ACTIVATED N-ras IN A HUMAN RECTAL CARCINOMA CELL LINE ASSOCIATED WITH CLONAL HOMOZYGOSITY IN myb LOCUS-RESTRICTION FRAGMENT POLYMORPHISM

Yasuhito YUASA,*1 E. Premkumar REDDY,*2 Johng S. RHIM,*3 Steven R. TRONICK*3 and Stuart A. AARONSON*3

*1Department of Hygiene, Gunma University School of Medicine, 3-39-22 Showa-machi, Maebashi, Gunma 371, Japan, *2Roche Institute of Molecular Biology, Nutley, NJ 07110, USA and *3Laboratory of Cellular and Molecular Biology, National Cancer Institute, NIH, Bethesda, MD 20892, USA

An N-ras transforming gene was detected in human rectal carcinoma-derived cells (7060) and molecularly cloned. The genetic lesion responsible for the transforming activity of the 7060 oncogene was localized to a single nucleotide transition from A to T in codon 61 of the predicted protein. This lesion in the second exon results in substitution of histidine for glutamine at this position. We also found an EcoRI restriction fragment length polymorphism, consisting of two alleles, of the human c-myb gene. The 7060-transformed epithelial cells showed the homozygous phenotype, while normal fibroblasts of the same patient showed the heterozygous phenotype. This suggests a relationship between the phenotypic change in the c-myb locus and the induction of the 7060 tumor.

Key words: Transforming gene — ras gene — Point mutation — Restriction fragment length polymorphism — Rectal carcinoma

Human cellular transforming genes have been detected by DNA-mediated gene transfer techniques with mouse NIH/3T3 cells.1,2) The majority of cellular transforming genes so far detected are related to a small family of evolutionarily conserved genes, designated as ras. The cellular ras family consists of three proto-oncogenes, c-Harvey (H)-ras, c-Kirsten (K)-ras and N-ras.3,4) Activation of ras oncogenes in human tumors is most commonly due to point mutations at codon 12 or 61 in their coding sequences.5-7)

It has been reported that transfection of rat embryo fibroblasts by a human ras oncogene does not convert them into tumor cells unless a second oncogene such as a myc gene is introduced together with the ras gene.5,4) Moreover, the activation of two oncogenes, N-ras and myc in the HL60 human promyelocytic leukemia cell line9) and K-ras and myc in lung carcinoma Lu-65 cells,10) has been demonstrated. The myc and myb genes have been reported to show structural and functional homology.12)

We report here the detection and cloning of an N-ras transforming gene in 7060 human rectal carcinoma-derived cells. The lesion responsible for the activation of the N-ras oncogene was identified. We also detected a restriction fragment length polymorphism (RFLP) of the human c-myb locus. A phenotypic change in the c-myb locus was found on comparison between 7060-transformed epithelial cells and normal fibroblasts in the same patient. The significance of the RFLP of the c-myb locus is discussed.

MATERIALS AND METHODS

Cells and Transfection Assays The 7060 cell culture was initiated from a metastatic liver tumor from a rectal carcinoma of a 39-year-old Caucasian female. The cells were obtained from Dr. Robert J. Huebner (National Cancer Institute, NIH, Maryland). DNA transfection analysis was carried out by the calcium phosphate coprecipitation method.12,13) For transfection of cloned DNA, 40 µg of normal calf thymus DNA was coprecipitated as a carrier. NIH/3T3 cells,4) seeded 24 hr earlier at 1.5×10⁵ per 10-cm dish in Dulbecco’s modified Eagle’s medium/10% calf serum, were exposed for 18–20 hr to the DNA precipitate. Cultures were maintained with twice weekly changes of Dulbecco’s modified Eagle’s medium/5% calf serum. The foci of transformed cells were scored at 14–21 days.
DNA Blot Analysis

High-molecular-weight DNA (20 μg) was digested with appropriate restriction endonucleases under the conditions suggested by the manufacturers. The digested DNAs were electrophoresed in 1% agarose gels which were blotted onto nitrocellulose filters as described by Southern.15) The filters were hybridized in 50% formamide/0.75M sodium chloride/0.075M sodium citrate at 42° for 24 hr. Human N-ras and c-myb clones were isolated from a human DNA library.7,16) From the phage DNA, plasmid subclones lacking human repetitive sequences were prepared and used as probes.

Molecular Cloning

The λgtWES-2B strain of the λ phage was propagated in Escherichia coli strain LE 392.17) Phage DNA and cellular DNA were digested with EcoRI and then purified separately by preparative sucrose gradient centrifugation. The purified phage arms and cellular DNA fragments were ligated at a molar ratio of 1:1 with T4 DNA ligase, and then packaged in vitro into phage particles. Positive plaques were identified through in situ hybridization of phage plaques.17)

DNA Sequence Analysis

Nucleotide sequencing was performed by the procedure of Maxam and Gilbert.18) DNA fragments were obtained by using various restriction endonucleases and were labeled either at their 5' ends with [γ-32P]ATP and polynucleotide kinase or at their 3' ends with cordycepin 5'-[α-32P] triphosphate and terminal deoxynucleotidyl transferase. The end-labeled DNA fragments were digested with appropriate restriction endonucleases, isolated by polyacrylamide gel electrophoresis and then used for sequence analysis.

RESULTS

Identification of the 7060 Oncogene

When high-molecular-weight DNA of the 7060 cells was subjected to transfection analysis with NIH/3T3 cells, we observed a low level of focus formation with a separately prepared DNA preparation (Table I). The 7060 cells used comprised a mixture of two morphologically different cell types, fibroblastic (about 80%) and epithelial cells (about 20%). Both types of cells were cloned using a cloning cylinder. The fibroblastic cells appeared to be normal, since they showed contact inhibition of growth and did not grow in soft agar. However, the epithelial cells overgrew on the cell monolayer and formed many colonies in soft agar. The epithelial cells also induced tumors in nude mice. These results show that the epithelial cells were cancerous cells originating from the carcinoma. DNAs from the fibroblastic cells and three clones of epithelial cells were assayed for transforming activity (Table I). Only the epithelial cell DNA showed a positive result, the transforming activity of the epithelial cell DNA being 3- to 4-fold higher than that of the 7060 mixed cell DNA. The transforming activity of all first-cycle NIH/3T3 transformants was retained through a subsequent cycle of transfection. Since isozyme analysis of the 7060 fibroblastic and epithelial cells showed that they had the same phenotypes (W. D. Peterson, unpublished data), these cells originated from the same person, with a probability of 99.91%. These results suggest that activation of the 7060 transforming gene was a somatic event.

EcoRI-restricted DNAs of individual transfectants were hybridized with a 300 base pair (bp) probe specific for human repetitive sequences of the Alu family.19) Each first-cycle transfectant tested showed numerous bands containing human repetitive sequences. Even third-cycle transfectants digested with EcoRI invariably retained more than one Alu family-related band (data not shown), implying that human sequences were linked to the transforming sequence.

Table I. Transforming Activity of DNAs Isolated from 7060 Mixed, Fibroblastic and Epithelial Cells

<table>
<thead>
<tr>
<th>Donor DNA</th>
<th>Transfection assay (no. of foci/no. of recipient cultures)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mixed cells</td>
<td>Preparation 1: 3/13 Preparation 2: 3/16</td>
</tr>
<tr>
<td>Fibroblastic cells</td>
<td>Preparation 1: 0/8 Preparation 2: 0/8</td>
</tr>
<tr>
<td>Epithelial cells</td>
<td>Preparation 1: 3/8 Preparation 2: 8/8</td>
</tr>
<tr>
<td>Clone A</td>
<td>Preparation 1: 8/8 Preparation 2: 6/8</td>
</tr>
<tr>
<td>Clone B</td>
<td>Preparation 1: 10/8 Preparation 2: 3/8</td>
</tr>
</tbody>
</table>

Two separately prepared DNAs from 7060 mixed, fibroblastic and epithelial cell clones were used to transfect NIH/3T3 cells. NIH/3T3 cells, plated 24 hr earlier, were transfected with 30 μg of high-molecular-weight DNA. The foci of transformed cells were scored at 14–21 days.
To determine if there was a relationship between the 7060 transforming gene and any known oncogene, we subjected EcoRI (or BamHI)-restricted DNA of first-cycle transfectants to molecular hybridization with various oncogenes of viral and human origin as probes. Only with N-ras specific probes, EcoRI-digested 7060 transfectant DNAs exhibited additional hybridizing bands not observed in mouse DNA (Fig. 1). Unique sequence probes, probe A (PauII, 900 bp) and probe B (XbaI, 800 bp) are specific for the N-ras 9 kilobase-pair (kbp) and 7 kbp EcoRI fragments, respectively (Fig. 1A and B, lane 3). With probe A, a 7.5 kbp mouse cell specific band (Fig. 1A, lane 1) was detected. An additional DNA fragment was detected for all transfectants tested with probe A (Fig. 1A, lane 2). With probe B, only a single extra band was detected in all transfectants (Fig. 1B, lane 2). These results show that the transforming gene in the 7060 cells is an activated N-ras gene. The 7060 mixed cell DNA (Fig. 1, lane 4) as well as DNAs of the 7060 fibroblastic and epithelial cell clones (data not shown) showed the same bands as the normal human placenta DNA, as regards size and density.

Molecular Cloning of the 7060 Oncogene

To characterize the N-ras oncogene associated with 7060 cells, we cloned its sequence from a first-cycle NIH/3T3 transfectant, designated as 90-5. EcoRI-digested DNA of this transfectant showed 9 and 7.5 kbp bands with probe A, and a 7 kbp band with probe B (Fig. 1A and B, lane 2). The 90-5 DNA was digested completely with EcoRI, and the fragments were separated by sucrose density gradient centrifugation. Fractions that hybridized with probe A or B were ligated separately to phage λgtWES arms, packaged in vitro, and amplified through infection of Escherichia coli strain LE
From 200,000 plaques screened, 4 and 2 positive clones were obtained using probes A and B, respectively. Following plaque purification, the cloned DNAs were digested with EcoRI and then subjected to agarose gel electrophoresis and Southern blotting analysis with probes A and B. Clones containing N-ras-related EcoRI inserts of either 9 or 7 kbp were obtained. Clones designated as λ7060-75 and λ7060-79 specifically hybridized with probe A, and clones designated as λ7060-51 and λ7060-56 hybridized only with probe B. N-ras specific EcoRI fragments were subcloned into plasmid pBR322 for further analysis. The subclones were designated as p7060-75, p7060-79, p7060-51 and p7060-56, respectively. Restriction enzyme analysis was carried out to compare the normal and 7060 N-ras genes, using HindIII, PvuII, SstI and XbaI (data not shown). Both DNAs showed the same restriction patterns (Fig. 2), indicating that the genetic lesion responsible for the acquisition of transforming activity was very likely rather subtle.

**Biologic Activity of the Cloned 7060 Gene**

The 7060 oncogene was cloned as two independent EcoRI fragments, neither of which showed transforming activity in transfection assays (data not shown). To assess the biologic activity of the gene, 9 kbp and 7 kbp EcoRI fragments purified from clones p7060-75 and p7060-56, respectively, were ligated, and the resulting concatamers were tested for ability to transform NIH/3T3 cells (Fig. 3). The reconstituted 7060

![Fig. 3. Transforming activities of ligated N-ras fragments. Two purified EcoRI fragments from the normal N-ras gene (■) and/or the 7060 oncogene (▲) were ligated and then their transforming activity was determined by means of the NIH/3T3 transfection assay. ffu, focus-forming units.](image)

![Fig. 4. Comparison of the DNA sequences and predicted amino acid sequences of the entire first and second exons of the 7060 oncogene and the N-ras (human) gene. A single base change (A to T) and the corresponding amino acid change of glutamine to histidine are boxed. The nucleotide sequence analysis was performed according to the procedure of Maxam and Gilbert.](image)
gene induced the morphological transformation of cells.

To determine the region(s) of the 7060 oncogene required for the transforming activity, we ligated the p7060-75 EcoRI fragment to the 7kbp EcoRI fragment of the normal N-ras gene and also ligated the 9 kbp EcoRI fragment of the normal N-ras gene to the fragment of p7060-56. The transforming activity of these molecules was compared with that of homologous constructs. As shown in Fig. 3, the recombinant containing the 7060 9 kbp and the normal N-ras 7 kbp EcoRI fragments was active, whereas neither the reciprocal recombinant nor the normal N-ras gene itself showed detectable transforming activity (Fig. 3). Thus, the site of malignant activation of the 7060 oncogene could be localized to the EcoRI 9 kbp fragment corresponding to the 5' half of the gene.

Identification of the Activated Lesion
Restriction enzyme analysis demonstrated that the 9 kbp EcoRI fragment of the 7060 oncogene, where the transforming activity was localized, contained the first and second exons of the N-ras p21 coding sequence. To identify the lesion responsible for the activation of the 7060 oncogene, we performed nucleotide sequence analysis of its first and second exons, and compared the sequences with those of the corresponding exons of the human N-ras proto-oncogene. The first exon was located within 300 bp HindIII fragment, whereas the second exon resided within a 450 bp PstI-BstEII fragment. No differences were observed in the corresponding sequences of the first exon (Fig. 4). However, analysis of the second exon revealed the presence of a single base change of A to T within the 61st codon of the predicted p21 coding sequence (Fig. 4). This results in a change from glutamine to histidine at this position in the protein. We conclude, therefore, that this single base change and the resultant amino acid change confer transforming activity on the 7060 oncogene.

RFLP of the c-myc Locus
To examine the possible involvement of other oncogenes in 7060 carcinoma induction, the 7060 cell DNA was hybridized with probes specific for the H-ras, K-ras, myc, myb, mos and sis genes. No oncogene probes showed amplification of proto-oncogenes in the 7060 mixed, fibroblastic and epithelial cells (data not shown). None of the probes other than the myb gene showed any rearrangement of proto-oncogenes in any of the 7060 cell types. When the 7060 mixed and fibroblastic cell DNAs were digested with EcoRI and hybridized with a 2.6 kbp EcoRI fragment of the human c-myc gene, they yielded three bands of 2.6 kbp, 1.6 kbp and 1 kbp (Fig. 5A, lanes 1, 2). However, all of the 4 epithelial cell clone DNAs showed only 1.6 and 1 kbp bands (Fig. 5A, lanes 3 and 4).

The sum of the 1.6 and 1 kbp fragments is 2.6 kbp. After digestion with HindIII, all of the 7060 cell types gave the same 7.1 kbp fragment (Fig. 5B, lanes 1 to 4). These data
suggest that one allele in the 7060 fibroblastic cells contains an extra EcoRI site in the 2.6 kbp fragment and hence this allele yields 1.6 and 1 kbp fragments. The 7060 epithelial cells appear to have lost one allele which does not contain the extra EcoRI site. Alternatively, the 7060 epithelial cells might obtain the extra EcoRI site in both alleles. In either case, the alteration of the EcoRI restriction site might be associated with induction of the 7060 carcinoma.

We examined the presence of the EcoRI restriction site in other human normal cells (2 cases) and tumor cells (25 cases) to determine whether or not the alteration of the EcoRI site is specific for the 7060 cells. Southern blot analysis showed three phenotypes (Fig. 5A, lanes 5 to 7), indicating that these EcoRI fragments represent an EcoRI RFLP. The RFLP can be explained by the existence of two alleles, a and b. Allele a is characterized by two fragments of 1.6 and 1 kbp, whereas allele b gives a 2.6 kbp fragment. Eight tumor and 1 normal cell DNAs were homozygotes aa (Fig. 5A, lane 5), 13 tumor cell DNAs were heterozygotes ab (Fig. 5A, lane 6), and 4 tumor and 1 normal cell DNAs were homozygotes bb (Fig. 5A, lane 7). On pooling the data for all the tumor and normal cells, the frequency of allele a was found to be 0.57, whereas the frequency of allele b was 0.43 (54 alleles analyzed).

**Discussion**

Transforming genes of the ras family have been detected on transfection analysis in a wide variety of human malignancies. Most of the transforming genes detected in colorectal carcinomas have been identified as activated c-K-ras 2 oncogenes. An activated N-ras gene has been identified in an adenocarcinoma of the colon. Our detection of an N-ras oncogene in 7060 human rectal carcinoma-derived cells is another example of an activated N-ras gene in colorectal malignancies.

The original 7060 cells comprised two types of cells, fibroblastic cells with a normal phenotype and transformed epithelial cells. Since isozyme analysis indicated that the two cell types had the same origin, it is most likely that the epithelial and fibroblastic cells originated from carcinoma cells and surrounding stromal cells, respectively. Transfection experiments showed that the epithelial cells contained the transforming N-ras gene but the fibroblastic cells did not. These results suggest that the 7060 N-ras oncogene is most probably activated by a somatic event. This conclusion is consistent with that of a previous study in which point mutations of ras oncogenes were found only in tumor cells, and not in normal cells in the same patients.

The activating lesion of the 7060 N-ras oncogene was identified as a single nucleotide substitution of thymine for adenine in the 61st codon. This results in substitution of histidine for glutamine at this position of the p21 coding sequence. This finding, together with those in previous studies, indicates that the activation of ras oncogenes in naturally occurring human malignancies is most commonly due to point mutations at one of two major "hot spots", codons 12 and 61, in the ras coding sequence.

We found RFLP of the human c-myb gene, which was detected with EcoRI. There seem to be two alleles, a and b. Allele a is characterized by 1.6 and 1 kbp bands and allele b by a 2.6 kbp band. This alteration in restriction fragment length is very likely due to the presence or absence of an extra EcoRI site in the c-myb 2.6 kbp fragment. Since the HindIII-digested pattern did not differ between DNAs, the alteration responsible for the presence or absence of the extra EcoRI site must be subtle, such as a small deletion or a base substitution. RFLPs are very useful as genetic markers in linkage studies on disease loci. For example, polymorphic DNA markers have been reported to be genetically linked to genetic diseases including Huntington's disease and cystic fibrosis. The human c-myb locus has been mapped to the q22-24 region of chromosome 6. The human c-myb locus can be used as a genetic marker of the region.

RFLPs of human proto-oncogenes have been detected by Southern blot hybridization with probes for H-ras, c-fms, c-mos and L-myc. These RFLPs raise the possibility of an association between human tumors and the alleles. A polymorphism of the human H-ras locus has been found more frequently in leukocyte and tumor DNAs of...
cancer patients than in leukocytes from a
nonaffected population.\(^{28}\) In the case of the
c-myb locus, more examples of normal and
tumor cells will have to be investigated to
determine the association between tumors
and the allele.

When we digested the 7060 fibroblastic
cell DNA with EcoRI and hybridized with
the human c-myb EcoRI 2.6 kbp fragment
as a probe, the DNA yielded 3 bands of 2.6,
1.6 and 1 kbp. This suggests that the patient
was originally a heterozygote. However, all
of the 7060-transformed epithelial cell clone
DNAs showed a homozygous pattern, i.e.
1.6 and 1 kbp bands. Since all 4 clones of the
7060 epithelial cells lacked allele \(b\), the
phenotypic change must be a very early
event. These data imply that the phenotypic
change in the c-myb locus may be associated
with the induction of the 7060 carcinoma.
The phenotypic change itself can be ex-
plained by the loss of allele \(b\) or the appear-
ance of an extra EcoRI site in allele \(b\). The
7060 epithelial cells may have lost the region
containing the q22-24 region of chromosome
6 or the entire chromosome 6. There may be
a gene(s) that suppresses tumorigenesis in
this region of chromosome 6, or the loss of
the region might simply be a nonspecific
chromosomal rearrangement, since many
chromosomal alterations occur in cancer
cells. Alternatively, the phenotypic change
might be due to the appearance of an extra
EcoRI site in allele \(b\), although this is less
likely. In this case, the genetic change may
have caused altered qualitative or quantita-
tive expression of the c-myb gene. It remains
to be determined whether or not the pheno-
typic change in the c-myb locus may have
contributed to the induction of the 7060
carcinoma.

While this paper was in preparation,
Yokota et al.\(^{29}\) reported that an allelic dele-
tion of the c-myb locus was observed in carci-
nomas and a sarcoma.

Acknowledgments

We wish to thank Ms. A. Chang, Mr. K. Sitz,
Mr. G. Kim and Ms. C. Y. Dunn for their
technical assistance and Ms. S. Fujii for prepara-
tion of the manuscript. We also thank Mr. M.
Oto for a critical review of the manuscript, Dr.
R. J. Huebner for the 7060 cells and Dr. W. D.
Peterson for the karyotype and isozyme analyses.

This work was supported in part by a Grant-in-
Aid for Cancer Research from the Ministry of
Education, Science and Culture of Japan.

(Received April 10, 1986/Accepted May 19, 1986)

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N-ras ONCOGENE AND RFLP OF c-myb
