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Three different mutations in codon 61 of the human N-*ras* gene detected by synthetic oligonucleotide hybridization

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ABSTRACT

The activation of ras genes in naturally occurring tumors has, thus far, been found to be due to mutations in codon 12 or 61 resulting in single amino acid substitutions. We have used highly labeled synthetic oligonucleotides to detect mutations in these codons and to determine the exact position of the mutation. Using this approach we have found three different mutations in codon 61 of the N-ras gene of various human tumor cell lines. In the fibrosarcoma line HT1080 the first nucleotide of the codon is mutated; in the promyelocytic line HL60 the second and in the rhabdomyosarcoma line RD301 the third nucleotide. For RD301 this implies that the normal glutamine residue at position 61 is replaced by histidine. In addition to the mutated N-ras gene the three cell lines have a normal N-ras gene which is indicative of the dominant character of the mutations.

INTRODUCTION

The human ras gene family consists of three members: the H-ras, K-ras and the N-ras gene (1). These genes code for related proteins of 21kD, which are located at the inner face of the cell membrane (2) and are thought to be involved in transducing signals from cell surface receptors to their intracellular targets (3). A significant portion of tumor cell lines and fresh tumor tissue has been found to possess an activated ras gene. Such genes are characterized by their ability to induce oncogenic transformation of mouse 3T3 cells. In all cases so far analysed the activation is due to a point mutation in the 12th or 61st codon of a ras gene resulting in a single amino acid substitution in the gene product (4-15).

The fixed position of the mutations makes it possible to use synthetic oligonucleotides to screen directly for the presence of activating mutations in genomic DNA of tumor tissue. This direct assay is based on the fact that a fully matched DNA hybrid has a higher thermal stability than a hybrid with a mismatched basepair. By using a selective washing procedure nonperfect hybrids can melt off, whereas a fully matched hybrid remains stable.

This type of assay has been introduced by Conner et al. (16) to detect a single base mutation in the β-globin gene. To detect possible mutations that lead to amino acid substitution at position 12 or 61 of the three ras genes we have synthesized 6 sets of 20-mers which can detect whether and at which position a mutation is present. Here we describe the assay system for mutations in codon 61 of the human N-ras gene.

MATERIALS AND METHODS

Cell lines and DNA

MOLT-4 was obtained from D.Valerio, HL60 from H. Janssen(Netherlands Cancer Institute), HT1080 and RD301 from Dr. A.Hall (London). Genomic DNAs from lines K562 and Rc-2a were gifts from Dr.G. Grosveld (Rotterdam) and H.Janssen, respectively. Plasmids pAT8.8 (containing part of the N-ras gene from HT1080 (17))and pSVN-ras (containing the N-ras gene of HL60 under control of the SV40 early promoter) were provided by Dr.A.Hall (London) and Dr.R.A.Weinberg (MIT), respectively. (α-^{32}P)dATP (specific activity 3000 Ci/mmole) was obtained from Amersham (U.K.)

Synthetic oligonucleotides

The oligomers were synthesized by the solid phase triester method as described (18). The 8-mer primer was synthesized with a 5'-terminal phosphate group (J.E. Marug and J.H. van Boom, submitted).

Labeling of oligomers

^{32}P-labeled oligomer probes were synthesized on 20-mer templates corresponding the antisense sequence of the N-ras gene by enzymatic extension of hybridized 8-mers (19). The reaction was in 10 µl containing 6 mM Tris HCl (pH 7.6), 6 mM MgCl, 6 mM β-mercaptoethanol, 50 mM NaCl, 20 µCi (α-^{32}P)dATP, 100 µM of each of the other dNTPs, 30 ng template, 10 ng primer and 3 units DNA polymerase I (large subunit) at 0° for 1 hr. The reaction was stopped by the addition of 10 µl formamide and the mixture was loaded onto a 10% polyacrylamide-7M urea sequence gel. Due to the 5' phosphate groups on the elongated strand, it can be separated from the unlabeled template on a 10% polyacrylamide-7M urea gel. The labeled oligomer was visualized by autoradiography, the excised band was eluted in 500 µl, 1 mM EDTA and the eluate was used directly for hybridization. The ^{32}P-labeled oligomers have specific activity of 6 x 10^9 dpm/µg.

Agarose gel electrophoresis and direct gel hybridization

Agarose gel electrophoresis and direct-gel hybridization were essentially as described by Studencki and Wallace(19). The DNA digests were

electrophoresed in a 0.5 cm thick 0.5% agarose gel. The gels were stained with ethidium bromide and photographed under UV-light. Subsequently, the DNA was denatured *in situ* in 0.4 M NaOH, 0.8 M NaCl (30 min at room temperature) and neutralized in 0.5 M Tris-HCl (pH 7.2), 1.5 M NaCl at 0° for 30 min. Gels were dried on to Whatman 3 MM paper using a Biorad gel-dryer. The dried gel was wetted with distilled water to remove the Whatman 3MM paper and sealed in plastic bags for hybridization. Hybridization took place in 5 ml 5xSSPE (1x SSPE=10 mM sodium phosphate pH 7.0, 0.18 M NaCl and 1 mM EDTA), 0.3% sodium dodecylsulfate containing 10 µg/ml sonicated, denatured *E.coli* carrier DNA (16 hr at 50°C). Hybridized gels were washed as followed: twice in 2xSSPE, 0.1% sodium dodecylsulfate (30 min) at room temperature, once in 5xSSPE, 0.1% sodium dodecylsulfate at 52°(15 min) and once the same solution at 59°C for 5 min. Gel membranes were covered with Saran wrap and autoradiographed at -70°C for 4 days using intensifying screens.

RESULTS

Principle of the method

To screen for mutations in codon 61 of the human N-*ras* gene we have synthesized four different groups of 20-mers (Fig.1). The first 20-mer (N61-I) has a sequence identical to a 20-long stretch covering the 61st codon (CAA) of the normal N-*ras* gene. N61-II is a mixture of three 20-mers identical to N61-I except that at the position of the first nucleotide of codon 61 the wild-type C has been replaced by A, G or T. As a consequence this group of oligomers can only form an imperfect hybrid with the normal N-*ras* gene. However, when codon 61 is mutated in its first position, one of the 20-mers of the N61-II group will form a complete match with the mutated gene. Similarly, in N61-III G, T and C substitute for the wild-type second base of the codon. Finally, the two 20-mers of N61-IV have T and C instead of A as the third nucleotide of the codon (substitution of G gives rise to a silent mutation). The oligonucleotide probes used in this study had the sense orientation and were of high specific activity. To attain the latter, we prepared anti-sense 20-mers and a sense 8-mer (complementary to the 3' end of the 20-mers) by chemical synthesis. The label was introduced by incubating this template-primer complex with(α-^{32}P)dNTPs and DNA polymerase I (large subunit). The 8-mer primer carried a 5'-terminal phosphate group which enabled us to separate the labeled oligomer from the unphosphorylated template on a polyacrylamide-urea gel.

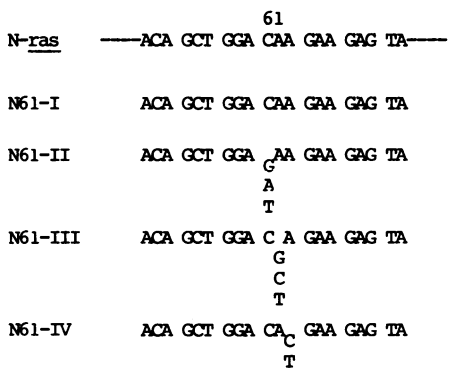

Fig.1. Sequence of the 20-mer probes containing codon 61 of the N-ras gene. Sequences are derived from Taparowsky et al. (6).

Hybridization conditions

Hybridization of the labeled oligomer probes to plasmid DNA or genomic DNA was performed in a dried agarose gel as described by Schinnick et al. (20) and Studencki and Wallace (19). To that end, DNA was digested, fractionated on a 0.5% agarose gel and denatured in situ, after which the gel was dried. The dried gel membrane was wetted in distilled water and subsequently hybridized with the labeled oligomers. According to Studencki and Wallace this procedure allows the detection of a single gene copy in a relatively small amount of genomic DNA (1-5 µg).(19).

To determine the melting temperature of a fully matched hybrid and of a one base pair -mismatched hybrid between one of the 20-mers and the N-ras gene the labeled oligomer probes were hybridized to plasmid DNAs containing a mutation in codon 61 of the N-ras gene. These plasmids, pAT8.8 and pSVN-ras,contain parts of the activated N-ras genes from a secondary NIH/3T3 cell transfectant of the fibrosarcoma cell line HT1080 (17; $CAA_{61} \rightarrow AAA$; 14) or from a tertiary transfectant of the promyelocytic cell line HL60 (21; CCA_{61}— CTA; J.M. Cunningham and R.A.Weinberg, pers.comm.), respectively. In 5xSSPE, the melting temperature for a fully matched hybrid was 61-62°C, for a hybrid with a one base pair-mismatch 55-56°C (data not shown). We found that hybridization at 50° and a final wash at 59° were suitable to specifically detect a fully matched hybrid. This is exemplified in Fig.2, lanes 7 and 8. A mixture of pAT8.8 and pSVN-ras digested with PstI was hybridized to the oligomers N61-I (panel I), N61-II (panel II), N61-III (panel III) and N61-IV (panel IV).A 4.4-kb PstI-fragment from pAT8.8 containing codon 61 with a mutation of the first nucleo-

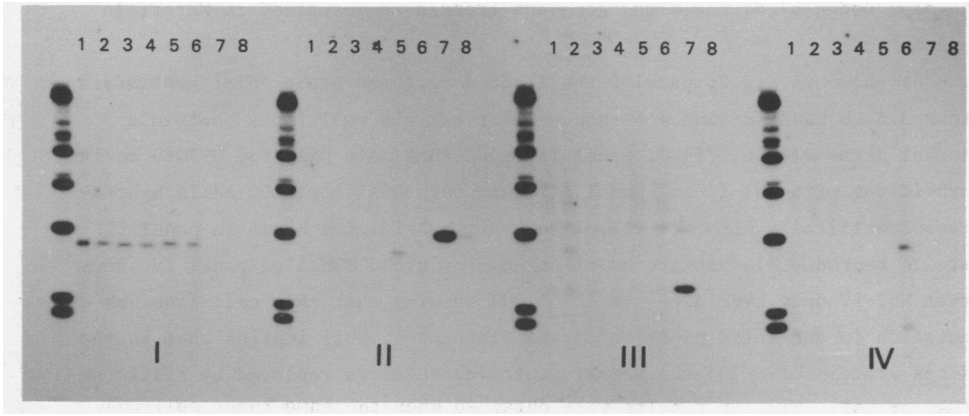

Fig.2. Hybridization of synthetic oligomers to genomic DNAs of human tumor cell lines. 10 µg genomic DNA was digested with PstI and electrophoresed on a 0.5% agarose gel. In addition, 10 and 0.5 haploid genome copies of the plasmids pAT8.8 and pSVN-ras digested with PstI were fractionated on the same gel. Four such gels were dried and hybridized to [^{32}P]-labeled oligomer probe N61-I (panel I), N61-II (panel II), N61-III (panel III) and N61-IV (panel IV) at 50°C in 5xSSPE. The gel membranes were washed and mismatched hybrids were washed off at 59°C in 5xSSPE. Lane 1: MOLT-4; lane 2: HL60; lane 3: Rc-2a; lane 4: K562; lane 5: HT1080; lane 6: RD301; lane 7: 10 haploid genomic copies of pAT8.8 and pSVN-ras; lane 8: 0.5 haploid genome copy of pAT8.8 and pSVN-ras. Marker: phage λ DNA digested with HindIII (23, 9.5, 6.4, 4.3, 2.3 and 2.1 kb).

tide hybridizes only with N61-II which implies that a fully matched hybrid is possible with one of the oligomers present in group N61-II. A PstI-fragment of 2.8 kb from pSVN-ras where codon 61 has a mutation at the second position hybridizes only with N61-III. None of the other oligomer probes hybridizes to the two fragments. This shows that our conditions are specific to detect only fully matched hybrids. The amount of plasmid DNA used (10 and 0.5 haploid genome copies) furthermore shows that we can detect a single genome copy.

Hybridization to genomic DNA

To test whether we can detect N-ras gene mutations in genomic DNA we have analysed DNA isolated from both HT1080 and HL60 cells. In addition, we have analysed DNA derived from four other cell lines, three of which have been shown to contain an activated N-ras gene by the 3T3 cell assay: the T-ALL cel line MOLT-4 (22), the AMMoL cell line Rc-2a (H. Janssen, personal communication), and the rhabdomyosarcoma cell line RD301 (17). The CML cell line K562 has not been reported to contain an activated ras gene. The DNAs were digested with PstI which generates a 3.6-kb fragment con-

taining codon 61 of the N-ras gene and treated as described in Materials and Methods.

As shown in Fig.2, panel I the labeled oligomer probe N61-I hybridizes to a 3.6-kb band in lane 1-6 showing that all six cell lines contain a normal N-ras allele. Fig.2, panel II shows that only DNA from HT1080 cells hybridizes with N61-II and panel III that only DNA from HL60 cells hybridizes specifically with N61-III. (The other hybridizing bands in panel III are a reproducible background of unknown origin). Finally, panel IV shows that N61-IV only hybridizes to RD301 DNA showing that this cell line has a mutation in the third position of the 61st codon. This implies that in the N-ras protein of RD301 the normal glutamine (CAA) is replaced by histidine (CAC or CAT). Similar results were obtained when the same tumor cell DNA were digested with EcoRI (not shown). From these results we conclude that genomic DNA of both HT1080 and HL60 contain the same point mutations in the 61st codon as the isolated genes from NIH/3T3 cell transfectans (the plasmids pAT8.8 (17) and pSVN-ras (21), respectively). Furthermore, we conclude that in the three cell lines containing a mutated N-ras gene a normal N-ras allele is also present.

Our oligomers failed to detect a mutation in codon 61 of the N-ras gene of the MOLT-4 cell line and the Rc-2a cell line which both have been reported to have an activated N-ras gene. In these cases the mutations may be located in codon 12 as recently found for the human teratocarcinoma cell line PA1 (12) but other positions can not be excluded (26).

DISCUSSION

We have used synthetic oligonucleotides to detect mutations in codon 61 of the human N-ras gene. We found such mutations in the genomic DNAs of HT1080, HL60 and RD301 at the three possible codon positions. In HT1080 the first nucleotide of codon 61 is mutated and in HL60 the second. This confirms the results obtained through sequence analysis of activated N-ras genes isolated from 3T3 cells transformed by DNA from these two cell lines (14;J.M.Cunningham and R.A.Weinberg,pers.comm.). In RD301 the third nucleotide is mutated(from A to C or T),which implies that the normal glutamine at position 61 is replaced by histidine. Furthermore, we found that in these three cell lines, in addition to a mutated N-ras allele, a normal allele is present, showing the dominant character of the mutations. The dominant character was already suggested by the ability of the mutated N-ras genes to transform NIH/3T3 cells (17,23). Similarly, both a normal and an affected K-ras gene are present in the lung carcinoma cell line Calu-1 (9). On

the other hand, several cell lines have been reported to contain only the mutated allele of an activated ras gene: H-ras in the bladder carcinoma cell line T24 (24) and K-ras in the colon carcinoma SW480 (9), the lung carcinoma A2182 and the bladder carcinoma A1698 (10).

The use of synthetic oligonucleotides for the detection of mutations in the family of ras oncogenes may have considerable advantages as compared to what is as yet the only alternative, the 3T3 cell assay. Firstly, the assay is performed directly on the genomic DNA avoiding the possibility of mutations introduced during the transfection procedure (23). Secondly, it detects both the mutated and the normal alleles. This opens the possibility of discriminating whether a mutation is present in one or in both alleles as discussed above. Furthermore, for those cases where both an activation and an amplification of a particular ras gene have been found (25) it can detect whether the normal or the mutated allele has been amplified. Answers to these questions may allow conclusions as to whether the ratio between affected and normal alleles plays a role in the tumorigenic properties of a cell. Thirdly, its sensitivity does not depend on the transforming capacity of the affected gene. Some mutations result in so called "weakly transforming alleles" (26) and may be difficult to detect in 3T3 cell assay. A final advantage of the method is its relative rapidity and easiness. It will allow the screening of large numbers of tumor tissues for activated ras genes to get a clearer insight into the significance of these activations for in vivo tumorigenesis and tumor progression. Thus far, our knowledge of the role of activated ras genes in these processes is only limited, particularly because most experiments have been done with established cell lines which could have acquired an activated gene after their isolation (12).

A disadvantage of the method is that its detection level is limited. Tumors that consist for a major part of non-neoplastic tissue may yield a hybridization signal which is to weak for unambiguous conclusions. Furthermore, the methods is limited to specific point mutations, on our case in the 12th or 61st codon of the three different ras genes. Recently, however, Fassano et al. (26) have found that mutations in codons 13, 59 and 63 of the H-ras gene can also activate this gene. Such mutations have not yet been found in naturally occurring tumors, suggesting that their contribution to tumor formation is only limited.

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