Mechanism of Activation of Human ras Genes Cloned from a Gastric Adenocarcinoma and a Pancreatic Carcinoma Cell Line

Bryan M. O'Hara, Marianne Oskarsson, Michael A. Tainsky, and Donald G. Blair

ABSTRACT

We have analyzed the mechanism of activation of two human ras oncogenes. We have also identified a rasN gene from a human gastric adenocarcinoma which efficiently induced both morphological transformation and tumorigenicity of NIH3T3 cells in a transfection assay. The rasN gene in tumor tissue DNA did not appear to be rearranged or amplified. A molecular clone, which contained an EcoRI fragment spanning the first and second rasN exons, was molecularly cloned directly from the human tumor DNA. Chimeric constructions and DNA sequencing defined the mechanism of activation of the gene as a mutation in the 61st amino acid codon substituting arginine for glutamine. Normal DNA isolated from Epstein-Barr virus immortalized lymphocytes derived from the same patient did not induce morphological transformation or tumorigenicity in NIH3T3 cells.

A cloned cell line isolated from the human pancreatic carcinoma cell line PANC1 had previously been shown to contain an activated rasK gene. Sequence analysis of the cloned transfected gene reveals a G to A change within codon 12, which is presumably responsible for its biological activity. This represents the first identification of a 12th asparagine acid substitution of a c-ras oncogene from a human tumor-derived cell line.

INTRODUCTION

Genomic DNA from a wide variety of human and rodent tumors and tumor-derived cell lines has been shown to transform NIH3T3 cells by morphological and other criteria in transfection assays. In the majority of cases analyzed to date, the transfected DNA sequences responsible for the transforming event have been shown to be members of the ras gene family. Two of these, rasK (1-3), and rasX (4-8), are the cellular homologues of the transforming oncogenes of the Harvey and Kirsten murine sarcoma viruses. The third, rasN (9-11), has only been identified as an activated cellular sequence. Although each member of the ras family represents a unique cellular gene, they code for an immunologically cross-reactive family of structurally similar proteins of approximately Mr 21,000 (12). The activated transforming cellular ras genes which have been identified differ from their normal cellular homologues at a single nucleotide, which results in an amino acid substitution at either the 12th or 61st codons of the ras sequence. Although under certain conditions both normal rasK (13) and rasX (14) will transform NIH3T3 cells, the presence of altered amino acids at the 12th or 61st positions allows these genes to function as efficient transforming genes in DNA transfection assays.

The cellular rasK gene has been found to be activated more frequently in tumors or lines of hematological origin (9, 15-17), and substitutions at positions 61 and 12 and more recently 13 (18) have been identified. Activated homologues of the Harvey murine sarcoma virus (rasK) have been found with low frequency in a wide variety of neoplastic types, although in certain experimental situations they are preferentially detected in carcinomas (19, 20). The activating mutation in rasK has been shown to occur at either the 12th (21, 22) or the 61st codon (23). The majority of activated ras genes found in carcinomas or carcinoma cell lines from any species have been cellular homologues of the Kirsten murine sarcoma virus oncogene (rasX). These genes have been shown to contain amino acid substitutions at position 12 (5, 7, 24) or 61 (25).

Many of the activated oncogenes have been derived from transformed cell lines and it is possible in these cases that the activation events could have occurred during culture in vitro. Activation of rasK and rasX genes during culture in vitro has in fact been shown to occur (26, 27); however, activated ras genes have been shown to be present in human tumor DNA in several instances. Two activated rasN genes have been found in human tumor tissues isolated directly from patients (15, 16). In the latter study, it was found that the normal tissue DNA from the patient was inactive in transfection assays (16).

In the course of screening DNA from a variety of human solid tumors for transforming activity, we have detected an activated rasN gene in DNA prepared directly from a fresh surgical specimen of gastric adenocarcinoma tumor tissue. This afforded the opportunity of examining an activated rasN gene isolated directly from a tumor of a patient whose normal DNA was available and on whom a medical history had been obtained. The present study describes the cloning and characterization of this activated gene and the identification of its activating lesion. This study represents the first analysis of an activated rasN cloned from solid human tumor material. We also describe the further analysis of activated rasN gene originally detected in a human pancreatic carcinoma derived cell line (28, 29) which, as we report here, contains an alteration at position 12.

MATERIALS AND METHODS

DNA Transfection. Transfections were performed by a modification of a previously described procedure (30). NIH3T3 cells were plated at 3.9 × 10⁵ cells/60-mm dish in Dulbecco's modified Eagle's medium with 10% calf serum and 18 h before transfection. For transfections of human tumor or cell DNA, 100 µg of DNA were precipitated and the precipitate was divided among 5 dishes. Cells were split from each 60-mm dish to two 100-mm dishes and foci were scored 10-14 days later. In some cases, 0.125 mm dexamethasone was added to the media to enhance focus detectability. For tumor induction assays 12 µg of supercoiled pSV2neo plasmid were added to the cell DNA prior to formation of the precipitate. Cells from each 60-mm dish were split to a 75-cm² flask 18 h after transfection and the selective drug Geneticin (Gibco) was applied at 400 µg/ml 24 h later. Cells were maintained under selection until colonies formed (10-14 days). The cells were then trypsinized, pooled, and tumorigenicity was measured as previously described (28). For transfections of in vitro ligated samples, DNAs were applied at approximately 50 ng/dish using sheared calf thymus DNA as carrier (20 µg/dish). The cells were either carried without trypsinization in the transfection dishes or trypsinized and replated each in three 100-mm dishes.

Tumor and Normal DNA. DNA was prepared from a fresh surgical sample of a primary gastric adenocarcinoma, designated HT54, and...
from lymphocytes immortalized by Epstein-Barr virus infection and designated 09-54 from the same patient. The patient, a 56-year-old male at the time of surgery, had not received chemotherapy. The tumor was a large (8 x 7.5 cm) well-differentiated adenocarcinoma of the stomach which showed invasion through the muscular wall to the serosal tissues but not into the periserosal adipose tissue. There was no evidence of tumor recurrence 1.5 years after surgery. Extraction from tissues and cells was as previously described (29).

Southern Analysis and Plasmid DNAs. Southern analysis was performed as described using 20 μg DNA/lane (31). The "R" probe specific for the 5' EcoRI fragment of the cellular ras" gene was obtained from M. Wigler (10). A 1-kilobase probe specific for the 3' EcoRI fragment was obtained following XbaI digestion of a plasmid (p3A-3) carrying the 3' EcoRI fragment of a human ras" gene derived from P1A cells (26). Probes were purified free of vector sequences by gel electrophoresis. The normal human ras" gene λNP13 was obtained from M. Wigler (11) and the 5' and 3' EcoRI fragments were subcloned into pBR322. myc-Specific DNA, a gift from Dennis Watson, covered most of the third exon of the human c-myc gene from the Clal to the RsaI sites (32). The fos-specific probe was the EcoRI-Neol 5' fragment of pHVV supplied by Miller (33).

Isolation of Human ras" Sequences. An L.47λ phase-mouse genomic library was prepared from partially MboI digested DNA of secondary transfected NIH3T3 cells, shown to contain human Alu repeats and ras" sequences. The library was screened with 1P-labeled BluR (34) or ras" HiiH3 (35) probes. Positive clones were purified and analyzed by restriction endonuclease digestion and hybridization to BluR and HiiH3 probes. EcoRI fragments have been subcloned into pBR322.

Cloning of ras" Gene Fragments from HT54. DNA prepared from HT54 was digested to completion with EcoRI and separated on a 10-40% sucrose gradient for 16 h at 24,000 rpm in a Beckman VTi 50 rotor. Appropriate fractions carrying the 9- or 7-kilobase ras" fragments shown. Coding exons are indicated by Roman numerals. K, EcoRI; B, BamHI; S, SacI; Ba, BamHI; Sm, SmaI; H, HindIII.

RESULTS

Transfected ras" from Human Pancreatic Carcinoma Cell Line with Single Base Change at Position 12. Panc1 is an epithelial cell line derived from a human pancreatic carcinoma of ductal cell origin. A subclone of this line (A1165F3c12) which showed improved growth in agar suspension was obtained from Dr. J. DeLarco, National Cancer Institute, and was shown to contain a transfectable activity identified by Southern blot analysis as a human ras" allele (29).

We prepared a φ phage library from a secondary NIH3T3 transfected and isolated the series of clones, shown diagrammatically in Fig. 1. Based on comparison with published restriction maps of this locus (35, 37), the clones we isolated spanned the first three coding exons of the ras" allele. Since previously published data indicated that single base changes at the 12th and 61st codons activated different members of the ras family, we initially sequenced the 450-base pair Sau3A fragment (Fig. 1) which spanned the 12th codon. The sequence we obtained was identical to the normal ras"-2 sequence (34) except at the 12th codon, where a G to A transition resulted in a substitution of aspartic acid (GAT) for glycine (GGT) at this position. It is reasonable to presume that this lesion is responsible for the biological activity we have detected and represents the first identification of an aspartic acid substitution at this position in the c-K ras of a cell line derived from a spontaneous human tumor.

Identification of Oncogene from a Human Gastric Adenocarcinoma. HT54. We had previously described the use of the nude mouse as a vehicle for screening for the presence of transformed cells in a transfected cell population. We (26) and others (14) have modified this assay by including a plasmid carrying a selectable drug resistance marker as a cotransfecting sequence with the tumor derived DNA and then applying drug selection to the transfected cell population to select for a cell population which expresses the transfected plasmid and which presumably carries transfected genomic sequences as well. This procedure appears to increase both the rate of tumor appearance and the number of tumors detected (data not shown), presumably due to the clonal expansion of any cell transformed by transfected genomic DNA during growth in selective media. This procedure also allows an internal measure based on the efficiency of genomic DNA transfection of the probability of detection of a single copy cellular gene. Based on an estimated average of 3 x 10⁵ kilobases of genomic DNA per cotransfected cell, 1 x 10³ colonies of NIH3T3 cells should contain a genomic equivalent of human DNA. Although this is only an approximate figure, it does permit limits of sensitivity to be placed on transfections in which no transforming activity is detected.

In the course of testing human tumor DNAs extracted from 60 solid tumor specimens for the ability to induce foci or tumorigenicity in transfected NIH3T3 cells, we identified DNA from a human gastric adenocarcinoma (HT54), active in both these assays. Table 1 shows the efficiencies of focus and tumor formation obtained with HT54 DNA. Foci were detected in the

Table 1 Transfection analysis of DNA from tumor HT54 and from lymphocytes of the same patient

<table>
<thead>
<tr>
<th>Assay</th>
<th>Number of Foci/Mg</th>
<th>Tumors</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HT54</td>
<td>15/19 (4-5)</td>
<td>0.09</td>
</tr>
<tr>
<td>MT181</td>
<td>4/4 (2)</td>
<td>0.41</td>
</tr>
<tr>
<td>05-94</td>
<td>2/11 (9)</td>
<td>Not tested</td>
</tr>
<tr>
<td>Human placental</td>
<td>1/13 (6)</td>
<td>Not tested</td>
</tr>
</tbody>
</table>

* Numbers in parentheses, time of appearance of tumors in weeks. Tumors obtained with 05-94 DNA appeared at a time consistent with normal background. They did not contain human N-ras sequences (not shown).
absence of cotransfection. The large foci consisted of refractile fibroblastic cells easily detectable against the nontransformed 3T3 cell background. Their morphology and growth characteristics initially suggested that HT54 might contain an activated member of the ras gene family. A second more highly invasive gastric adenocarcinoma tested in these assays was found to be inactive (not shown).

Southern blots of DNA extracted directly from tumors were hybridized to probe prepared from sonicated total human placental DNA, and all tumors were found to contain large numbers of hybridizing bands (data not shown). This was expected because the initial drug selection should result in all injected cells containing cotransfected human DNA. The presence of discrete bands, however, suggested that the tumor had arisen from a small number of cells within the mass population of transfected cells. DNA from one of these primary tumors (Table 1, MT181 and Fig. 2, lane 5) was used in a second round of transfection and was found to induce foci in NIH3T3 cells with a slightly increased efficiency relative to the HT54 parent.

Several foci and tumor explants obtained following transfection with HT54 DNA were examined with a variety of onc gene probes to determine if a transferred onc gene could be identified. Hybridization to ras<sup>nu</sup> and ras<sup>sk</sup> specific probes revealed no hybridizing sequences other than the bands expected for the endogenous mouse cellular homologue of these two genes; however, hybridization with a probe specific for ras<sup>nu</sup> revealed a new band with size consistent with that of the human cellular ras<sup>nu</sup> gene. Fig. 2 shows such an analysis of EcoRI-digested DNAs using the ras<sup>nu</sup> 5' fragment-specific R probe. This indicated that the activated gene in HT54 DNA as an ras<sup>nu</sup> gene and suggested that no obvious rearrangement of ras<sup>nu</sup> had occurred in that primary tumor.

Recent reports (14) have indicated that the presence of large numbers of copies of the normal human ras<sup>nu</sup> gene may be sufficient to induce NIH3T3 cells to form tumors in nude mice. Amplification of c-myc has also been reported in gastric adenocarcinomas maintained as xenografts in nude mice (39). Comparison of the intensities of the signals obtained in HT54 DNA using ras<sup>nu</sup> and myc probes to the signals obtained in the DNAs in Fig. 2, lanes 1-3, and to the signal obtained with the fos probe (Fig. 2B) showed that the HT54 ras<sup>nu</sup> and myc genes were not amplified or grossly altered. This assumes that the tumor sample (which appeared homogenous on the basis of pathological examination) was not grossly contaminated with normal tissue.

Normal DNA from Same Patient Free of Activated ras<sup>nu</sup> Sequences. Following the identification by Southern blot analysis of ras<sup>nu</sup> activity associated with the HT54 DNA sample, we obtained a sample of normal peripheral blood from the patient, who at the time had suffered no recurrence of metastatic disease and had received no chemotherapeutic treatment. Lymphocytes were prepared and infected with Epstein-Barr virus to establish a B-lymphocyte culture for subsequent analysis. DNA was prepared from these cells approximately 6 weeks after infection and used to transfect NIH3T3 cells in our standard cotransfection tumor assay. HT54 DNA was transfected in parallel as a positive control. Although mice given injections of HT54 DNA-transfected cells again developed tumors within 5 weeks, no tumors arose in mice given injections of lymphocyte-transfected cells until 9 weeks (Table 1). We occasionally see tumors in mice given injections of normal 3T3 cells or cells transfected with normal human mouse DNA at about 10 weeks, which most likely represent spontaneously transformed 3T3 cell background tumors (28). In this case, DNA was extracted from the two tumors and analyzed with a ras<sup>nu</sup> specific probe. No new hybridizing bands were detected, indicating transfected ras<sup>nu</sup> sequences were not responsible for the tumorigenesis of these cells. The 13 mice were each given injections of 2 x 10<sup>6</sup> cells derived from approximately 4 x 10<sup>5</sup> transfected colonies; thus, it appears likely that the normal lymphocyte DNA did not contain activated ras<sup>nu</sup> sequences, and as has been observed in other cases, the oncogene activation event has not occurred in normal somatic cells and most probably represents an event unique to tumor tissue.

Cloning of ras<sup>nu</sup> from HT54 Tumor DNA. In order to verify the presence of an activated N-ras gene in the HT54 tumor itself and to identify the activating lesion, we undertook to clone ras<sup>nu</sup> sequences directly from HT54 DNA. DNA from HT54 was digested to completion with EcoRI and was fractionated on a sucrose gradient. Fractions were analyzed by Southern analysis using the 5' and 3' EcoRI fragment-specific probes for the presence of the expected 9- and 7-kilobase ras<sup>nu</sup> specific bands (11). The appropriate fractions were pooled, used to construct a library in λgtWES.AB, and the library screened using both the 5' and 3' EcoRI fragment probes simultaneously. Several clones were obtained, and two have been characterized in detail. One of these λ54-1, hybridized exclusively to the 5'...
specific probe and carried a 9-kilobase insert, as expected from the known restriction map of the normal ras<sup>N</sup> gene and from the Southern analysis shown. The other, λ54-2, hybridized exclusively to the 3'-specific probe and contained the expected 7-kilobase insert.

Fragment of Activated ras<sup>N</sup> Gene Carried by λ54-1. To determine if λ54-1 or -2 contained fragments representative of the activated ras<sup>N</sup> gene present in HT54, the cloned DNAs were digested with EcoRI, ligated to the appropriate EcoRI fragment obtained from the normal gene, and transfected into NIH3T3 cells. Fig. 3 shows the results of these experiments. The 9-kilobase EcoRI fragment induced the formation of foci when ligated to the 3' 7-kilobase EcoRI fragment of the normal gene (Fig. 3, A). Three foci were picked, grown up, and DNA was prepared from them. Southern analysis revealed that each culture carried a novel 9-kilobase EcoRI fragment hybridizing to the ras<sup>N</sup> 5'-specific probe, as expected. Southern analysis of one of these is shown in Fig. 2A, lane 9. The 7-kilobase fragment of λ54-2 did not induce foci in NIH3T3 cells after ligation to the normal 5'-9-kilobase EcoRI fragment (Fig. 3, B).

To facilitate further analysis, the λ54-1 insert was subcloned into the EcoRI site of pBR322, the plasmid being designated as p54-1. In order to further narrow the lesion in the 9-kilobase isolate, combinations of portions of it with portions of the normal gene were made by in vitro ligation of gel-purified fragments, and these were then tested by transfection into NIH3T3 cells. As expected, the BstEII fragment spanning the expected positions of the first two exons was active (Fig. 3, E). The PsI-EcoRI fragment spanning the expected position of exon II was also active (Fig. 3, H). The reciprocal ligations were inactive, indicating that the PsI-BstEII fragment spanning the expected position of exon II contained sufficient information to activate the ras<sup>N</sup> gene in a transfection assay.

p54-1 ras<sup>N</sup> Coding Sequences: Difference from Normal ras<sup>N</sup> in Only One Nucleotide. To identify any changes in the ras<sup>N</sup> coding regions of p54-1, exons I and II were sequenced. When compared to the published sequence of the normal gene, exons I and II and their immediate flanking areas showed no changes except at codon 61 in exon II; there, the triplet CGA was found in place of the normal CAA, leading to a glutamine to arginine change at this position. This was in full agreement with the in vitro ligation data, and it is reasonable to conclude that the change in the 61st codon is responsible for the activity detected in the NIH3T3 transfection assay.

**DISCUSSION**

We have analyzed the activated ras genes identified by DNA transfection in DNA from a long-established human pancreatic carcinoma cell line and a primary human gastric adenocarcinoma. The detection of an amino acid change at position 12 of the pancreatic carcinoma derived ras<sup>K</sup> sequence is consistent with this change being responsible for the biological activity we have detected. The long culture history of this cell line and the fact that this represented an agar clone of the original tumorderived cell line which was selected for its aggressive growth in agar suspension make it impossible to determine if this lesion was present in the original primary tumor.

In the second case, however, we have demonstrated the presence of an activated ras<sup>N</sup> gene in cell DNA isolated directly from fresh human gastric adenocarcinoma tumor tissue. The ras<sup>N</sup> gene was cloned from this tumor DNA and the activating lesion was mapped to the second coding exon by constructing a series of ras<sup>N</sup> hybrids between the gastric carcinoma ras<sup>N</sup> and normal ras<sup>N</sup> sequences. This was confirmed by direct sequence analysis, which revealed a single base change in the 61st codon, resulting in the substitution of an arginine in place of glutamine at this position. This presumably is responsible for its ability to express a transforming phenotype. It is important to note that the activated gene was cloned directly from unmanipulated tumor material, thus strengthening the supposition that the mutation was present and could have played a role in tumor development in vivo. We have confirmed that normal DNA from established Epstein-Barr virus-infected lymphocytes from the same patient does not contain an activated ras sequence.

The biochemical roles of the ras genes are unknown, although a number of observations have been made which suggest how they might function. They bind guanine nucleotides (40) and are stimulated in guanine nucleotide binding by epidermal growth factor or insulin (41). There is also limited amino acid homology between viral ras<sup>K</sup> and transducin, a guanine nucleotide-dependent regulatory protein that is structurally homologous to the hormone-sensitive adenylate cyclase regulatory G proteins (42). These observations suggest a role for ras proteins in signal transduction, possibly involving adenylate cyclase. Since a mutation conferring increased oncogenic potential at ras<sup>N</sup> also impairs its GTPase activity (43), the only known biochemical difference between normal and mutant ras alleles, this reduced activity may form the basis for the functional differences between normal and mutant proteins.

Activated ras<sup>K</sup>, ras<sup>H</sup>, and ras<sup>N</sup> genes have been found in a wide variety of tissues, and no strict correlation between the identity of an activated ras gene and the tissue in which it is found has been seen, although correlations have been observed in certain experimental situations involving carcinogen-induced mouse tumors (19, 20). To date, the great majority of mutations identified in activated human cellular ras genes involve the 12th and 61st codons, with the majority of activated ras<sup>K</sup> genes showing mutations at codon 12, whereas those of ras<sup>N</sup> show an even distribution. Activated ras<sup>N</sup> genes in which the activating lesion has been identified show mutations at position 61 (11, 44) and at position 12 (16, 26). Of the 6 possible substitutions at position 61 which would result from single base changes in the CAA codon, only two have been detected in activated genes.

---

**Fig. 3. Localization of the alteration in the HT54 N-ras 5' EcoRI fragment using transfection assays.** Chimeric constructions were made using 9-kilobase (kb) 5' EcoRI and 7-kilobase 3' EcoRI fragments cloned from HT54 DNA. B in top line, expected positions of exons 1 and II; J, restriction sites. In 4-11, I, restriction sites used for ligation. _____, appropriate fragments from the normal N-ras gene; □, N-ras fragments cloned from sources as follows: A, λ54-1; B, λ54-2; C, an unrestricted activated N-ras gene cloned from human PA-1 cells and included for comparison (pMT-J) (14); E-H, p54-1. Restriction sites are E, EcoRI; B, BstEII; P, PstI.
from human cells or tumors; similarly, alterations at position 12 appear to show a tendency towards aspartic acid residues. Whether mutation at particular codons or to the encoding of particular amino acids represent preferential patterns in the activation of ras genes will only become apparent as more data are accumulated. Detection of these activated genes has relied on the NIH transfection focus assay for their characterization and this assay may have a bias towards detecting particular mutations. Seeberg et al. (45), have shown that although all amino acid substitutions, except proline, at codon 12 of H-ras activated the gene, different substitutions gave varying degrees of transformation. The use of alternative screening systems such as tumorigenesis in nude mice (26, 28) may increase the detectability of some mutations (14, 18).

The mechanism leading to inducing the mutational change is impossible to determine. The A→G change we have observed, however, represents a transitional change which is characteristic of chemical mutagens. Nitrous acid, for example, oxidatively deaminates adenine and can lead to an AT→GC transition. It is possible in these cases that the mutations arose during tissue culture cell replication as a result of errors in DNA replication. The role of the activated ras \(^*\) gene we have identified in the initiation or maintenance of the gastric adenocarcinoma from which it was isolated cannot be directly ascertained. The fact that it was readily detectable and isolatable from an unmanipulated surgical specimen, however, strongly suggests it was present in this population of tumor cells and would tend to support the contention that it played a role in tumor initiation or development.

ACKNOWLEDGMENTS

We thank F. Propst for instruction in sequencing techniques and K. J. Dunn for excellent technical assistance. We wish to acknowledge the cooperation of Dr. James Whitman (Advanced Biotechnologies, Inc.) for his help in obtaining tumor specimens and pathological data.

REFERENCES


Mechanism of Activation of Human ras Genes Cloned from a Gastric Adenocarcinoma and a Pancreatic Carcinoma Cell Line

Bryan M. O'Hara, Marianne Oskarsson, Michael A. Tainsky, et al.


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/46/9/4695

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.