Amino-acid substitutions at codon 13 of the N-*ras* oncogene in human acute myeloid leukaemia

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DNAs from four out of five patients with acute myeloid leukaemia (AML) tested by an in vivo *selection assay in nude mice using transfected mouse NIH 3T3 cells were found to contain an activated N-*ras *oncogene. Using a set of synthetic oligonucleotide probes, we have detected a mutation at codon 13 in all four genes. The same codon is mutated in an additional AML DNA that is positive in the focus-formation assay on 3T3 cells. DNA from the peripheral blood of one patient in remission does not contain a codon 13 mutation.*

THE use of focus assays to detect morphological transformation of NIH 3T3 cells in DNA transfection experiments has shown that between 10 and 30% of human tumours contain altered forms of either the c-Ha-*ras*-1 gene, the c-Ki-*ras*-2 gene or the N-*ras* gene. These genes encode GTP-binding proteins of 188 and 189 amino acids that are located at the inner surface of the cell membrane and which are evolutionarily highly conserved[1,2] In the 15 or so cases of altered *ras* genes in human tumours that have been analysed so far, activation has been shown to result in a single base pair mutation which leads to the substitution of either a glycine at amino acid 12 or glutamine at amino acid 61 (refs 3-17). However, *in vitro* mutagenesis experiments have shown that mutations at amino acids 13, 59 and 63 can also lead to transforming activity[18].

We have studied the presence of activated *ras* genes in acute myeloid leukaemia (AML), a disease characterized by abnormal proliferation and differentiation of cells of the myeloid, monocytic and erythroid lineage. This disease is subdivided into six groups (FAB M1-M6; ref. 19) according to which immature cell is dominant in the population. Using a direct *in vivo* selection assay in nude mice of transfected 3T3 cells[20], we find that four out of five AML DNAs tested contain an activated N-*ras* gene. An altered N-*ras* oncogene was also detected in another AML sample using the focus-formation assay. We analysed these activated N-*ras* genes for the presence of mutations using a set of synthetic oligonucleotide probes and report here that all five activated N-*ras* genes are found to contain a mutation at codon 13.

Transfection of NIH 3T3 cells

In initial experiments using focus formation on NIH 3T3 cells as an assay for the transfer of transforming genes, DNAs prepared from five samples of AML cells were found to have low, if any, transforming activities (D.T. and C.J.M., unpublished results). However, in a different laboratory one out of three AML DNA samples was found to have transforming activity[38]. Because focus formation may not detect all transforming genes (for example, some alterations to *ras* genes lead to only a minimal change in morphology of the transformed cells[21]), we sought to increase the sensitivity of the transformation assay. Previously, Blair *et al.*[20] have shown that direct *in vivo* selection of transformed cells can be used as an assay for the transforming genes in transfected cells. As an additional step to ensure that all the injected cells had been transfected with donor DNA, DNA samples were co-transfected with the plasmid pSV2Neo (ref. 22) and the transfected cells selected in antibiotic G418. A similar approach has been described by Fasano *et al.*[23], but we injected a smaller number of cells than these authors. We found that tumours arose 30-55 days after inoculations of NIH 3T3 cells transfected with four out of five preparations of DNA from AML samples (AML 33, 49, 73, 77). Of 15 other human tumour DNA samples tested in the same way, 10 failed to give any tumours, even within 90 days, whereas 5 other samples gave tumours 32-80 days after inoculation of transfected cells (Table 1). Normal DNA prepared from either mouse livers or human lymphocytes gave a very low incidence of tumours—0/3

Table 1 Co-transfection and nude mouse tumorigenicity of AML DNAs

DNA + pSV2Neo	FAB classification of AML	Primary tumour incidence per co-transfection of 20 μg DNA	Latent period (days)	Secondary tumour incidence per co-transfection	Latent period (days)
Normal mouse DNA		3/28	94	NT	
Normal human		0/3			
HT1080		3/3, 1/3	30-70	NT	
AML 33	M1	1/3	50	3/4	39
AML 49	M5	1/3, 2/3, 1/3	37	3/3	27
AML 73	M2	1/3	30	1/4	34
AML 77	M4	1/3	55	NT	
AML 50	M5	0/3	90	NT	
15 Human tumours (non-AML)		10×0/3, 4×1/3, 1×2/3	32-80	NT	

20 μg Cellular DNA and 300 ng pSV2Neo were precipitated with calcium phosphate into each of three 60-mm plates seeded 1 day previously with 2×10^5 NIH 3T3 cells; 22-24 h later, the co-precipitate was washed off and the cells incubated in normal growth medium (Dulbecco's modified Eagle's medium + 10% calf serum) for 20 h. Each dish was then trypsinized and seeded to a 150-mm dish containing selective medium with 1 mg ml^{-1} G418. After 7-10 days of growth in selective medium, each dish contained $1\text{-}1.5\times10^3$ G418^R colonies and a total of $3\text{-}4\times10^6$ cells. 1.5×10^6 cells were then injected into each of two inguinal subcutaneous sites of *nu/nu* mice. NT, not tested.

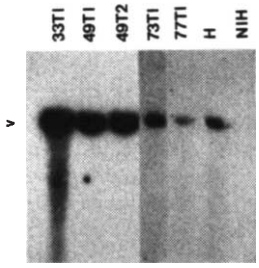

Fig. 1 Southern blot analysis to show the presence of human N-*ras* in NIH 3T3 transfectants from AML DNAs. 33T1, tumour in nude mice derived from co-transfection of NIH 3T3 cells with pSV2Neo and AML 33 DNA; 49T1 and 49T2, two tumours that arose from separate experiments with AML 49 DNA; 73T1 and 77T1, tumours that arose from experiments with AML 73 and 77 DNA, respectively. Control samples are DNAs from normal human fetal lung and NIH 3T3 cells. In each lane 5 μg *Eco*RI-digested DNA was separated. Arrow, 8.8 kb.
Methods. DNAs were digested with *Eco*RI, electrophoresed in a horizontal (0.7%) agarose gel and blotted to nitrocellulose. The filter was hybridized (5×SSC, 20 mM sodium phosphate, 5× Denhardts' solution, 10% dextran sulphate, 50 μg ml^{-1} denatured salmon sperm DNA, 50% formamide) at 42 °C for 16 h with 10^8 c.p.m. ml^{-1} of a ^{32}P-labelled probe obtained by nick-translation of the 800-bp *Eco*RI/*Sst*I fragment of N-*ras* contained in pAT8.8 (ref. 24). This fragment does not hybridize to mouse DNA and detects a single 8.8-kilobase (kb) fragment in *Eco*RI digests of human DNA. After hybridization the filters were washed twice in 0.1×SSC, 0.1% SDS at 60 °C and then exposed to Kodak XAR-5 film at −70 °C with intensifying screens.

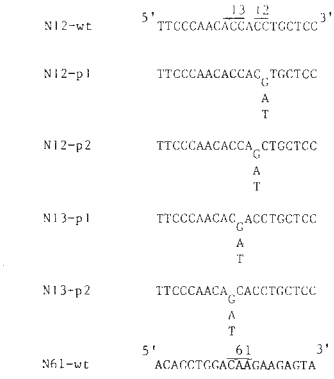

Fig. 2 Sequence of the 20-mer probes used to detect mutations in codon 12, codon 13 and codon 61 of the N-*ras* gene. Sequences are derived from Taparowski *et al.*[12].
Methods. The N12 and N13 probes were synthesized using as template chemically synthesized 20-mer and an 8-mer primer complementary to the 3′ end of the 20-mer. This primer-template complex was incubated with [α-^{32}P]dCTP (3,000 Ci $mmol^{-1}$; Amersham), cold dGTP, dATP and TTP, and DNA polymerase I as described by Bos *et al.*[17]. The 8-mer primer carried a 5′-terminal phosphate group which enabled us to separate the labelled oligomer from the unphosphorylated template on a polyacrylamide urea gel. The N61 probe was synthesized similarly but now using [α-^{32}P]dATP (see ref. 17). The oligonucleotides were prepared by a phosphotriester approach[37] using a fully automatic synthesizer (Biosearch SAM-I).

(human) and 3/28 (mouse)—and any tumours which did appear only arose 90 days after inoculation. DNA prepared from HT1080 cells, known to contain an activated N-*ras* oncogene[24], produced tumours 30–70 days after inoculation in 4/6 co-transfections. The transforming activity detected in the first transfection with AML DNAs could be passaged serially with similar or slightly greater efficiencies (Table 1).

To examine whether any of the tumours that arose in nude mice resulted from the acquisition of a human *ras* oncogene, DNA prepared from the tumours was analysed by Southern blotting with probes for N-*ras*, Ha-*ras* and Ki-*ras*. It was found that DNA prepared from each of the primary transfectants from the four AML DNAs detected by *in vivo* selection (Fig. 1) and the one detected by the focus assay contained the human N-*ras* gene. In some, but not all, transfectants the human N-*ras* gene was amplified 20–30-fold. There was no evidence for transfer of any of the other *ras* oncogenes, and tumours that arose after co-transfection of non-AML DNA did not contain human *ras* genes (data not shown).

Mutations at codon 13

To analyse the transfected N-*ras* genes in the NIH 3T3 transfectants derived from AML DNAs, we used synthetic 20-mer oligonucleotide probes. Using these probes we are able to detect single base pair (bp) substitutions in the N-*ras* gene based on the fact that a fully matched hybrid between the oligomers and genomic DNA is more stable than a 1-bp mismatched hybrid[25]. For the positive detection of mutations, we have synthesized groups of oligomers varying at one position in a codon (see Fig. 2). One oligomer of each group will then hybridize to form a fully matched hybrid with each of the possible mutations in a base pair. For example, the oligomer group N12-p2 consists of three oligomers, one of which will form a fully matched hybrid with an N-*ras* gene mutated at the second base of codon 12.

When *Pst*I-digested DNA from four AML transfectants (33T, 49T, 77T and 1T) was hybridized to the probe corresponding to the sequence of the normal N-*ras* gene around codon 61 (N61-wt) and washed under conditions in which only a fully matched hybrid is stable, a strongly hybridizing signal was seen (Fig. 3*A*). In two transfectants (49T and 33T) the signal was very intense, showing that the N-*ras* genes were highly amplified (see also Fig. 1). No hybridization was seen with the oligomer groups corresponding to mutations at codon 61, indicating that none of the N-*ras* genes in the transfectants contained mutations at codon 61 (data not shown). In contrast, the oligomer corresponding to the normal sequence of the N-*ras* gene at codon 12 (N12-wt) failed to form a fully matched hybrid with any of the AML transfectant DNAs (Fig. 3*B*), although it did hybridize to human cellular DNAs containing single-copy representations of the wild-type sequences at position 12 (lanes *a*, *b*). However, when the transfectant DNAs were hybridized with the groups of probes corresponding to mutations at each of the base pairs of codon 12 (N12-p1, N12-p2), fully matched hybrids were not detected (Fig. 3*C*, *D*).

The absence of fully matched hybrids with either normal or mutant probes led us to investigate whether any mutations occurred in the sequence surrounding codon 12. Recently, Fasano *et al.*[18] have shown by *in vitro* mutagenesis that mutations in codon 13 (Gly) can lead to transforming activity of the c-Ha-*ras*-1 gene. Therefore, we analysed the transfectant DNAs by hybridization with probes (N13-p1 and N13-p2) that could recognize mutations at the first or second position of codon 13. The oligomer group N13-p2 hybridizes with each of the AML transfectant DNAs (Fig. 3*F*). Those transfectants with the highest copy number of the N-*ras* gene showed the greatest signal with the N13-p2 probe, but hybridization was also seen in a transfectant (77T1) that only contains one or a few copies of N-*ras* (compare Figs 1 and 3*F*), whereas no hybridization was detected with control DNAs having the wild-type sequence (Fig. 3, lanes *a*, *b*). Thus, we conclude that the N13-p2 probe forms a fully matched hybrid with the AML transfectant DNAs. No hybridization was detected with the N13-p1 probe to the transfectant or control DNAs (Fig. 3*E*). Therefore, our results show that the transfectant DNAs contain N-*ras* genes with a mutation at the second position of codon 13. Recently, we have found that the N-*ras* gene in transfectant DNA of AML 73 also

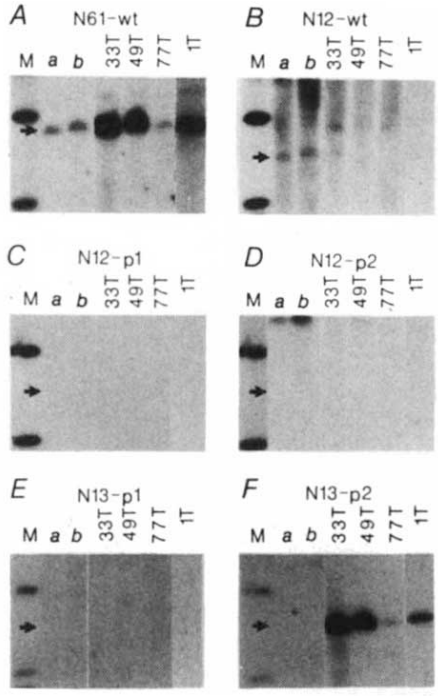

Fig. 3 Hybridization of synthetic oligomer probes to genomic DNA of 3T3 cell transfectants of four different human AMLs. The panels are replicates of each other with DNAs of HL60 (*a*) and HT1080 (*b*) as control and the DNAs of the AML transfectants 33T, 49T, 77T and 1T. The panels were hybridized to different oligomer probes: *A*, N61-wt; *B*, N12-wt; *C*, N12-p1; *D*, N12-p2; *E*, N13-p1; *F*, N13-p2; M, fragments of phage λ DNA digested with *Hin*dIII (4.3 and 2.3 kb). Arrows indicate the position of the N-*ras* fragment.

Methods. Hybridization was essentially as described by Bos *et al.*[17]. Genomic DNA (10 μg) was digested with *Pst*I and electrophoresed on a 0.5% agarose gel. The gels were denatured in 0.4 M NaOH, 0.8 M NaCl, neutralized in 0.5 M Tris-HCl (*p*H 7.2), 1.5 M NaCl and dried. The dried gel membranes were hybridized at 50 °C with N61-wt and at 53 °C with the other probes in 5 × SSPE (SSPE = 10 mM sodium phosphate *p*H 7.0, 0.18 M NaCl, 1 mM EDTA), 0.3% SDS and 10 μg ml^{-1} sonicated *Escherichia coli* DNA. Hybridized gels were washed in 2 × SSPE, 0.1% SDS at room temperature, in 5 × SSPE, 0.1% SDS at 53 °C for 15 min and finally in the same solution at either 59 °C (for N61-wt) or 63 °C (for the N12/13 probes) for 5 min. Gel membranes were autoradiographed for 2–4 days using intensifying screens.

hybridizes with the N13-p2 probe (data not shown) and thus contains a mutation at the same position.

Mutation to valine or aspartic acid

The use of mixed oligonucleotide probes allows the detection of the position of a mutation in a codon rather than establishing the precise mutant sequence. To analyse the exact nature of the mutations present in the N-*ras* gene of the AML transfectants, we hybridized DNAs to the individual oligonucleotide probes corresponding to the three possible mutations at the second position of codon 13. To prepare these individual probes, the labelled oligonucleotides of group N13-p2 were electrophoresed on a polyacrylamide gel (Fig. 4, insert 1), the individual bands excised and the separated oligomers further purified by electrophoresis on cellulose acetate strips (Fig. 4, insert 2).

To identify the separated oligomers, they were sequenced as described in Fig. 4 legend. The slowest-running oligomer (N13-p2/a) on the polyacrylamide gel had the sequence TTCCCAACATCACCTGCTCC and thus identifies a GAT codon 13 (Asp) in a fully matched hybrid. The second oligomer (N13-p2/b) identifies a GTT codon (Val), whereas the third and fastest-running oligomer (N13-p2/c) identifies a GCT codon (Ala). The separated oligonucleotide probes were then hybridized to *Pst*I-digested DNA from four AML transfectants. As shown in Fig. 4*A*, oligomer N13-p2/a forms a fully matched hybrid with DNA of transfectants from AML 1 and 33, whereas oligomer N13-p2/b forms a fully matched hybrid with DNAs from transfectants of AML 49 and 77 (Fig. 4*B*). The probe N13-p2/c did not hybridize with any of the transfectants (Fig. 4*C*) or with any of the separated oligomers, to control MOLT-4 DNA or to 10 genomic copies of the plasmid pAT8.8 (refs 13, 24), which contains the wild-type sequence at codons 12 and 13 (lane p). From these results we conclude that the N-*ras* gene in transfectants of AML 1 and 33 DNA contains a GAT codon 13, substituting Asp for Gly, whereas the transfectants from AML 49 and 77 contain a GTT codon 13, encoding Val. Recently, we found that AML 73 also hybridized specifically with N13-p2/b and thus contains a Val at position 13 (data not shown). The fact that the transfectant DNAs form fully matched hybrids with different members of the N13-p2 group is further evidence for the specificity of the hybridization, as each DNA now serves as a control for the other. There is no apparent correlation between the classification of the AMLs and the amino-acid substitution: AMLs 1 and 33 (Asp) were classified on the FAB scheme as M4 and M1 respectively, whereas AMLs 49, 73 and 77 (Val) were classified as M5, M2 and M4 respectively.

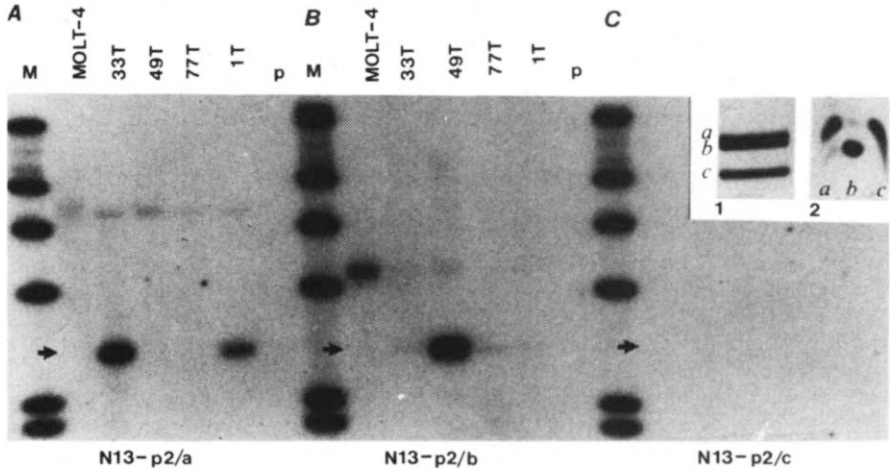

Fig. 4 Hybridization of separated N13-p2 oligomer probes to genomic DNAs of the AML transfectants. The panels are replicates of each other, with *Pst*I-digested DNAs of MOLT-4 as control and the DNAs of AML 33T, 49T, 77T and 1T. The panels were hybridized to N13-p2/a (TTCCCAACATCACCTGCTCC; panel *A*), to N13-p2/b (TTCCCAACAACACCTGCTCC; panel *B*) and to N13-p2/c (TTCCCAACAGCACCTGCTCC; panel *C*). Arrows indicate the position of the N-*ras* fragment; M, phage λ DNA digested with *Hin*dIII; p, 10 genomic copies of plasmid pAT8.8. This plasmid contains an 8.8-kb fragment of the activated N-*ras* gene HT1080 (CAA_{61}-AAA; ref. 24).

Methods. The three members of oligomers N13-p2 (*a*, *b* and *c* in inserts 1 and 2) were separated on a 20% polyacrylamide gel (mono/bis = 62.5:1) in 0.5 × TBE (TBE = 90 mM Tris, 90 mM boric acid, 2.5 mM EDTA) (see insert 1). To eliminate contamination, the isolated oligomers were further purified by electrophoresis on cellulose acetate strips in 7 M urea, 5% acetic acid, 5 mM EDTA *p*H 3.5 at 5,000 V (see insert 2). The fragments were eluted in 1 mM EDTA and used directly for hybridization. The sequence of each oligomer was determined by synthesizing N13-p2 with an end-labelled primer and cold deoxynucleoside triphosphates. This end-labelled probe was separated into the three members of N13-p2 and sequenced according to Maxam and Gilbert[26]. Gel separation and hybridization were as described in Fig. 2 legend.

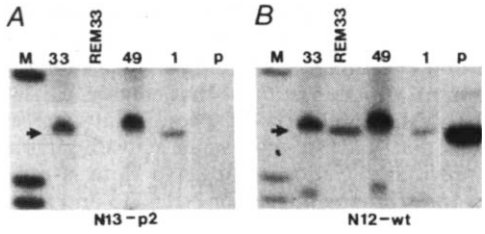

Fig. 5 Hybridization of N13-p2 (*A*) and N12-wt (*B*) oligomer probes to genomic DNA of isolated blast cells of AML patients 33, 49 and 1, and to genomic DNA of blood cells from AML patient 33 after remission (REM33). The differences in hybridization signal between the lanes result from loading differences and not from amplification of the N-*ras* gene. Arrows indicate the position of the N-*ras* band. Lane p, 20 genomic copies of the plasmid pAT8.8 containing the wild-type sequence at codons 12 and 13 (refs 13, 24). Gel separation and hybridization were as described in Fig. 3 legend.

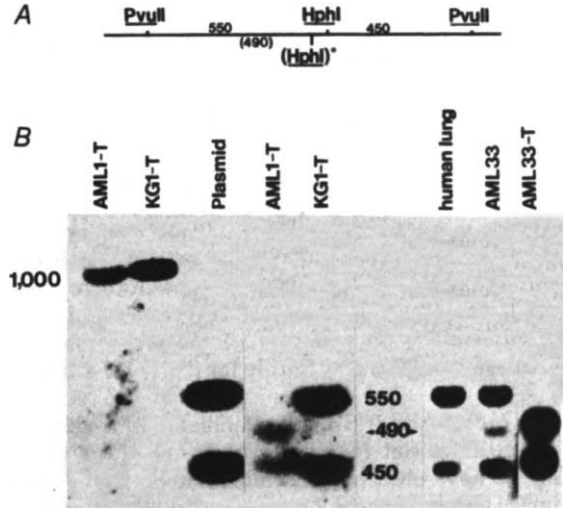

Fig. 6 *A*, Map of *Pvu*II and *Hph*I restriction enzyme sites in and around the first exon of N-*ras* to show the sizes of fragments generated after digestion of the normal N-*ras* gene and N-*ras* with Asp at codon 13. Restriction enzyme sites are taken from Brown *et al.*[13], Hall *et al.*[24] and A. Hall (personal communication). *B*, Southern blot analysis to show the altered *Pvu*II/*Hph*I fragments resulting from the presence of a new *Hph*I site generated by the Asp 13 mutation in the original leukaemic DNA from patient AML 33 and in transfectant DNAs of AML 1 and AML 33. This new *Hph*I site results in a *Pvu*II/*Hph*I fragment of 490 bp compared with 550 bp in wild-type DNA (see map).
Methods. DNA was digested either with *Pvu*II or *Pvu*II and *Hph*I, electrophoresed on a 1.4% low gelling temperature agarose gel and transferred to a nitrocellulose filter. The filter was hybridized with the nick-translated 1.0-kb *Pvu*II/*Pvu*II fragment of plasmid pAT7.8 (refs 13, 24) (see map). DNAs: pAT7.8 is a plasmid containing the *Bam*HI/*Eco*RI fragment with the first two exons of N-*ras* cloned from normal DNA[13], AML 33 is the original leukaemic sample, AML 33T a tumour derived after co-transfection into NIH 3T3 cells, AML 1T is a tumour derived from a focus isolated after transfection of NIH 3T3 cells with AML 1 DNA (note that this is a different transfectant from that used in Figs 3, 4 and 5 and contains fewer copies of N-*ras*), KG1-T (a transfectant from KGI DNA that contains a position 12 mutation; J.W.G.J. and J.L.B., unpublished).

Somatic mutation in leukaemic cells

To exclude the possibility that the mutations at codon 13 were acquired during transfection and selection for transformed cells, the DNAs from three leukaemias were analysed. DNA was digested with *Pst*I, electrophoresed on an agarose gel and hybridized *in situ* to the oligonucleotide probes N12-wt and N13-p2. Hybridization of the gel to the mutant-specific N13-p2 oligomer group showed that all three AML DNAs hybridized, but no hybridization was detected to a sample containing 20 copies of a plasmid carrying the wild-type 12 and 13 sequences (Fig. 5*A*). Thus, the DNAs from these original leukaemic cells also contain the mutation at codon 13, eliminating the possibility that the mutations had arisen as a result of experimental manipulation.

The availability of DNA prepared from the peripheral blood of one patient (no. 33) in remission (REM33) allowed us to investigate whether the codon 13 change was present in normal cells. Figure 5*A* shows that REM33 DNA did not hybridize with the N13-p2 mutant-specific probes. As a control, we rehybridized the gel with the N12-wt probe and obtained a signal with the normal-sequence probe hybridized to REM33 DNA (Fig. 5*B*). Therefore, we conclude that this DNA (and thus the normal tissues of the patient) does not contain an altered N-*ras* gene hybridizing with N13-p2, and that the altered N-*ras* gene has arisen by somatic mutation. Hybridization with the normal-sequence probe was also obtained with the three DNAs from leukaemias, which could result from the presence of normal cells in the leukaemic sample. However, the high white blood cell counts of the leukaemic samples ($54–140 \times 10^9$ cells per l), coupled with the high proportion of cells with blast morphology (95–100%), makes it likely that the hybridization signal with the N12-wt probe is a result of the leukaemic cells containing both a normal and a mutant N-*ras* gene. The presence of amplified copies of the N-*ras* gene in some transfectants suggested that the position 13 mutations might act in concert with an increased copy number of the N-*ras* gene, but examination of the AML DNAs by Southern blotting shows no evidence of N-*ras* gene amplification (data not shown).

Restriction site polymorphism

The N-*ras* 13 mutation in AML 33 and AML 1 (Gly–Asp) changes the sequence GGTGG to GGTGA, which is the recognition site for the restriction enzyme *Hph*I. This new *Hph*I site will shorten a 550-bp *Pvu*II/*Hph*I fragment by 60 bp (see Fig. 6). We have analysed the original leukaemic DNA of AML 33 and transfectant DNAs from AMLs 1 and 33 for the presence of this extra *Hph*I site by Southern blot analysis. To that end we have compared the length of the *Pvu*II/*Hph*I fragments in these DNAs with the lengths of fragments in N-*ras* genes containing the wild-type sequence at and around codon 12 and in a codon 12 mutant (for details see Fig. 6 legend). The digestion of a plasmid, pAT7.8, containing the first and second exons of a normal N-*ras* gene[13] and of a transfectant, KG-1T, mutated at codon 12 (J.W.G.J. and J.L.B., unpublished results) generates a 550-bp *Pvu*II/*Hph*I fragment and a 450-bp *Hph*I/*Pvu*II fragment when hybridized with a probe of the *Pvu*II/*Pvu*II fragment of pAT7.8 (Fig. 6). However, when DNA from AML 33 is digested with *Pvu*II and *Hph*I, a novel 490-bp fragment, as well as the 450- and 550-bp *Hph*I/*Pvu*II fragments, is detected. The presence of both the 550-bp and the novel 490-bp fragment confirms our observation with oligonucleotide probes that this DNA contains N-*ras* genes with both a wild-type codon 13 and an Asp 13 mutation (compare Fig. 5). Southern blotting of a transfectant from AML 33 (AML33-T) and a transfectant of AML 1 (AML1-T) shows that these contain only the novel 490-bp and the 450-bp *Hph*I/*Pvu*II fragments as expected, because transfectants will contain only the mutated transforming N-*ras* allele. As a new *Hph*I site could not have been generated by a mutation in codon 12, the restriction enzyme analysis confirms the observations with oligonucleotide probes that AML 1 and AML 33 contain a codon 13 mutation of Gly to Asp.

Discussion

We have found that DNAs isolated from AML contain altered N-*ras* genes. These activated oncogenes have also been detected in three other samples of AML cells from patients[27–29]; in three AML cell lines, HL60 (ref. 28), Rc2a and KG-1 (ref. 38); and

recently in three more AML samples (D.T. and C.J.M., unpublished results). Why there should be a bias towards activation of N-*ras* genes in these leukaemias is not clear, but it is noteworthy that also in cell lines of acute lymphoblastic leukaemia, N-*ras* normally seems to be the activated *ras* oncogene[27,30] N-*ras*, rather than the other *ras* genes, may be critically involved in the control of proliferation and differentiation of cells in the haematopoietic lineage. Alteration of this gene may then lead to the malignant state of the cells. The activation of the same *ras* oncogene in multiple isolates of similar tumours is reminiscent of findings that there is a reproducible pattern of *ras* gene activation in tumours induced by the same carcinogenic agent[31-33]. Therefore, a specific carcinogenic agent may be responsible for the N-*ras* activations in AML.

Unlike previously described examples of *ras* gene activation[3-17], we have not found mutations in codons 12 or 61. Instead, we have observed that mutations occur in codon 13, resulting in a replacement of Gly by either Val or Asp. One example of a codon 12 and a codon 61 mutation in AML cells has been noted previously[17,34] but, as only ~10-20% of malignancies seem to contain *ras* genes with mutations at codon 12 or 61 (for example, see ref. 16), the absence of codon 12 or 61 mutations in the samples we have analysed may result from the comparatively small sample size. The detection of codon 13 mutations in our samples of AML raises the question of why they have not been seen before. If codon 13 changes are restricted to AML, then they may not have been reported previously because only two mutant N-*ras* genes have been characterized from AML[17,34]. Furthermore, most of our samples were of FAB subtypes M1, M4 and M5, which have not been studied before. A restriction of codon 13 mutations to AML would imply that there is some constraint on the type of N-*ras* mutation in AML. Such a constraint could arise through the mechanism of mutation or through selection for the type of alteration in p21 protein. If codon 13 mutations are found in other malignancies, it is surprising that they have not been reported previously.

Our experience indicates that N-*ras* codon 13 mutants can be relatively inefficient in inducing foci. Although foci were detected in one laboratory with a DNA, AML 1, containing Asp 13 (ref. 38), they were not detected in another laboratory with DNA containing the same mutation, AML 33 (D.T. and C.J.M., unpublished results). This discrepancy has now been resolved by the observation that when the focus assays were kept for longer, foci induced by DNA derived from AML 33 were apparent 19-20 days after transfection but not at the usual time of examination at 15-16 days. However, foci from DNAs containing Val 13 were still not apparent even after leaving the assays for 20 days. Cells cultured from tumours resulting from co-transfection with DNAs containing the Asp 13 mutation also look more transformed than those that contain Val 13 mutants (D.T. and C.J.M., unpublished). These results are consistent with the idea that Val 13, which is an activating mutation not identified previously[18], and perhaps Asp 13 in N-*ras*, are relatively weak transforming alleles for NIH 3T3 and may be more difficult to detect than codon 12 or 61 mutations.

Our observation of multiple cases of mutations at codon 13 suggests that the activation of the N-*ras* gene at this site is an essential step in causing these AMLs. However, it is unlikely that the activation of the N-*ras* oncogene is the only step involved in the genesis of AML, because much evidence favours the argument that changes to more than one gene are involved in neoplastic transformation (see ref. 1 for review). Furthermore, specific chromosome translocations and other aberrations are associated with AML[35]. These chromosome aberrations occur at sites distinct from the 1cen-1p21 chromosomal site of N-*ras*[36] and separate aberrations are found in the different subgroups of AML. For example, a translocation, t(8;21), is found in AML M2 and an inversion of chromosome 16, inv16, in AML M4 with increased eosinophils (see ref. 35 for review). In contrast, we have found activation of N-*ras* in samples of AML from four different subgroups (M1, M2, M4 and M5); thus, the activation of N-*ras* seems to be affecting a process that is essential to all cells in the myeloid lineage, whereas the chromosome aberrations affect genes whose activities are more limited.

For the analysis of the N-*ras* mutations, we have used a set of specific oligonucleotide probes. This assay system is not only powerful in the detection of mutations in transfectant DNAs but also in the detection of specific mutations in tumour DNA. Using this assay system and the *Hph*I polymorphism generated by the Asp 13 mutation, we are currently analysing a large number of AML DNAs to gain more precise insight into the occurrence of different *ras* gene mutations in this leukaemia.

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