to their preferential exposure and/or sensitivity to specific carcinogens.

Accurate estimation of the amount of mutant c-K-*ras* genes in these tumors is difficult because of the incomplete cleavage of the single base mismatches present in the DNA:RNA hybrids. However, by comparing the relative intensities of their mismatch-specific cleaved bands with those generated using DNA from homogenous populations of SK-CO-1 cells that contain an amplified (3- to 4-fold) mutant c-K-*ras* allele (Forrester et al., 1988), and using our histological data to calculate the fraction of tumor cells within each tissue section (Figure 3 and data not shown), it is possible to assume that the mutant gene was present in most of the neoplastic cells from many of these tumors. It seems also likely that significant amounts of tumor cells from some of these specimens (cases 5, 6, and 7, for instance) contained increased ratios of mutant versus normal c-K-*ras* alleles. A more extensive analysis will be required to determine whether or not there are correlations between the nature of the mutations and/or the differences in mutant c-K-*ras* gene dosage and expression levels and the clinical manifestations of the corresponding tumors.

The ubiquitous presence of the mutant c-K-*ras* gene in most of these tumors (in different tumor regions, in the majority of the tumor cells, and in both primary tumors and their corresponding metastases), either from autopsies or from surgical resections, suggests that c-K-*ras* mutational activation is an early event in cancer of the exocrine pancreas. These results, especially the persistence of the activated oncogene in the metastases, also support the concept that mutant *ras* genes are actively involved in the maintenance of the tumor phenotype and continuosly contribute to the process of tumor progression, including the metastatic process. In conclusion, our studies suggest that the reproducible and therefore predictable somatic mutational activation of the c-K-*ras* gene in pancreatic carcinoma plays a direct and critical role in the genesis of this common type of human cancer.

Experimental Procedures

Cell Lines and Tumors

The origins of the human normal foreskin fibroblasts (HNF) and the SK-CO-1 colon carcinoma cell line (Fogh and Trempe, 1975) and their culture conditions have been previously described (Perucho et al., 1981; Winter et al., 1985). Frozen specimens of nonfixed pancreatic tumors were obtained from the Tissue Cooperative Human Network of the University of Alabama at Birmingham (cases 1 and 3) and from the University Hospital at Stony Brook (cases 2 and 4). The paraffin-embedded tissues from 1971 to 1987 were obtained from the files of the LAC-USC Medical Center (pancreatic carcinomas 5 through 17 and gall bladder carcinomas) or the Winthrop University Hospital at Mineola, Long Island, NY. (pancreatic carcinomas 18–22). Surgical resections and biopsies were fixed in 10% buffered formalin immediately upon removal. Autopsies were performed within 30 hr after death. All tissues were routinely paraffin embedded. The pancreatic tumors were all ductal adenocarcinomas. The gall bladder tumors were all adenocarcinomas.

Plasmids and Oligonucleotide Primers

RNA probes were generated using pAKINgly (Winter et al., 1985), a pSP65 (Promega) derivative containing normal sequences from the first coding exon of the c-K-*ras* gene from the Sau3A site at position +97 to the PstI site at approximately 180 in the first intron, relative to the ATG initiation codon (Nakano et al., 1984; McGrath et al., 1983). For in vitro transcriptions, pAKINgly was linearized with AatI, located at the splicing junction, 10 bp upstream of the ATG codon (Figure 1).

The primers used for PCR amplification of c-K-*ras* sequences were 5′CATGTTCTAATATAGTCACA3′(A) and 3′GTTATCTCCATTTAGAACAA 5′(B). Primer A hybridizes to the noncoding DNA strand from positions −53 to −23 and primer B hybridizes to the coding strand from positions +104 to +123 (Nakano et al., 1984, McGrath et al., 1983). The antisense RNA probes generated by linearizing the plasmid with AatI span from +97 to −10 and are therefore included in the amplified region (see Figure 1).

In Vitro Transcriptions

Uniformly labeled RNA probes were synthesized with the SP6 polymerase in vitro transcription system (Melton et al., 1984) using 50 μCi of α-[^{32}P]CTP (Amersham, 10 μCi/ml, 400 Ci/mmol) as described (Winter et al., 1985) in the presence of 30 μM of unlabeled CTP. Synthesized RNA probes were resuspended in 30 μl of loading buffer (97% formamide; 0.1% SDS; 10 mM Tris-HCl [pH 7.0]; 0.01% bromophenol blue and xylene cyanol) and electrophoresed in 1.5 mm thick denaturing urea-polyacrylamide gels (Maniatis et al., 1982). The gels were exposed to XAR-5 Kodak film for 30 sec, the radioactive bands were excised from the gel, and the RNA probes were eluted by incubation for 3 hr at 37°C with gentle shaking in 375 μl of 2 M ammonium acetate, 0.1% SDS. The eluted probes were precipitated with ethanol after adding 30 μg of yeast tRNA, dried, dissolved in 50 μl of hybridization buffer (85% formamide, 0.4 M NaCl, 1 mM EDTA, 40 mM PIPES [pH 6.7]) and stored at −70°C.

Hybridizations and RNAase A Digestions

DNA:RNA hybridizations were performed in 30 μl of hybridization buffer for 12–16 hr at 40°-45°C. Purified probes (25 × 10^3 cpm) were hybridized to PCR amplified DNA in the presence of 20 μg of yeast tRNA, after heating at 90°C for 10 min. RNAase A digestions were performed at 30°C by adding to the samples 300 μl of a buffer containing 20 μg/ml of RNAase A (type III A, Sigma), 10 mM Tris-HCl (pH 7.5); 1 mM EDTA; 0.2 M NaCl, and 0.1 M LiCl (Myers et al., 1985). The reactions were terminated by adding 10 μl of 20% SDS and 5 μl of Proteinase K (10 mg/ml, Beckman) and incubated for 15 min at 37°C. The samples were extracted with phenol:chlorophorm:isoamyl alcohol after adding yeast tRNA (10 μg), ethanol precipitated, and analyzed in 0.5 mm thick urea-polyacrylamide gels for 2 hr at 250 volts.

PCR-Mediated Amplification of c-K-*ras* Sequences from Paraffin Sections

We used the preparation method of Shibata et al. (1988). A single 5–10 μm section cut from the block was placed in a 500 μl microtube. Subsequent sections were stained with hematoxylin and eosin to confirm the exact tissue analyzed. The average surface area of the tissue was 1.0 cm^2, although smaller fragments were used successfully. The sections were deparaffinized by adding 400 μl of xylene, and then centrifuged for 5 min. The xylene was decanted and its residue removed with 400 μl of 95% ethanol. The tissue pellets were dessicated and 100 μl of the PCR mix was added directly to the tubes. The PCR mix contained 10 mM Tris (pH 7.5), 50 mM NaCl, 10 mM $MgCl_2$, 1.5 mM of each dNTP and 1 μmol of each primer (Saiki et al., 1985). The tubes

were heated at 100°C for 10 min and then subjected to 40 cycles of amplification on a modified liquid handling apparatus (Pro/Pette, Cetus Corporation, Emeryville, CA) which sequentially denatured, cooled, and added DNA Polymerase I (Klenow, US Biochemicals, Cleveland, OH) to the samples.

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