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Analysis of *RAS* gene mutations in acute myeloid leukemia by polymerase chain reaction and oligonucleotide probes

(DNA transfection/tetramethylammonium chloride hybridization/DNA fingerprinting/alkylating agents/myelodysplastic syndromes)

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ABSTRACT ***In vitro*** **DNA amplification followed by oligonucleotide dot blot analysis were used to study** ***RAS*** **gene mutations in acute myeloid leukemia (AML). Fifty-two presentation AML DNAs were screened for mutations in codons 12, 13, and 61 of** ***NRAS*** **and in codons 12 and 61 of** ***KRAS*** **and** ***HRAS*****. Fourteen (27%) contained mutations—all in** ***NRAS*** **and predominantly in codon 12. The most common amino acid substitution identified was of glycine by aspartic acid at codon 12 (7/18), with a G → A transition being the most common base change (11/18). No particular correlation was observed between disease subtype and the incidence or type of** ***NRAS*** **mutation. In DNA samples from four patients, 2** ***NRAS*** **mutations were found to coexist. NIH 3T3 focus-formation assays revealed that in each case the mutations were present in different** ***NRAS*** **alleles. We also report the absence of a mutated** ***RAS*** **gene in relapse DNAs of four patients in which a** ***RAS*** **oncogene had been detected at presentation. These observations suggest that** ***RAS*** **mutations arise as part of the evolution of neoplastic transformation.**

Mutations leading to the activation of the transforming potential of cellular *RAS* genes have been implicated in the development of many human malignancies of diverse origin. Normal mammalian cells contain three closely related *ras* protooncogenes, *HRAS*, *KRAS*, and *NRAS*, encoding 21-kDa proteins that bind guanine nucleotides, possess intrinsic GTPase activity, and are located at the inner surface of the plasma membrane (1). Activating mutations of *RAS* genes that have been detected *in vivo* occur in codons 12, 13, and 61 (2–4). These codons fall within two regions of homology, which appear to form important components of the GTP-binding site (5, 6). *In vitro* mutagenesis studies on *HRAS* have revealed additional sites at which amino acid substitutions can activate the transforming potential of p21: in the region neighboring codon 61, the substitution of threonine for alanine at position 59, and the mutation of glutamic acid to lysine at position 63 (7). Substitutions at positions 116 (asparagine) and 119 (aspartic acid), two residues that determine the guanine specificity of nucleotide binding (5, 6), also increase the transforming capacity of p21 (8, 9). Mutations at codons 59, 63, 116, and 119 have yet to be found as activating lesions *in vivo*.

Induction of tumors in experimental animals by chemical carcinogenesis leads in some systems to reproducible activation of the same *RAS* oncogene, often with the same mutation (10–13). In some types of human cancer, there also appears to be a distinct bias for transforming mutations affecting a particular member of the *RAS* family. For example, only activation of *HRAS* has been reported in human bladder and urinary carcinomas (ref. 14; K. Visvanathan and I. C. Summerhayes, personal communication); mutations in *KRAS* are most common in lung and colon carcinomas (15–19), whilst activated *NRAS* predominates in hematopoietic malignancy (3, 20, 21).

Recently, Saiki and colleagues (22) have used synthetic oligonucleotide probes to identify point mutations and allelic sequence variation in DNA enzymatically amplified by the polymerase chain reaction (PCR) technique (23). In the present study, we have applied this approach to the detection of *RAS* gene mutations in acute myeloid leukemia (AML) DNAs. With this *in vitro* amplification technique, specific regions of DNA can be amplified $>10^5$-fold after 25 cycles of *in vitro* DNA synthesis. These highly amplified sequences are then a suitable substrate for oligonucleotide probing experiments using probes of moderate specific activity and short autoradiographic exposures.

MATERIALS AND METHODS

Materials. DNA was extracted from leukapheresed blood taken at the time of initial diagnosis (prior to chemotherapy) and cryopreserved. Eight of the 52 patients screened were followed up as the disease progressed and samples were obtained during remission and/or relapse states.

Synthetic Oligonucleotides. The oligomers were synthesized by the solid-phase triester method. The oligonucleotide probes were 5′-end-labeled by phosphorylation with [γ-^{32}P]ATP (Amersham; specific activity, >5000 Ci/mmol; 1 Ci = 37 GBq) and T4 polynucleotide kinase (Amersham), and they were routinely purified by spin dialysis over 1-ml Bio-Gel P4 Fine (Bio-Rad) columns. The procedure gave probes with specific activities of $\approx$4.0 μCi/pmol. Where probes of identical specific activity were required, the kinased oligomers were purified over a 10% polyacrylamide/7 M urea gel, which allows separation of the labeled 20-mer from its unlabeled precursor (3, 24).

PCR and Oligonucleotide Probe Hybridization. Genomic DNA (1 μg) was subjected to 25 cycles of PCR amplification as described (22). Twenty microliters of the final reaction mixture (equivalent to 100 ng of the original DNA) was adjusted to 0.4 M NaOH/25 mM EDTA in a 200-μl vol and applied to a Genetran-45 nylon filter (Plasco) under vacuum with a Bio-Dot apparatus (Bio-Rad). Replicate filters were prepared and the DNA was fixed by baking or by UV illumination.

The filters were prehybridized individually for 30 min at 55°C–56°C in 3 M tetramethylammonium chloride/50 mM Tris·HCl, pH 7.5/2 mM EDTA/0.3% $NaDodSO_4$/denatured sonicated salmon sperm DNA (100 μg/ml)/5× Denhardt's

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Abbreviations: AML, acute myeloid leukemia; PCR, polymerase chain reaction.

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Table 1. Sequences of the ^{32}P-labeled 20-mer *NRAS* probes

Probe *NRAS*	Sequence (sense)	
N12 wt	GGA GCA **GGT** GGT GTT GGG AA	
N12 p1	*n*GT	*n* = A,C,T
N12 p2	G*n*T	*n* = A,C,T
N13 wt	GGA GCA GGT **GGT** GTT GGG AA	
N13 p1	*n*GT	*n* = A,C,T
N13 p2	G*n*T	*n* = A,C,T
N61 wt	ACA GCT GGA **CAA** GAA GAG TA	
N61 p1	*n*AA	*n* = A,G
N61 p2	C*n*A	*n* = C,G,T
N61 p3	CA*n*	*n* = C,T
N59 Thr	CTG GAT ACA **ACT** GGA CAA GA	
N63 Lys	GGA CAA GAA **AAG** TAC AGT GC	

wt, Wild type.

solution (1× Denhardt's solution = 0.02% Ficoll/0.02% polyvinylpyrrolidone/0.02% bovine serum albumin). Approximately 1 pmol of probe (2 × 10^6 cpm/ml) was then added and hybridization was continued at the same temperature for 1 hr. Filters were washed twice in 2× SSPE (1× SSPE = 10 mM sodium phosphate, pH 7.2/0.18 M NaCl/1 mM EDTA)/0.1% $NaDodSO_4$ for 10 min at room temperature. Then, the filters were rinsed in 3 M tetramethylammonium chloride hybridization buffer minus carrier DNA and Denhardt's solution, and washed in this solution for 30–60 min at 60°C. Finally, the filters were autoradiographed at −70°C (Kodak XAR) for 1–12 hr. Quantitative analyses of dot blots were performed by densitometry of preflashed x-ray film (25).

DNA Transfection. NIH 3T3 focus-formation assays were carried out as described (26).

RESULTS

***NRAS* Mutations in Presentation AML DNAs.** DNA samples from 52 patients with AML were screened for the presence of *N*-, *K*-, and *HRAS* gene mutations using synthetic oligonucleotide probes and an *in vitro* amplification step. Since the activating mutations so far identified *in vivo* are at or near the 12th or 61st codon, amplified DNAs were analyzed with a set of oligomers each designed to be complementary to a different point mutation within sequences around the two regions (Table 1). This approach allows for the identification of all possible single base-pair substitutions in these codons because a fully matched hybrid between the oligomer and genomic DNA is thermally more stable than a single base-pair mismatch hybrid.

The sensitivity of this approach is dramatically increased by the introduction of an enzymatic amplification step (22, 23, 27). In the PCR analysis of each *RAS* gene, two sets of 20-base oligonucleotide primers are used, which hybridize to opposite strands of the genomic DNA flanking the target sequence around either codons 12/13 or 61. After repeated cycles of denaturation, annealing, and chain elongation, DNA target sequences of 112–115 bases are generated (Fig. 1).

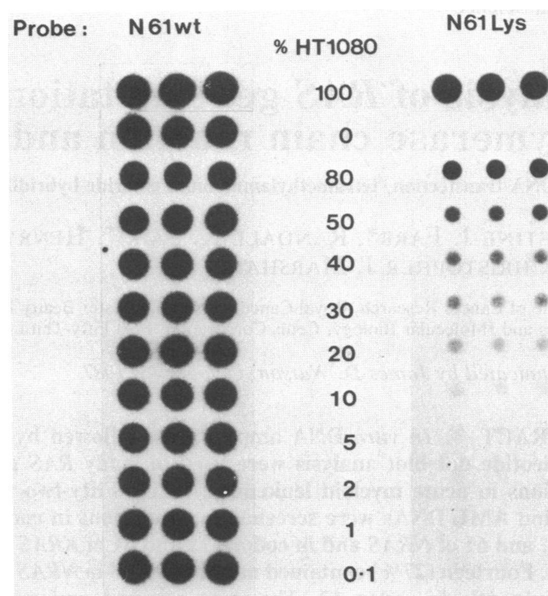

FIG. 2. Sensitivity of the PCR/oligonucleotide approach. DNA from the HT1080 cell line (heterozygous for N61 lysine) was diluted with normal human placental DNA, such that a series of samples was amplified, representing from 100% to 0.1% of the cells from which DNA was extracted containing the mutant allele. Samples were spotted out in triplicate and hybridized with the N61 wild-type (wt) and N61 lysine oligomers.

Since in AML the numbers of circulating blasts vary considerably, the sensitivity of our standard PCR/oligonucleotide protocol was assessed. DNA from cell lines carrying a known *RAS* mutation (HT1080, heterozygous for N61 lysine; and EJ, homozygous for H12 valine) was diluted with normal human placental DNA, such that a series of samples was amplified, which represented from 100% to only 0.1% of the cells from which DNA was extracted containing the mutant allele. Similar results were obtained from both dilution series (Fig. 2). Using our standard protocol it is possible to clearly identify a mutation present at only the 10% level. Below this, the signal approaches background.

Of the 52 initial presentation AML samples screened [FAB subtypes (French–American–British classification) M1, M2, and M4], 14 contained *RAS* mutations (Table 2, Fig. 3). These mutations were all in *NRAS* and predominantly at codon 12. The most common amino acid substitution identified was aspartic acid for glycine (9/18). The most common base change was a G → A transition (11/18). Mutations appear to occur randomly in the various subtypes of the disease (M1, 1/8; M2, 5/22; M4, 8/22). In contrast to our previous analysis of transfectants generated from AML

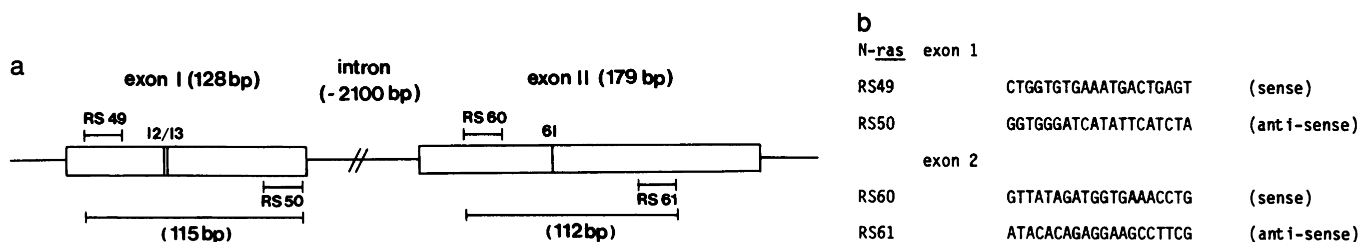

FIG. 1. (*a*) Position of amplifying primers in relation to the *NRAS* gene. bp, Base pairs. (*b*) PCR primer sequences.

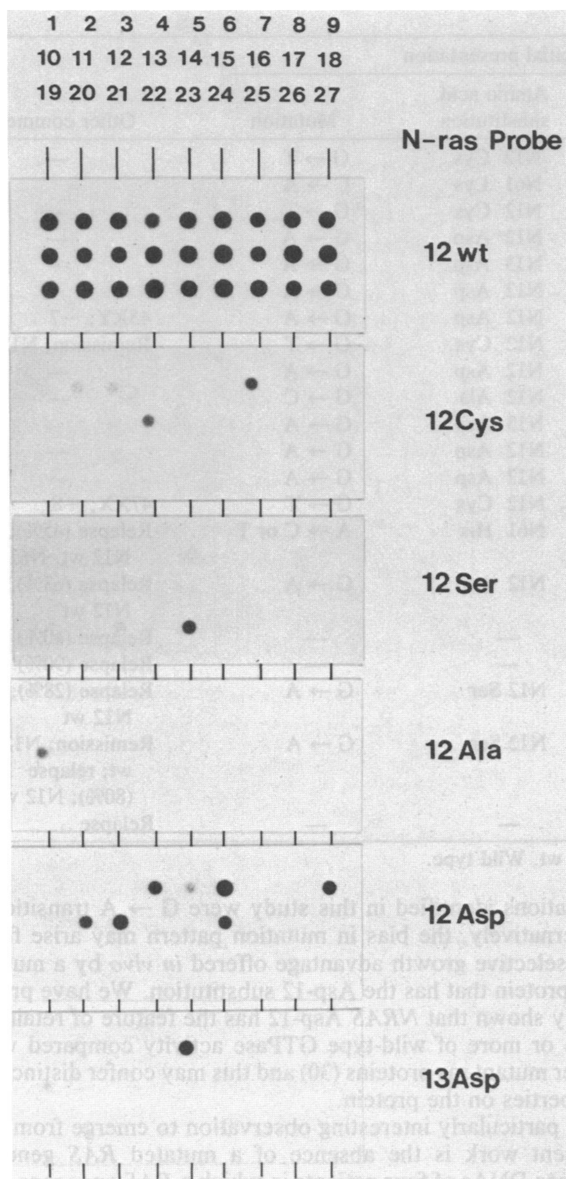

FIG. 3. Dot blot analysis of AML DNA samples. DNA from 52 presentation samples was enzymatically amplified and screened for mutations in codons 12, 13, and 61 of *NRAS* and in codons 12 and 61 of *KRAS* and *HRAS*. Shown are 14 presentation samples in which mutations at either codons 12 or 13 of *NRAS* were detected together with all remission and relapse samples screened. (Using the PCR/dot blot analysis, the normal allele is always present because of inevitable contamination with normal cells and/or the normal allele of a heterozygous cell.) Grid numbers and the corresponding amplified DNAs are as follows: 1, human placental DNA; 2, AML-10; 3, AML-17; 4, AML-34; 5, AML-35; 6, AML-40; 7, AML-43; 8, remission 43; 9, AML-54; 10, AML-67; 11, AML-71; 12, AML-89; 13, AML-96; 14, relapse 96; 15, AML-126; 16, relapse 126; 17, AML-318; 18, relapse 318; 19, AML-908; 20, relapse 908; 21, AML-921; 22, relapse 921; 23, AML-951; 24, remission 951; 25, relapse 951; 26, AML-961; 27, relapse 961. Samples 35 and 67 each contain *NRAS* mutations in both codons 12 and 13. The N12 cysteine mutations in AML-10 and -96 coexist with *NRAS* codon 61 substitutions. No *N*-, *H*-, or *KRAS* mutations were detected in the other AML samples screened.

DNAs, only 2 codon 13 mutations were detected (both aspartates). In two patients who had gone into remission after therapy, analysis of a peripheral blood sample taken during remission showed that the *RAS* gene mutation found at initial presentation (951/N12 serine, 43/N12 cysteine) was undetectable.

The presentation samples were also screened for threonine-59 and lysine-63 substitutions, two activating mutations that have been identified by *in vitro* mutagenesis studies (7). No mutations at these sites were found in either *NRAS* or *HRAS*.

To determine the relative strength of mutant and normal *RAS* gene signals in relation to the percentage of peripheral blasts in the various samples, densitometry measurements were taken. These studies show that in some leukemic samples with a high proportion of blast cells, the mutant *RAS* gene appears to be present in only a fraction of the circulating leukemic blasts (e.g., AML-67, -71).

Presence of More Than One *NRAS* Mutation in Some AML DNAs. Amplified DNA samples from four AML patients (AML-10, -35, -67, and -96) formed stable hybrids with two different mutant-specific probes. These samples therefore appear to contain more than one mutation. To confirm these results, the original DNA samples were used in an NIH 3T3 transfection assay and a series of independently transformed foci was isolated. Analysis of these transfectant DNAs showed that different foci contained one or the other of these *RAS* gene mutations but never both (Fig. 4). Thus, these DNAs contain two mutant *RAS* genes, but the mutations are not in the same allele of *NRAS*. Furthermore, in the case of mutations occurring simultaneously in codons 12 and 13 in the same leukemic genome, their very detection using this approach indicates that they cannot be present in the same allele, as this would obviously result in thermal instability of the DNA hybrid. Comparison of the strengths of the hybridization signals from these samples showed that in most cases the signals from the two mutant probes were similar. However, in one case, AML-35, the N12 aspartate mutation was considerably weaker than either the N13 aspartate or the normal *NRAS* signal (Fig. 3). Such data argue that the leukemic sample contains different clones of cells bearing *RAS* mutations.

Absence of *RAS* Gene Mutations in Relapse DNAs from Patients Whose Presentation Sample Contained a Mutation. We screened for *N*-, *K*-, and *HRAS* mutations in samples from seven patients, who had relapsed after a period in remission (Table 2, Fig. 3). In three cases (presentation/relapse pairs: 961/13, 318/788, and 908/948), no *RAS* mutation had been detected in the presentation samples and no mutation was identified on relapse. In a further four cases, *RAS* mutations had been detected in the presentation samples (one N12 Asp-126; two N12 Ser-921, -951; one N12 Cys-96; one N61 His-96). Strikingly, no *RAS* mutation could be identified in the relapse DNAs. Since AML is sometimes treated by bone marrow transplantation, the absence of a mutation in relapse could arise from leukemia in the donor marrow. However, in these cases, bone marrow transplantation had not been performed. To preclude the possibility of inadvertent confusion of clinical samples, the presentation/relapse pairs were subjected to DNA fingerprinting (data not shown). This confirmed that they were derived from the same individual in each case.

DISCUSSION

We have used a sensitive technique based on enzymatic amplification of target DNA sequences followed by oligonucleotide dot blot analysis to study *RAS* gene mutations in human AML. This approach has the advantages that large numbers of samples can be screened, it is sensitive, and it allows detection of all possible single-base mutations at codons 12, 13, and 61. Fourteen of 52 (27%) presentation samples contained mutations of *NRAS*. This confirms our (and others) previous experience that mutations in *NRAS* are the most frequently found in AML and mutations in *HRAS* and *KRAS* occur but are less common (3, 28, 29). No

Table 2. Classification of AML samples

			Initial presentation			
Patient	Age, yr (sex)	Subtype(s)	% peripheral blasts	Amino acid substitution	Mutation	Other comments
10	31 (M)	M2	60	N12 Cys	G → T	—
				N61 Lys	C → A	
17	33 (F)	M2	64	N12 Cys	G → T	—
34	34 (F)	M1	84	N12 Asp	G → A	—
35	49 (M)	M4	7	N13 Asp	G → A	—
				N12 Asp	G → A	
40	39 (M)	M2	70	N12 Asp	G → A	45XY; −7
43	22 (M)	M4	71	N12 Cys	G → T	Remission; N12 wt
54	32 (F)	M5/M2/M4	30	N12 Asp	G → A	—
67	35 (M)	M4	96	N12 Ala	G → C	—
				N13 Asp	G → A	
71	48 (F)	M4	97	N12 Asp	G → A	—
89	56 (M)	M4/M5	96	N12 Asp	G → A	—
96	38 (F)	M4	10	N12 Cys	G → T	47XX; +8
				N61 His	A → C or T	Relapse (45%); N12 wt, N61 wt
126	47 (M)	M2/M6	3	N12 Asp	G → A	Relapse (63%); N12 wt
318	17 (F)	M4	NA	—	—	Relapse (40%)
908	50 (M)	M4	91	—	—	Relapse (90%)
921	54 (F)	M4	3	N12 Ser	G → A	Relapse (28%); N12 wt
951	36 (F)	M2	50	N12 Ser	G → A	Remission; N12 wt; relapse (80%); N12 wt
961	20 (F)	M4	95	—	—	Relapse

All mutations listed were confirmed by reamplification of the AML DNAs. wt, Wild type.

particular correlation was observed between FAB subtype and the incidence or type of *NRAS* gene mutations. Mutations were equally common in undifferentiated M1 and in the more differentiated M4.

The amino acid substitution detected most frequently (7/18) was that of an aspartate for glycine at codon 12, resulting from a G → A mutation. The incidence of Asp-12 substitutions may reflect the involvement of a particular agent in leukemogenesis. For example, the G → A mutation is that most frequently found with alkylating agents. In addition to the 7 Asp-12 substitutions, 4 more of the 18 mutations identified in this study were G → A transitions. Alternatively, the bias in mutation pattern may arise from the selective growth advantage offered *in vivo* by a mutant ras protein that has the Asp-12 substitution. We have previously shown that *NRAS* Asp-12 has the feature of retaining 40% or more of wild-type GTPase activity compared with other mutant ras proteins (30) and this may confer distinctive properties on the protein.

A particularly interesting observation to emerge from the present work is the absence of a mutated *RAS* gene in relapse DNAs of four patients in which a *RAS* oncogene had

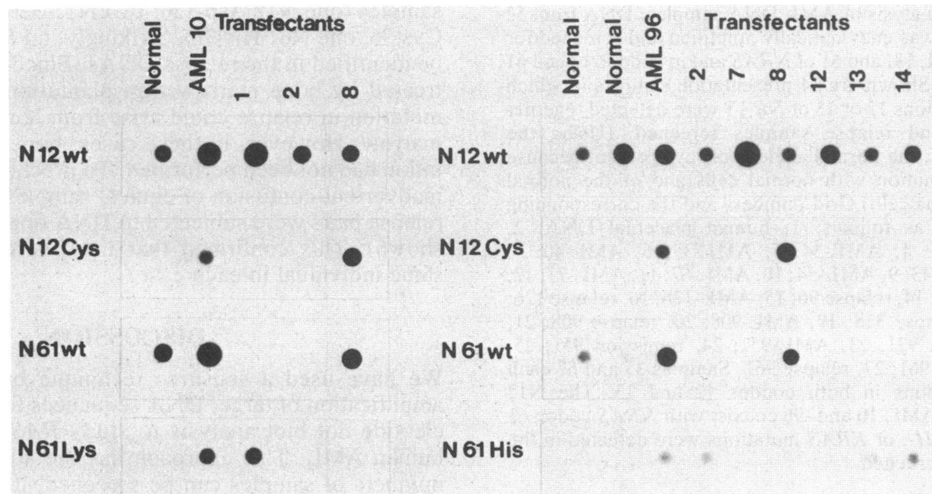

FIG. 4. The presence, on different alleles, of *NRAS* mutations in both codons 12 and 61 in two AML samples. NIH 3T3 focus-formation assays were undertaken on DNAs from AML-10 and -96. DNA from foci of transformed cells was amplified *in vitro*, dot blotted, and hybridized with N12 and -61 wild-type (wt) oligomers and with the appropriate mutant probes. Due to the close homology of the murine and human *NRAS* genes in exon 1, some amplification of mouse N-*ras* has occurred, which is detected by the oligomer probe spanning codon 12, the sequence of which is identical in the two genes.

been detected at presentation. (In one of these patients, AML-96, two *RAS* gene mutations were detected at initial presentation.) The data suggest treatment was able to eradicate the clone of leukemic cells carrying the *RAS* gene mutation but that when the leukemia reappeared, the *RAS* gene mutation did not recur. The phenomenon of remission followed shortly afterward by relapse is thought to result from fully leukemic cells being eradicated from the marrow, but the generation of altered cells in a "preleukemic" state persists (31, 32). The results therefore argue that the bone marrow in remission did not contain cells bearing the *RAS* gene mutations. In these four cases, therefore, it is unlikely that the mutations of the *RAS* genes were the initiating event in leukemogenesis; otherwise, the relapse samples would be expected to bear the same mutation. Moreover, some leukemic samples appear to contain *NRAS* genes mutated at two different sites, and in some samples with a large fraction of leukemic blasts the hybridization signal from the mutant probe is much reduced relative to the wild-type signal. These observations also suggest that some mutations are arising after the initial transforming event and are part of the clonal evolution of the disease. The simultaneous presence of activated *NRAS* and *KRAS* oncogenes in the same tumor DNA has been reported elsewhere (19).

Such an interpretation of the time at which *RAS* gene mutations occur is at variance with the view derived from experimental tumor models in rodents (10–13, 33) and from studies on human colon villous adenomas/carcinomas (18, 19), which argues that *RAS* gene mutations are an early initiating event in tumorigenesis. Furthermore, Hirai and colleagues (34) and others (R. A. Padua, G. Carter, J. Gow, D. Hughes, C. J. F., F. M., and A. Jacobs, unpublished data) have shown the presence of *NRAS* gene mutations in bone marrow from patients with myelodysplasia, a condition that predisposes to acute myeloid leukemia. It therefore seems likely that *RAS* gene mutations can occur at a number of stages in transformation, either as initiating events and/or later as part of the evolution of neoplastic transformation.

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