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ACTIVATED c-*raf* GENE IN A RAT HEPATOCELLULAR CARCINOMA INDUCED BY 2-AMINO-3-METHYLIMIDAZO[4,5-*f*]QUINOLINE

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SUMMARY: A rat hepatocellular carcinoma, IQ7, induced by 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ) gave two transformants of NIH 3T3 cells on DNA mediated gene transfer. One of these transformants was examined further and secondary and tertiary transformants were obtained. The secondary transformant was tumorigenic in nude mice. The activated oncogene in this primary transformant was identified as rat c-*raf* by Southern blot analysis.

Genomic DNAs from human and experimental neoplasms have been tested for their transforming activity by NIH 3T3 cell transfection assay and in these extensive studies the activated oncogenes have mainly been identified as member of the *ras* gene family with single point mutation (1). But several activated oncogenes other than those of the *ras* gene family have been detected by this transfection assay (2,3,4). Recent studies revealed that c-*raf* genes in a Japanese specimen of gastric cancer and in a glioblastoma line had transforming activity on NIH 3T3 cells (5,6). IQ, a mutagen isolated from broiled sardines, induced hepatocellular carcinomas in rats (7). Previously, we reported that one of these hepatocellular carcinomas, IQ4, contained two transforming genes. One of these was identified as c-H-*ras*. The other was not a member of the *ras* gene family or *neu* (8). DNA from another hepatocellular carcinoma, IQ7, induced two transformants, IQ7-1 and IQ7-2, which also contained transforming genes other than those of *ras* family or *neu*. Here we report that activated c-*raf* is the transforming gene in IQ7-2.

Abbreviation used: IQ, 2-amino-3-methylimidazo[4,5-*f*]quinoline

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MATERIALS AND METHODS

Tumors A hepatocellular carcinoma, IQ7, was used. It was induced in a male Fischer 344 rat by diet containing 300 ppm of IQ (7).

Cells NIH 3T3 cells and transformed cells were maintained in Dulbecco's modified Eagle's medium with 10% and 5% calf serum (GIBCO), respectively.

DNA transfection High molecular weight DNAs were prepared from solid tumors and cultured cells as described (9). They were transfected into NIH 3T3 cells by the calcium phosphate precipitation method (10). Transformed foci were obtained after 14 to 21 days.

Southern blot analysis Restriction endonucleases were purchased from Takara Shuzo Co. or from Böhringer Mannheim and used according to the manufacturers' instructions. DNAs were digested with restriction endonucleases, subjected to agarose gel electrophoresis and transferred to nitrocellulose membrane filters by the method of Southern (11). The filters were hybridized in solution containing 40% formamide, 0.65 M NaCl, 0.1 M sodium Pipes (pH 6.8), 10% dextran sulfate, 5 × Denhart's solution, 0.1% SDS, 5 mM EDTA and 100 μg/ml salmon sperm DNA at 40°C with nick-translated ID4 (a rat repetitive sequence isolated by T. Sekiya, this Institute) for 18 hrs, and washed four times with 2 × SSC (1 × SSC is 0.15 M NaCl/0.015 M sodium citrate), 0.2% sodium pyrophosphate and 0.1% SDS for 20 min each at 60°C. With other probes, the hybridization conditions were the same, except that the formamide concentration was increased to 50% and the temperature was 42°C. Washing was performed at 50°C.

Probes ID4, BS9 (v-H-*ras*) (12), HiHi380 (v-K-*ras*) (13), the *Sal*I-*Nco*I fragment of p6a1 (cDNA of human N-*ras*) (14), the *Eco*RI-*Bam*HI fragment of pRIBam (v-*erb*-*B*) (15) and the *Xho*I-*Bst*EII fragment of cloned v-*raf* (16) were used. Plasmids were digested with appropriate restriction endonucleases, and subjected to electrophoresis in agarose slab gels. The inserts were eluted from the gels by electrophoresis and labeled with ^{32}P by nick translation. The specific radioactivities were about 1-2 × 10^9 cpm/μg.

Tumorigenicity About 1 × 10^6 cultured cells of a second transformant and control NIH 3T3 cells were tested by subcutaneous injection of the cells into two nude mice (BALB/C, nu/nu).

RESULTS

Sixty μg of high molecular weight DNA from a tumor, IQ7, was transfected into NIH 3T3 cells and two primary transformants, IQ7-1 and IQ7-2, were isolated.

The presence of rat genomic DNA in these transformants was analyzed by Southern blot hybridization using ID4 as a probe. ID4 is a cloned rat repetitive sequence with the size of 294bp, which was obtained by *Taq*I digestion of rat genomic DNA (T. Sekiya, unpublished data). Fig. 1 shows that ID4 strongly hybridized with DNA of rat liver but not of mouse fibroblast. The two primary transformants showed several clear bands, suggesting that both transformants were induced by rat DNA. We further tested the transforming activity of DNA from IQ7-2 and found that it produced secondary and tertiary transformants; 120 μg of DNA of the IQ7-2 clone induced 45 secondary transformants and 60 μg

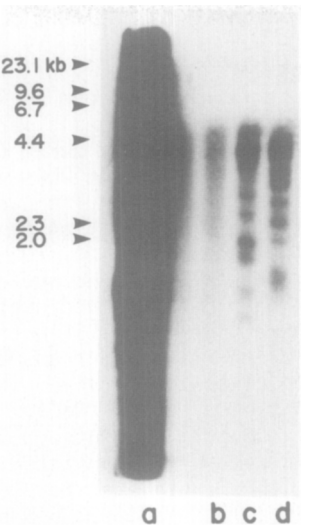

Fig. 1.: Detection of the rat specific repetitive sequence ID4 in the *Eco*RI digest of DNA of the primary transformant of IQ7. (a) normal F344 rat liver; (b) NIH 3T3 cells; (c) IQ7-1; (d) IQ7-2. The positions of fragments of λcI857 DNA digested with *Hind*III are shown by arrows.

of DNA of one secondary transformant induced 97 tertiary transformants. Among seven secondary transformants examined, only two clones had rat specific ID4 sequences (data not shown). The copy number of the ID4 sequence in rat genomic DNA is not high, being about 12,000 copies/haploid (personal communication by T. Sekiya) and this sequence was suggested not to be closely linked to the activated oncogene.

The DNAs of the primary transformants (IQ7-1 and IQ7-2), and the secondary and tertiary transformants of IQ7-2 were digested with appropriate restriction endonucleases and examined by Southern blot analysis with v-H-*ras*, v-K-*ras* and cDNA of human N-*ras* as probes. No extra-band of *ras* genes other than mouse specific ones was detected in these transformants (data not shown). We also tested for the presence of *neu* (4), which was recently reported as a new transforming gene, under the hybridization conditions described by Schechter et al. (4), except that we used a 1.7 kb *Eco*RI-*Bam*HI v-*erb*-*B* insert of pRIBam as a probe. Again no involvement of an *erb*-*B* related *neu* gene was found (data not shown).

Next we examined the involvement of c-*raf* in transformation. As shown in Fig. 2, *Taq*I digests of normal F344 rat liver DNA gave 7.3 and 2.6 kb frag-

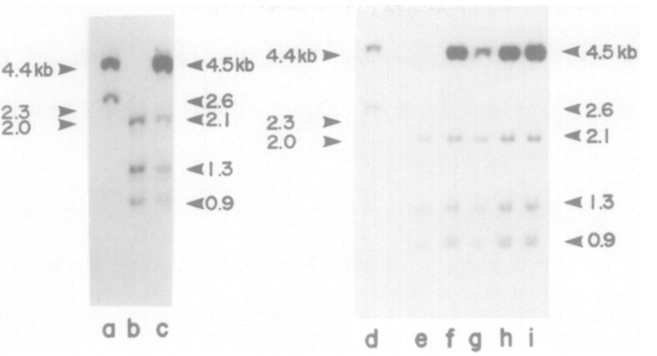

Fig. 2.: Rat specific c-*raf* sequences in the *Taq*I digests of DNAs of IQ7-2 and its secondary transformants. (a,d) normal F344 rat liver; (b,e) NIH 3T3 cells; (c) IQ7-2; (f-i) secondary transformants of IQ7-2. The calculated sizes of fragments are indicated on the right side.

ments and mouse fibroblast DNA gave 2.1, 1.3 and 0.9 kb fragments. DNAs from IQ7-2 and its secondary transformants contained a 7.3 kb but not a 2.6 kb fragment besides mouse specific fragments. To confirm that these fragments of IQ7-2 were derived from rat c-*raf*, we examined *Eco*RI, *Bam*HI and *Sst*II digests of IQ7-2 DNA. As shown in Fig.3, the 7.5 kb fragment of IQ7-2 obtained with *Eco*RI, the 9.5 kb fragment obtained with *Bam*HI and the 14 kb fragment obtained with *Sst*II were equal in size to those of normal F344 rat liver DNA. However, a 5.3 kb fragment of rat liver DNA obtained with *Eco*RI and an 8.5 kb fragment in an *Sst*II digest were not obtained from this transformant. Therefore, we thought that the 2.6 kb fragment in the *Taq*I digest, the 5.3 kb fragment in

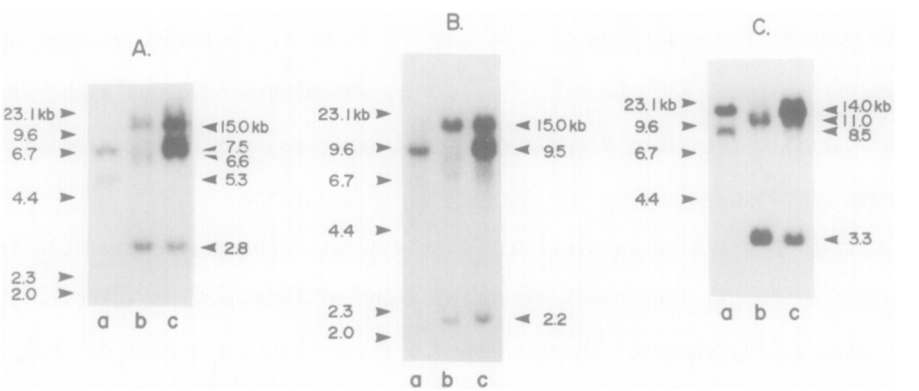

Fig. 3.: Rat specific c-*raf* sequences in the *Eco*RI, *Bam*HI and *Sst*II digests of DNA of IQ7-2. (a) normal F344 rat liver; (b) NIH 3T3; (c) IQ7-2. A. *Eco*RI, B. *Bam*HI, and C. *Sst*II digests.

the *Eco*RI digest and the 8.5 kb fragment in the *Sst*II digest of normal rat liver DNA were derived from a pseudogene and concluded that rat c-*raf* is responsible for the transformation of IQ7-2. In IQ7-1, no involvement of the *raf* gene was detected (data not shown).

The morphological characteristics of IQ7-2 and its secondary transformants did not differ from those of transformants induced by a1-1 (17), which has an activated human c-H-*ras* of T24 bladder carcinoma. The two were similarly refractile and lost contact inhibition under phase contrast microscopy. However, the appearance of transformed foci after DNA transfection was always earlier with DNAs from IQ7-2 and its secondary transformants (about 10 days) than with DNAs from a1-1 (14 days). When a secondary transformant was injected subcutaneously into two nude mice both developed tumors in 21 days, while normal NIH 3T3 cells of the similar treatment did not.

DISCUSSION

We have demonstrated that a transformant obtained by transfecting DNA of a rat hepatocellular carcinoma, IQ7, had the rat c-*raf* sequence and c-*raf* of this hepatocellular carcinoma was seemed to be activated.

Under our experimental conditions *Taq*I, *Eco*RI and *Sst*II digests of normal rat liver DNA gave two bands homologous to v-*raf*. Only one band was found in each digest of the transformant DNA. Bonner et al. mentioned that there was no pseudogene of c-*raf* in mouse, whereas there was in human (18). Our results suggest the existence of a pseudogene in rats. However, another possibility is that not all the rat c-*raf* exons, which have homology with v-*raf*, are necessary to transform NIH 3T3 cells and hence may not be transfected into the transformants.

Little information is available on the genetic changes responsible for activation of c-*raf*. The exact extent of exons of this gene is unknown. Mölder et al. (19) reported that enhanced transcription of a part of this large gene by LTR insertion is sufficient to transform fibroblast cells. Bonner et al. (18) also suggested that the transforming activity is not due to

mutation, but to an elevated level of expression. We are now cloning the activated c-*raf* gene found in this study and comparing it with the normal counterpart.

So far we have examined 5 hepatocellular carcinomas induced by IQ in rats, and found that two carcinomas, IQ4 and IQ7, showed transforming activity in NIH 3T3 cells. We have identified the transforming gene in the transformant induced by IQ4 as c-H-*ras* (8). Both IQ4 and IQ7 were suggested to contain other transforming genes. Long-term feeding of IQ seems to activate various oncogenes, unlike a single dose of *N*-nitroso-*N*-methylurea, which specifically activated Ha-*ras* by direct base substitution (20).

The activation of c-*raf* does not seem uncommon in experimental animal and human cancers. Because IQ and its related compounds have been found to be environmental carcinogens, and are thought to be important in human carcinogenesis (21), this experimental system may provide an insight into the mechanisms of induction of human cancers.

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