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Molecular cloning of an activated human oncogene, homologous to v-*raf*, from primary stomach cancer

(transfection/human transforming gene/gastric tumor)

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ABSTRACT **Transfection with high molecular weight DNA from a primary stomach cancer induced foci of transformed NIH 3T3 cells, and the transformed cells were tumorigenic in nude mice. By screening with a human *Alu*-family probe, we isolated the human DNA sequence from the secondary transformant cells. This transforming sequence encompasses about 60 kilobase pairs and is unrelated to known human transforming genes. Examination of homologies between this sequence and retroviral oncogenes revealed that the human transforming sequence is closely related to the v-*raf* oncogene of murine transforming retrovirus 3611-MSV.**

Morphological transformation of NIH 3T3 cells by transfection with genomic DNA has been used to detect dominantly acting transforming genes in human tumors and tumor-derived cell lines (1–5). Most naturally occurring transforming genes thus far detected have been identified as activated members of the *ras* gene family, either Ha-, Ki-, or N-*ras* (6–11). These activated *ras* genes have single point mutations in the *ras* coding regions, at either the 12th or the 61st codons (12–17).

Two other transforming genes, B-*lym*, which has been isolated from human B-cell lymphoma (18), and *oncD*, which has been identified in human colon tumor 2033 (19), do not appear to be related to known viral oncogenes, and the mechanisms of their activation are unclear. Another gene, *met*, recently isolated from a human osteosarcoma-derived cell line, appears to be activated by chemical mutagenesis (20).

Little is known of the nature of the oncogenes activated in the case of stomach cancer. Stomach cancer is the most common cancer in Japan and some European and South American countries.

We now report detection and molecular cloning of a transforming gene from a surgically removed stomach cancer of a Japanese patient. DNA hybridization experiments showed that this gene is closely related to v-*raf*, the oncogene of 3611-murine sarcoma virus (MSV) (21).

MATERIALS AND METHODS

Cells. NIH 3T3 cells (22) obtained from M. Wigler (Cold Spring Harbor Laboratory) were maintained at low cell densities in Dulbecco's modified Eagle medium (GIBCO) containing antibiotics (streptomycin at 100 μg/ml and penicillin at 100 units/ml) and 10% calf serum (Flow Laboratories).

Preparation of DNA. DNA was prepared from cultured cells by $NaDodSO_4$/proteinase K lysis and phenol/chloroform extraction, as described (4). Preparation of DNA from solid tumors was as described (23). Plasmid and bacteriophage DNAs were prepared as described (24, 25). The following plasmids were generously provided [directly or through T. Sekiya (National Cancer Center Research Institute) and M. Wigler] by the indicated scientists: pErbA, pErbB, pY6271(*yes*), pv-*myc*, and pABsub3(*abl*) (T. Yamamoto); pHT10(*mos*) (G. F. Vande Woude); pBR-FSV(*fps*) (H. Hanafusa); pSRA2(*src*) (J. M. Bishop); pMyb (T. Kawakami); pHB11(Ha-*ras*) and pKBE2(Ki-*ras*) (E. M. Scolnick); pSSV-11(*sis*) (S. Aaronson); pc(hu)-*fos* (I. Verma); pv-*fgr* 1700 (K. C. Robbins); p*ros* (L. H. Wang); pRev-T3(*rel*) (H. M. Temin); pv*ski-1* (E. Stavnezer); pSM-FeSV(*fms*) (C. J. Sherr); pBB3(*ets*) (P. H. Duesberg); p149(*raf*) (U. R. Rapp); pMT2.5(*int-1*) (H. E. Varmus); pHuB-*lym-1* (G. M. Cooper); BLUR8 (C. Schmid); and pHC79 (B. Hohn).

DNA Transfection. For all DNA transfers, we used a modified calcium phosphate precipitation method (26) with NIH 3T3 cells as the recipients. Focus assays were performed as described (4).

Enzymes. Restriction endonucleases were purchased from Nippon Gene (Toyama, Japan) or from New England Biolabs and used according to the accompanying instructions. *Escherichia coli* DNA polymerase I and T4 DNA ligase were from New England Biolabs and Takara Shuzo (Kyoto, Japan), respectively.

Southern Blot Hybridization. DNA samples were digested with restriction endonucleases and subjected to agarose gel electrophoresis and filter-blot transfer by the method of Southern (27). Filter-blotted DNAs were hybridized with a nick-translated ^{32}P-labeled DNA probe, under two sets of conditions. Less stringent hybridization conditions entailed hybridization in a mixture containing 40% (vol/vol) formamide, 0.9 M NaCl, 0.09 M sodium citrate (pH 7.0), 1× Denhardt's solution (0.02% polyvinylpyrrolidone/0.02% Ficoll/0.2% bovine serum albumin), and denatured salmon sperm DNA (20 μg/ml) at 42°C. More stringent hybridization conditions entailed hybridization in a mixture containing 50% formamide, 0.3 M NaCl, 0.03 M sodium citrate (pH 7.0), 1× Denhardt's solution, 10 mM EDTA, and denatured salmon sperm DNA (20 μg/ml) at 43°C. Hybridized DNA was visualized by autoradiography on Kodak XAR-5 film at −70°C, using an intensifying screen.

Molecular Cloning. Genomic libraries of the secondary transformants were constructed in a double amber derivative of λL47.1 (28) from DNA partially digested with either *Eco*RI or *Bam*HI and fractionated in a sucrose gradient, as described (4). We screened these libraries for the presence of a human sequence, using the method of Benton and Davis (29); the probe was initially BLUR8, a clone of the ubiquitous

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Abbreviation: kbp, kilobase pair(s).

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repeated human *Alu* family sequences (30). At later stages, we used portions of isolated clones as specific probes for isolating contiguous clones.

Cosmid libraries of the same DNAs were constructed in pHC79 (31) from DNA partially digested with *Eco*RI and size-fractionated as above. Screening of the cosmid library was performed as described (32), with purified fragments of the gene from phage recombinants as probes.

RESULTS

Detection of the Transforming Sequence. High molecular weight DNAs were prepared from surgically removed human stomach cancers of three Japanese patients. These DNAs were used to transfect mouse NIH 3T3 cells by the calcium phosphate coprecipitation technique (4). One DNA preparation from a Borrmann II primary cancer of a 69-year-old male patient gave rise to transformed foci, albeit with a low efficiency (Table 1). DNAs of the metastasized lymph node tumor and of normal mucosa from the same patient and DNAs from other patients did not induce foci upon transfection under the same experimental conditions. Reproducible occurrence of foci induction in two independent experiments suggests that the gene was already activated in the primary stomach cancer.

DNAs of the primary foci thus obtained were analyzed by Southern blot hybridization, with BLUR8 as a probe for the presence of human-specific repeated sequences. Seven out of seven primary transformants retained abundant amounts of human DNA sequences (Fig. 1, lane B). We observed three slightly different classes in the Southern blot hybridization profile with the DNAs from these seven primary transformants and thereby inferred that they arose from at least three independent transforming events (data not shown).

DNAs of the primary transformants were then used in the next round of transfection to obtain secondary transformants. Five secondary transformants were obtained independently from two different primary transformants. The transformation efficiency was still very low (1 focus per 48 μg of DNA), whereas the positive control (an active N-*ras* clone) gave rise to several thousands of foci per μg of DNA, in the same experiment. This finding, which is in contrast to the observation that the efficiency of second-cycle transfection is higher than that of initial transfection in many cases, suggests that this transforming sequence might be very large.

To characterize further the transforming sequence, we analyzed the DNAs of the secondary transformants for the presence of human DNA sequences. After two serial transfection cycles, only a small amount of human DNA remains in the secondary transformants; any human DNA fragments conserved in all secondary transformants would therefore be closely linked to or contained in the transforming sequence. Southern blot hybridization profiles of such conserved restriction fragments with human repeated sequences

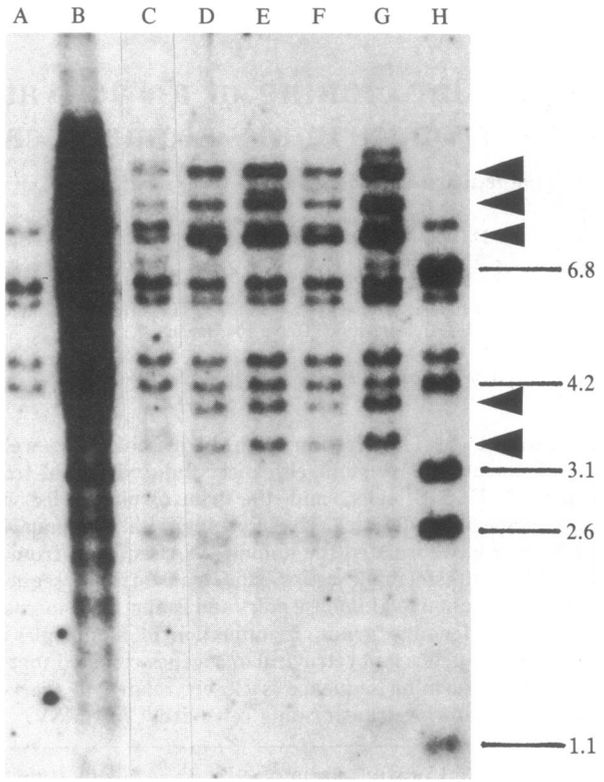

FIG. 1. Presence of human sequences in NIH 3T3 transformants. Ten micrograms of each cellular DNA was digested with *Eco*RI, subjected to electrophoresis in 1% agarose gel, and analyzed by blot hybridization with BLUR8 as probe under less stringent conditions (see *Materials and Methods*). Lanes: A, NIH 3T3 cell DNA; B, DNA from one of the seven primary transformants; C–G, DNAs of five different secondary transformants; H, a mixture of NIH 3T3 DNA (10 μg) and two recombinant λ phage DNAs (total 200 pg) containing the human c-Ki-*ras-2* gene (16), used as internal size markers [indicated in kilobase pairs (kbp) at right]. Arrowheads at right indicate human-specific *Eco*RI fragments commonly retained in these secondary transformants.

Table 1. Transformation of NIH 3T3 cells with DNAs from stomach cancer

DNA preparation	Source of DNA	Primary foci No./no. of dishes* Exp. 1	Primary foci No./no. of dishes* Exp. 2	*Alu*†	Secondary foci No./no. of dishes*	Secondary foci *Alu*†
I-1	Tumor of patient A	5/6	2/6	7/7	5/24	5/5
I-2	Lymph node‡ of patient A	0/6	0/6	—	—	—
I-3	Normal mucosa of patient A	0/6	0/6	—	—	—
II-1	Tumor of patient B	0/6	0/6	—	—	—
III-1	Tumor of patient C	0/6	0/6	—	—	—
III-2	Lymph node‡ of patient C	1/6	0/6	0/1	0/24	—
III-3	Normal mucosa of patient C	0/6	0/6	—	—	—
IV	λGNS35 (positive control)§	2000/6	1500/6	—	—	—
V	NIH 3T3 (negative control)	0/12	0/12	—	—	—

*Total number of dishes after split; to one initial transfection dish was added 30 μg of DNA and the preparation was split into three dishes 36 hr after transfection (4).

†Presence of human *Alu* sequences in the DNA of each transformant was determined by Southern blot hybridization with BLUR8 as a probe.

‡Metastasized tumor.

§Positive control experiments with cloned DNA of the active N-*ras* gene (17) gave rise to about 2–5 $\times$ 10^3 foci per μg of DNA.

have been used to distinguish different transforming genes (3–5). Thus, DNAs of the secondary transformants were digested with *Eco*RI and probed on Southern blots with nick-translated BLUR8 DNA, under the less stringent hybridization conditions (Fig. 1). At least five *Eco*RI fragments with human *Alu* sequences were conserved in all secondary transformants. This result clearly demonstrates that the transforming sequence is of human origin.

Tumorigenicity. Primary and secondary transformants exhibit typical properties characteristic of transformed fibroblasts; they are very refractive under a phase-contrast microscope and grow well in medium with 5% serum over the monolayer of untransformed NIH 3T3 cells. They are similar to NIH 3T3 cells transformed with active *ras* genes. Two independently arisen secondary transformants (SCT102 and SCT104) induced by DNAs from different primary transformants were further purified in a semisolid medium and then tested for the ability to form tumors in nude mice.

Approximately 2×10^6 cells of either normal NIH 3T3 cells or purified secondary transformants were injected subcutaneously into athymic nude mice (BALB/c origin, 10–13 weeks old). In mice injected with transformed cells, visible tumors developed within 1 week and grew very rapidly thereafter (≈20 mm mean diameter by 2 weeks), whereas normal NIH 3T3 cells did not induce tumors, even during 2 months observation.

Human Transforming Sequences. DNAs were extracted from these tumors and subjected to Southern blot hybridization with the BLUR8 probe under the more stringent conditions (Fig. 2). The DNAs from tumors in nude mice retained the same human DNA sequences as in the original secondary transformant cells. This implies that the human transforming sequence is responsible not only for cellular transformation but also for tumorigenicity in nude mice. The human DNA sequences in these tumors appear to be significantly amplified. In this experiment, because of a lowered background of cross-hybridization to mouse sequences, we detected six conserved *Eco*RI fragments with human repeated sequences; the sizes of these fragments were 13, 10, 8, 4.7, 3.8, and 3.3 kbp. Uncommon but human-specific fragments are seen in lanes D–G. We assume that these fragments are located near the site where the human sequence is integrated into a mouse chromosome.

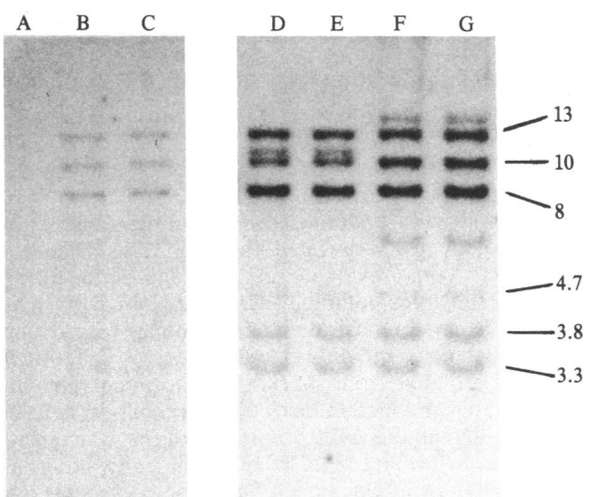

FIG. 2. Conserved human sequences in induced tumors. Ten micrograms of each of six different DNA preparations, from four tumors (two SCT102-derived and two SCT104-derived) and from two independent cultures of a secondary transformant (SCT104), were digested with *Eco*RI and analyzed for the presence of human sequences, as described in the legend to Fig. 1 but under the more stringent hybridization conditions (see *Materials and Methods*). Lanes: A, NIH 3T3 DNA; B and C, DNAs from SCT104 cells; D and E, DNAs from SCT102-derived tumors; F and G, DNAs from SCT104-derived tumors. The sizes of the conserved human fragments are shown in kbp.

Since the six *Eco*RI fragments were conserved in all secondary transformants, the approximate minimum size of the transforming sequence could be estimated, by summing the sizes of all six fragments, to be 42.8 kbp.

Molecular Cloning. For the molecular cloning of the transforming sequence (gene) of the stomach cancer, we first constructed λ phage libraries by inserting size-fractionated *Eco*RI partial digests of DNA from either a secondary transformant (SCT102) or SCT102-derived tumors grown in nude mice. From libraries containing a total of 10^6 recombinant phage clones, we obtained 10 isolates that possessed human repeated DNA sequences and covered about 45 kbp of the transforming sequence (Fig. 3). The clones contained five out of seven *Eco*RI fragments (including an 11-kbp flanking fragment) with human *Alu* sequences observed in the DNA of SCT102 cells. The 10- and 3.3-kbp fragments were not isolated from these libraries. From a λ phage library prepared from *Bam*HI partial digests of the same DNA, we obtained a portion of the 3.3-kbp *Eco*RI fragment, but the 10-kbp fragment was not cloned.

Then we constructed a cosmid library by inserting DNA fragments of 35–43 kbp, prepared by *Eco*RI partial digestion of the same DNA, into *Eco*RI-cleaved, dephosphorylated DNA of pHC79. From the cosmid library of 2×10^5 clones, we obtained six isolates that together covered most of the transforming sequence. Several clones of the λ and cosmid isolates contained the flanking human fragments and even some mouse DNA sequences, which should derive from joint regions where the human sequence is integrated.

Combining these results, we constructed the composite restriction endonuclease map of this transforming sequence shown in Fig. 3. In the secondary transformant SCT102, the human transforming sequence encompasses about 57 kbp. According to Southern blot analysis, the transforming sequence in another secondary transformant SCT104 spans about 75 kbp (unpublished observation). Here, a region of 57 kbp in DNA of the SCT104 cells appears to be common with that of the human sequence in SCT102, as illustrated in Fig. 3. This implies that the maximum estimate of the transforming sequence would be 57 kbp.

Relatedness to the v-*raf* Oncogene. The size and restriction endonuclease map, including analysis of regions containing human *Alu* sequences, of this human transforming sequence (Fig. 3) are clearly different from those of three members of the human *ras* gene family (7–11, 16, 17), B-*lym* (18), *oncD* (19), *met* (20), and *mcf*-2 and *mcf*-3 (23). Furthermore, Southern blot hybridization with human *ras* gene probes revealed that this sequence has no homology to *ras* sequences (data not shown).

We therefore searched for homologies to known viral oncogenes. A total of 40 kbp of the cloned transforming sequence (λS2-29, λS1-4, and λS2-24) was nick-translated and used as probes for the homology tests. Prior to the test, we verified that portions of this region (8- and 13-kbp *Eco*RI fragments) contain coding sequences; a polyadenylylated mRNA was detected with these probes specifically in the transformed cells (data not shown). A nearly complete set of known oncogene clones was digested with appropriate restriction endonucleases, electrophoresed, blotted, and then hybridized to the above probes under the less stringent conditions. Out of 22 oncogene clones tested, only clone p149 of v-*raf* (21) gave a positive signal (data not shown). The homology to v-*raf* was further confirmed, in a reversed manner; that is, nick-translated p149 was used to probe *Eco*RI-digested blotted DNA of the isolated human transforming sequence (Fig. 4). The v-*raf* probe p149 recognized 8- and 10-kbp *Eco*RI fragments of the sequence. This result

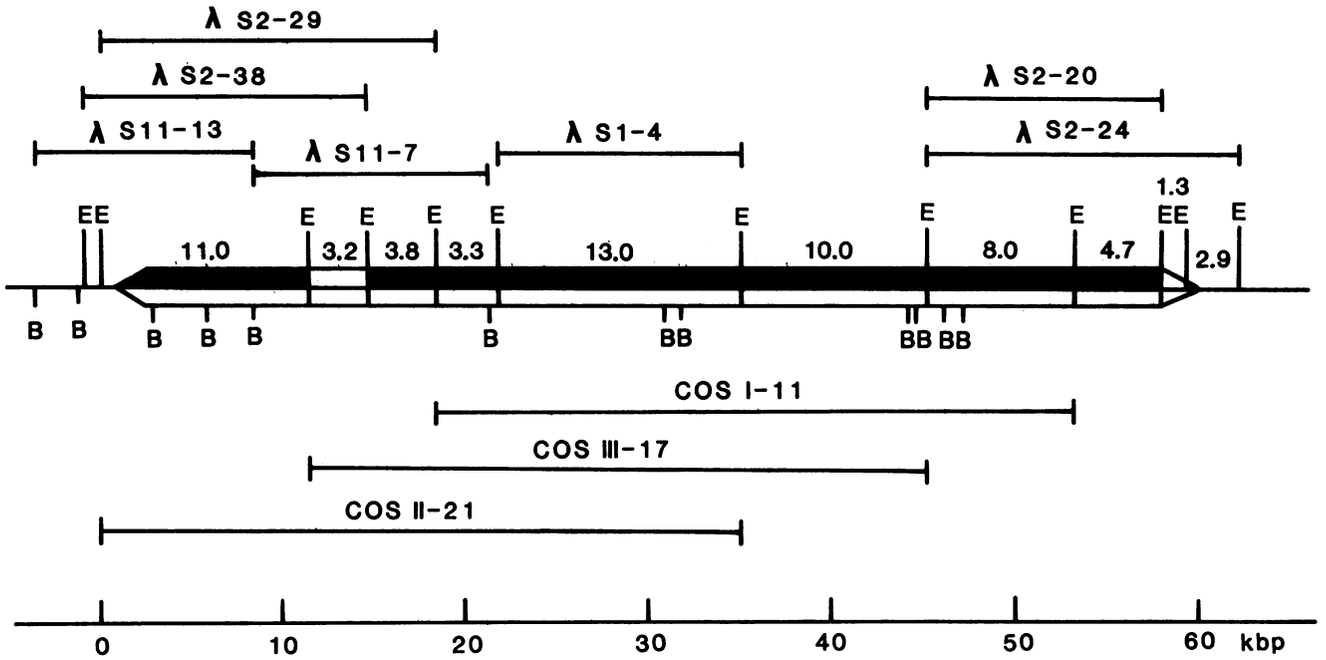

FIG. 3. A composite partial restriction endonuclease map of the human transforming sequence from a stomach cancer. Representative λ phage clones isolated from either *Eco*RI- or *Bam*HI-partial-digest libraries of SCT102 DNA are shown above the map. In the map, boxes represent human DNA sequences and lines at both sides represent mouse DNA sequences. Solid boxes indicate the regions containing human-specific repeating sequences. Sizes of restriction fragments are shown in kbp. Restriction endonuclease sites: E, *Eco*RI; B, *Bam*HI. Below the map are shown representative cosmid clones isolated from a library of *Eco*RI partial digests of SCT102 DNA.

coincided well with the recently obtained information on the c-*raf-1* gene, a functional human homologue of the v-*raf/mil* oncogene (34). In view of all these findings, we conclude that the active transforming sequence obtained from the human stomach cancer contained the c-*raf-1* gene.

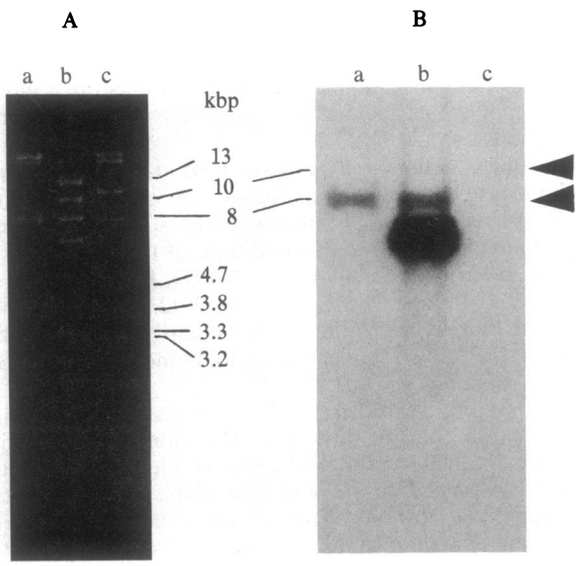

FIG. 4. Homology of the human transforming sequence to the v-*raf* oncogene. A 0.5-μg sample of each of three recombinant clones that together cover the entire region of the transforming sequence were digested with *Eco*RI, subjected to electrophoresis, blotted, and probed with nick-translated p149 plasmid DNA that contained 370 bp (*Xho* I–*Sph* I) of the v-*raf* sequence (33). Hybridization was performed under the less stringent conditions at 43°C. (*A*) Ethidium bromide staining of the gel. Lanes: a, λS2-24; b, cos I-11; c, λS2-29. (*B*) Autoradiograph of the hybridized blot. Arrowheads indicate two human *Eco*RI fragments with *raf/mil* homology. The dense signal at 6.4 kbp is due to the hybridization of pHC79 vector fragment to the probe.

DISCUSSION

We identified a heretofore unknown human transforming gene in a primary stomach cancer. DNAs of normal mucosa and of metastasized lymph node tumor from the same patient did not induce foci on transfection. We isolated the entire region of the human transforming sequence from DNA of secondary transformants of NIH 3T3 cells. The overall size of this sequence is nearly 60 kbp. To our knowledge, this is the largest transforming sequence so far detected in human DNA. Such a huge sequence may account for the poor efficiency of the first- and second-cycle gene transfers. If such is indeed the case, it could be that the failure of most tumor DNAs to induce foci after transfection (3–5) is, at least in part, related to the size of the DNA molecules in the preparations used. The average size of DNAs prepared from primary tumors has been around 50 kbp.

Both the restriction endonuclease map of the sequence and direct homology tests revealed that the transforming sequence differs from previously reported active transforming human genes. Direct homology tests for known retroviral oncogenes clearly demonstrated that the human transforming sequence contains v-*raf/mil*-related sequences. The *raf* oncogene was isolated from murine transforming retrovirus 3611-MSV, a virus which induces fibrosarcomas in newborn mice (33), whereas the *mil* (or *mht*) oncogene was isolated from avian transforming virus MH2, which causes liver and kidney carcinomas in chickens (35). These oncogenes were shown to be homologous ones derived from different species (36). Recently, two human homologues of the *raf/mil* oncogene have been cloned; one, c-*raf-2*, is a processed pseudogene and the other, c-*raf-1*, contains exons homologous to both v-*raf* and v-*mil* (34). The c-*raf-1* gene is located on chromosome 3, and c-*raf-2* is on chromosome 4 in the human genome (37). The c-*raf-1* sequence that Bonner *et al.* (34) isolated corresponds to the 3′-half, 22-kbp region (35–57 kbp on the scale in Fig. 3) of our 57-kbp transforming sequence. It was pointed out by Bonner *et al.* (34) that there is a part of the *raf/mil* coding sequences in this 3′-half region. Our

preliminary analysis of the cDNA clone revealed that, in addition to the 3'-half region, there are exons in the 5'-half region (unpublished data). The direction of transcription of this gene is from left to right on the map in Fig. 3, as based on the analysis of our cDNA clone (unpublished data) and which is also consistent with the evidence of Bonner *et al.* (34). Taken together, it is reasonable to assume that the functional unit of the c-*raf-1* gene encompasses a region of about 42–57 kbp on the human chromosome.

The putative active c-*raf-1* gene appears to act in a dominant manner in NIH 3T3 cells. Since the primary foci appeared more than once and the Southern blot hybridization profiles of the DNAs with a human *Alu* probe varied slightly, we suspect that this gene was activated in the primary cancer of the patient.

The mechanism of activation by which the normal gene was converted to the transforming one has not been elucidated. An apparent difference between the transforming c-*raf-1* gene and the normal c-*raf-1* sequence (34) is observed at the most 3'-terminal region: the size of the 3'-terminal *Eco*RI fragment in our clone is 4.7 kbp (Fig. 3), whereas that of normal human c-*raf-1* is 3.0 kbp (34).

It is of special interest to clarify the nature of the gene product and the activating mechanism(s) of this gene, because the predicted amino acid sequence of v-*raf* is distantly related to the *src* family of oncogenes (38), although the *raf* protein itself has no tyrosine-specific protein kinase activity (33).

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