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Detection of a *raf*-related and two other transforming DNA sequences in human tumors maintained in nude mice

(transfection assay/human transforming gene)

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ABSTRACT **High molecular weight DNAs prepared from a variety of human tumors maintained in nude mice were assayed for their ability to transform NIH 3T3 cells. DNAs from 4 of 21 tumors tested induced transformed foci in cultures of NIH 3T3 cells. They were from a Ewing sarcoma line, a glioblastoma line, a leiomyosarcoma line, and a lung carcinoma line. Hybridization analyses of the NIH 3T3 transformant DNAs with a human repetitive sequence as probe revealed that four distinct transforming DNA sequences were transferred to NIH 3T3 cells from the four tumor lines. The transforming DNA in a lung carcinoma line was a human homologue of the oncogene of Kirsten murine sarcoma virus (Ki-*ras*). On the other hand, the three other transforming DNAs showed no similarity to any known human transforming gene detected by the NIH 3T3 transformation assay. Further analyses with a series of cloned oncogenes as probes revealed that the transforming DNA in a glioblastoma line was a human homologue of the oncogene of 3611-murine sarcoma virus (*raf*). However, the two transforming DNAs in a Ewing sarcoma line and a leiomyosarcoma line had no sequence homology to any of the cloned oncogenes.**

The development of DNA-mediated gene transfer techniques has made it possible to detect specific genes capable of inducing phenotypic changes in recipient cells (1–3). In cancer research, Shih *et al.* (4) first reported that DNA from 3-methylcholanthrene-transformed mouse fibroblasts could induce morphologically transformed foci in cultures of NIH 3T3 mouse fibroblasts by the calcium phosphate precipitation technique. Subsequently, this transfection assay system with NIH 3T3 cells as recipients has been used to detect transforming genes in a variety of human tumor cell lines (5–11) and human tumors (12–14).

Most human transforming genes so far analyzed were found to be members of the *ras* gene family—namely, Ha-*ras*, Ki-*ras*, and N-*ras* (13, 15–22). They were demonstrated to have a similar exonic structure, and all the activated *ras* genes examined had nucleotide changes that accounted for their transforming activity (23–28). Other transforming genes were detected in a human mammary carcinoma cell line (8) and human B- and T-cell lymphomas (12, 29).

The actual roles of these transforming genes in the tumor cells themselves are unknown, but some genes capable of transforming NIH 3T3 cells may be involved in induction and maintenance of the malignant phenotypes of human tumors. Here we report the results of attempts to obtain such transforming genes by NIH 3T3 transformation assay. Four distinct transforming DNA sequences were detected in four human tumor lines.

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MATERIALS AND METHODS

Cell Lines. NIH 3T3 cells (30) were obtained from M. Wigler. For transfection assays, a subclone of NIH 3T3 cells was selected for a flat morphology and a low incidence of spontaneous transformation. The cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% calf serum (GSL), 100 units of penicillin per ml, and 100 μg of streptomycin per ml and were stored frozen in liquid nitrogen. Seven days before transfection experiments, frozen cells were thawed and propagated. The human tumor lines listed in Table 1 have been maintained by transplantation in nude mice in the Central Institute for Experimental Animals (Kawasaki, Japan).

Preparation of DNA. Cultured cells were washed twice with phosphate-buffered saline, and lysed by overnight incubation at 37°C in lysis buffer consisting of 0.5% $NaDodSO_4$/20 mM EDTA/40 mM Tris·HCl, pH 7.6/0.1 M NaCl/200 μg of proteinase K (Merck) per ml. The lysate was extracted twice with an equal volume of buffer-equilibrated phenol and then twice with a mixture of chloroform/isoamyl alcohol (24:1). Then the solution was mixed with 2 vol of ethanol, and precipitated DNA was wound onto a glass rod, washed with 70% ethanol, and dissolved in 1 mM Tris·HCl, pH 7.6/1 mM EDTA. For preparation of DNA from human tumors propagated in nude mice, frozen tumor tissues were minced with a razor blade, homogenized in phosphate-buffered saline in a loose-fitting Dounce homogenizer, washed twice with phosphate-buffered saline, and resuspended in the lysis buffer. Then, DNA was obtained as described above.

Transfection Assays. DNA transfection of NIH 3T3 cells was performed by the calcium phosphate precipitation technique with minor modifications (1, 9, 31). Each cellular DNA (75 μg) was dissolved in 2.5 ml of transfection buffer (0.7 mM $Na_2HPO_4 \cdot 7H_2O$/21 mM Hepes/0.145 M NaCl, pH 7.0). To the solution was added 125 μl of 2.5 M $CaCl_2$ with gentle shaking to form DNA–calcium phosphate precipitate. After 30–50 min, 1.25 ml of this DNA solution was introduced into a 100-mm culture dish containing 10 ml of Dulbecco's modified Eagle's medium with 10% calf serum into which 6×10^5 NIH 3T3 cells had been seeded the day before. Cells were incubated for 15–22 hr with the DNA precipitate and refed with 10 ml of Dulbecco's modified Eagle's medium containing 5% calf serum. Culture fluids were changed twice weekly and morphologically transformed foci were counted after 14–21 days.

Restriction Endonuclease Digestions. Cellular DNAs were digested with restriction endonucleases (Takara Shuzo, Kyoto, Japan) under the conditions recommended by the supplier. The completeness of digestions was monitored by adding bacteriophage λ DNA to portions of the reaction mixture and examining them by electrophoresis on agarose gel.

Abbreviation: kbp, kilobase pair(s).

Hybridization Analysis. DNAs were digested with restriction endonucleases, subjected to electrophoresis at 30 V for 20 hr on 1% agarose gel, and blotted onto a nitrocellulose filter by the method of Southern (32). The blots were hybridized with nick-translated (33) ^{32}P-labeled probes in 50% formamide/4× NaCl/Cit (1× NaCl/Cit = 0.15 M NaCl/0.015 M Na citrate, pH 7.0)/4× Denhardt's solution (1× Denhardt's solution = 0.02% polyvinylpyrrolidone/0.02% Ficoll/0.02% bovine serum albumin)/50 μg of salmon testis DNA per ml/20 μg of yeast RNA per ml/50 mM Hepes, pH 7.0, at 42°C for 40 hr. The filters were washed under appropriate conditions and the ^{32}P-labeled blots were detected by autoradiography.

RESULTS

Transforming Activity of DNAs from Human Tumor Lines. High molecular weight DNAs were extracted from 21 human tumors from various tissues that had been maintained in nude mice. Uncut DNAs [>50 kilobase pairs (kbp)] were used to transfect NIH 3T3 mouse cells by the calcium phosphate precipitation technique, and morphologically transformed foci were counted 2–3 weeks later. DNAs from 4 of 21 tumors tested induced transformed foci in NIH 3T3 cell cultures (Table 1). These DNAs were obtained from a Ewing sarcoma line (NB-1-JCK), a glioblastoma line (GL-5-JCK), a leiomyosarcoma line (FH), and a lung carcinoma line (LC-12-JCK). Control human placenta DNA and NIH 3T3 DNA did not transform NIH 3T3 cells. Transformed foci consisted of round or spindle-shaped highly refractile cells and were easily distinguishable from spontaneous overgrowth of NIH 3T3 cells.

On primary transfection, the efficiencies of focus formation by these DNAs (0.0044 to 0.022 foci per μg of DNA) were 1/2 to 1/10th those in similar transfection experiments reported by others (9, 10). However, in the second cycle of transfection, DNAs from these NIH 3T3 primary transformants induced foci in NIH 3T3 cell cultures at higher efficiencies (0.075–0.12 foci per μg of DNA). In the third cycle of transfection, the efficiencies of focus formation were comparable with those in the second cycle, except in the case of transformant DNAs derived from GL-5-JCK glioblastoma DNA; in the latter case, DNAs from two independent clones of secondary transformants exhibited 20 to 30 times higher transforming activity than in the second cycle transfection. No obvious gene amplification was detected by Southern blot analysis of these DNAs.

Table 1. Transforming activity of DNA from human tumor lines and NIH 3T3 primary transformants

Donor DNA	Type of tumor	Total foci per recipient cultures	
		Primary	Secondary
UCC-5-JCK	Cervix carcinoma	0/6	
CC-1-JCK	Choriocarcinoma	0/6	
COL-1-JCK	Colon carcinoma	0/6	
TE	Embryonal carcinoma	0/6	
Epe-1-To	Ependymoma	0/6	
NB-1-JCK	Ewing sarcoma	1/6	23/6
GL-4-JCK	Glioblastoma	0/6	
GL-5-JCK	Glioblastoma	3/6	28/6
Hpb-1-JCK	Hepatoblastoma	0/6	
HC-1-JCK	Histiocytoma	0/6	
FH	Leiomyosarcoma	5/6	24/6
LS-2-JCK	Leiomyosarcoma	0/6	
Hp-1-JCK	Liver carcinoma	0/6	
LC-4-JCK	Lung carcinoma	0/6	
LC-12-JCK	Lung carcinoma	5/6	17/6
LC-13-JCK	Lung carcinoma	0/6	
LC-14-JCK	Lung carcinoma	0/6	
SEKI	Melanoma	0/6	
MNB	Neuroblastoma	0/6	
OSS-6-JCK	Osteosarcoma	0/6	
RCC-9-JCK	Renal carcinoma	0/6	
Human placenta		0/24	
NIH 3T3		0/12	

NIH 3T3 cells, seeded at 6 × 10^5 cells per 100-mm culture dish 1 day before transfection, were transfected with 37.5 μg of DNA. In each transfection, 75 μg of DNA was prepared and applied to two recipient cultures. Morphologically transformed foci were counted 14–21 days after transfection.

Analyses of NIH 3T3 Transformant DNAs with a Human Repetitive Sequence Probe. DNAs derived from primary and representative secondary foci were examined for the presence of human DNA sequences by Southern blot analyses with the human *Alu* repetitive sequence probe BLUR-8 (34). The results showing that each primary and secondary transformant DNA contained various numbers of DNA fragments hybridizable to the human *Alu* sequence probe (data not shown) confirmed that serial transmission of human DNA sequences was responsible for the transformation of NIH 3T3 cells. Repeated cycles of transfection remove most of the human DNA sequences that are not closely associated with the transforming gene (7). Southern blot analyses of representative secondary and tertiary transformant DNAs were performed again using BLUR-8 as a probe. Fig. 1 shows the results of analyses of three secondary and three tertiary NB-1-JCK-derived transformant DNAs digested with *Eco*RI. Of the DNA fragments detected in each lane, eight were

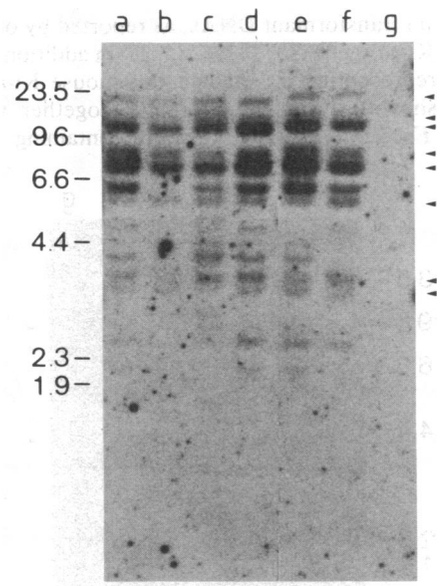

FIG. 1. Presence of human DNA sequences in NIH 3T3 secondary and tertiary transformants derived from NB-1-JCK Ewing sarcoma DNA transfection. Each DNA (13.5 μg) was digested with *Eco*RI, subjected to electrophoresis on 1% agarose gel, and blotted onto a nitrocellulose filter by the method of Southern. The filter was incubated with 4 × 10^7 cpm of nick-translated ^{32}P-labeled BLUR-8 probe (specific activity, >10^8 cpm/μg) for 40 hr and washed at 65°C for 2 hr with four changes of 2× NaCl/Cit/0.05% $NaDodSO_4$, and then for 30 min with 1× NaCl/Cit/0.05% $NaDodSO_4$. ^{32}P-labeled blots were exposed to Kodak XAR-5 film with an intensifying screen at −70°C. Lanes: a–c, independent secondary transformant DNAs; d–f, independent tertiary transformant DNAs; g, NIH 3T3 DNA. Arrows indicate *Eco*RI-generated DNA fragments with human *Alu* sequences common to all secondary and tertiary transformant DNAs. The sizes of marker fragments of *Hin*dIII-digested λ DNA are indicated in kbp.

common to all the digests; namely, human DNA fragments of 18.5, 13.0, 11.2, 8.2, 7.3, 5.6, 3.5, and 3.2 kbp. Similar analyses of GL-5-JCK-derived transformant DNAs digested with *Eco*RI revealed that four common human DNA fragments (9.4, 7.7, 4.7, and 4.1 kbp) were conserved in all six transformants (Fig. 2). Analyses of FH-derived transformant DNAs digested with *Eco*RI showed that at least 18 common human DNA fragments were conserved in all five transformants (Fig. 3*A*); namely, those of 25.0, 15.5, 11.4, 9.2, 8.3, 7.2, 5.8, 5.4, 4.9, 4.7, 4.2, 3.3, 2.9, 2.5, 2.3, 2.2, 1.9, and 1.3 kbp.

These results of analyses showing distinct restriction patterns conserved in secondary and tertiary transformant DNAs revealed the presence of three different genes with distinct sizes and structures in the three different tumor lines. Moreover, these distinct restriction patterns of the three genes appeared to be different from those of known members of the *ras* gene family, namely, Ha-*ras*, Ki-*ras*, and N-*ras*.

On the other hand, analyses of digests with *Eco*RI of five LC-12-JCK-derived secondary transformant DNAs revealed five *Alu*-containing DNA fragments (6.7, 4.6, 4.2, 3.2, and 2.6 kbp) that were common to all five transformants (Fig. 3*B*). This pattern of *Alu*-containing DNA fragments was similar to that reported for the Ki-*ras* gene (13, 18, 26, 35).

Relationship of Detected Transformant DNAs to Known Human Transforming Genes and Viral Oncogenes. To confirm the observations described above, we examined transformant DNAs derived from the four human tumor lines by Southern blot analyses, using specific probes for v-Ha-*ras* (BS-9) (36), v-Ki-*ras* (HiHi-3) (37), and N-*ras* (pNP1 and pNP5) (17).

First, analyses of digests with *Eco*RI of five LC-12-JCK-derived secondary transformant DNAs using HiHi-3 as a probe revealed the presence of two DNA fragments (6.7 and 3.1 kbp) in all transformant DNAs, as reported by others for the human Ki-*ras* gene (13, 18, 26, 35, 37) in addition to DNA fragments representing the endogenous mouse Ki-*ras* gene (data not shown). From these results, together with the pattern of *Eco*RI-generated fragments containing *Alu*, we concluded that the transforming gene of LC-12-JCK lung carcinoma line was a human homologue of the oncogene of Kirsten murine sarcoma virus.

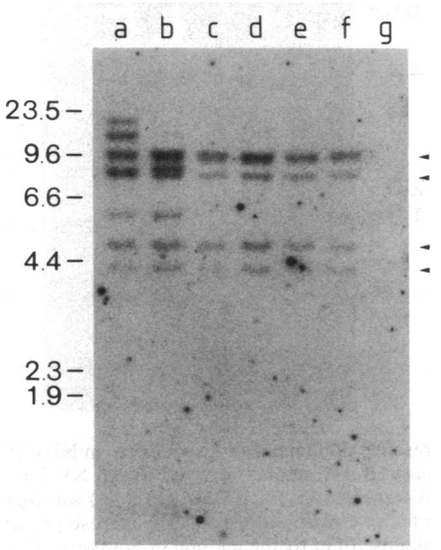

FIG. 2. Presence of human DNA sequences in NIH 3T3 secondary and tertiary transformants derived from GL-5-JCK glioblastoma DNA transfection. Each DNA (13.5 μg) was digested with *Eco*RI, subjected to electrophoresis on 1% agarose gel, and probed with ^{32}P-labeled BLUR-8 as described for Fig. 1. Lanes: a–c, independent secondary transformant DNAs; d–f, independent tertiary transformant DNAs; g, NIH 3T3 DNA. Arrows indicate *Eco*RI-generated DNA fragments with human *Alu* sequences common to all secondary and tertiary transformant DNAs. Sizes of marker fragments are indicated in kbp.

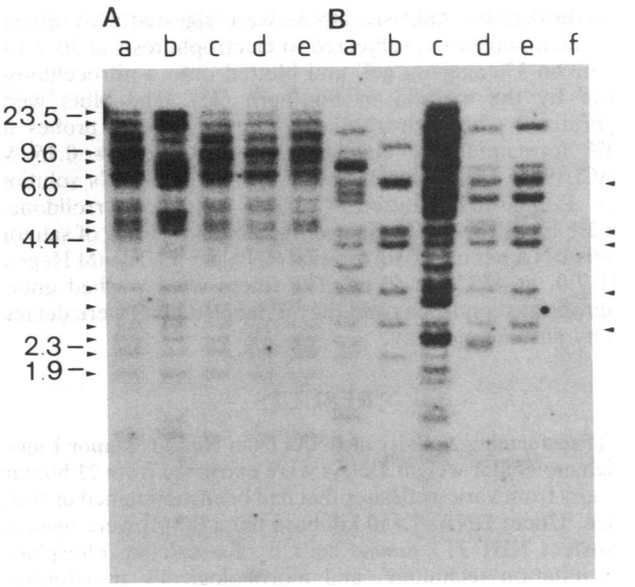

FIG. 3. Presence of human DNA sequences in NIH 3T3 secondary and tertiary transformants derived from FH leiomyosarcoma DNA transfection (*A*) or LC-12-JCK lung carcinoma DNA transfection (*B*). Each DNA (13.5 μg) was digested with *Eco*RI, subjected to electrophoresis on 1% agarose gel, and probed with ^{32}P-labeled BLUR-8 as described for Fig. 1. (*A*) Lanes: a–c, independent secondary transformant DNAs; d and e, independent tertiary transformant DNAs. Arrows indicate *Eco*RI-generated DNA fragments with human *Alu* sequences common to all secondary and tertiary transformant DNAs. (*B*) Lanes: a–e, independent secondary transformant DNAs; f, NIH 3T3 DNA. Arrows indicate *Eco*RI-generated DNA fragments with human *Alu* sequences common to all secondary transformant DNAs. Sizes of marker fragments are indicated in kbp.

On the other hand, none of the four *ras*-specific probes detected *ras*-related sequences other than endogenous mouse *ras* gene sequences in the transformant DNAs derived from FH, NB-1-JCK, and GL-5-JCK (data not shown). These three transformant DNAs were examined further by Southern blot analyses with specific probes for the following oncogenes: *Blym*, v-*abl*, v-*erbA*, v-*erbB*, v-*fgr*, v-*fms*, v-*fos*, v-*fps*, v-*mos*, v-*myb*, v-*myc*, v-*raf*, v-*rel*, v-*ros*, v-*sis*, v-*src*, and v-*yes*.

Fig. 4 shows the results of representative analyses of the three transformant DNAs using v-*raf*-specific XB probe (38). The DNAs were digested with *Hin*dIII (Fig. 4, lanes a–e) or *Pst* I (lanes f–j). In the transformant DNA derived from FH or NB-1-JCK, only the DNA fragments identical to those detected in normal NIH 3T3 DNA were detected in each case of the endonuclease digestion. In contrast, GL-5-JCK-derived transformant DNA was found to contain additional DNA fragments hybridizing to the *raf*-specific probe that were not detected in normal NIH 3T3 DNA. When the transformant DNA was digested with *Hin*dIII, three additional DNA fragments (4.0, 3.4, and 1.9 kbp) were present in the DNA (lane e), and the latter two fragments were identical to those detected in human placenta DNA (lane b). In case of the *Pst* I digestion of the transformant DNA, two additional fragments (5.8 and 2.4 kbp) were present in the DNA (lane j), and the two fragments were identical to those detected in human placenta DNA (lane g). These additional fragments that could hybridize to the *raf*-specific probe were also observed in analyses of several other independent GL-5-JCK-derived transformant DNAs. We concluded that the

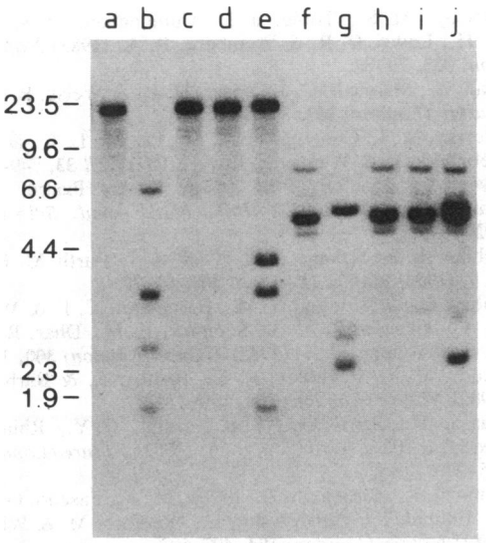

FIG. 4. Analyses of *raf*-related sequences in NIH 3T3 secondary transformants derived from transfection with FH leiomyosarcoma DNA, NB-1-JCK Ewing sarcoma DNA, or GL-5-JCK glioblastoma DNA. Each DNA (13.5 μg) was digested with *Hin*dIII (lanes a–e) or *Pst* I (lanes f–j), subjected to electrophoresis on 1% agarose gel, and blotted onto a nitrocellulose filter by the method of Southern. The filter was incubated with 2×10^7 cpm of nick-translated ^{32}P-labeled v-*raf*-specific XB probe (specific activity, $>10^8$ cpm/μg) for 40 hr and washed at 50°C for 2 hr with four changes of 0.1× NaCl/Cit/0.1% NaDodSO$_4$. ^{32}P-labeled blots were exposed to Kodak XAR-5 film with an intensifying screen at −70°C. Lanes: a and f, NIH 3T3 DNA; b and g, human placenta DNA; c and h, FH-derived secondary transformant DNA; d and i, NB-1-JCK-derived secondary transformant DNA; e and j, GL-5-JCK-derived secondary transformant DNA. The sizes of marker fragments of *Hin*dIII-digested λ DNA are indicated in kbp.

transforming gene of GL-5-JCK glioblastoma line was a human homologue of the oncogene of 3611-murine sarcoma virus (*raf*) (38). In addition, among the *Alu*-containing *Eco*RI fragments in the GL-5-JCK-derived transformant DNAs (shown in Fig. 2) only the 7.7-kbp fragment could be detected by the *raf*-specific probe.

In a series of analyses with the oncogene probes other than the *raf*-specific probe, the sequences detected in the transformant DNAs derived from FH, NB-1-JCK, and GL-5-JCK were identical to those detected in normal NIH 3T3 DNA.

Effect of Restriction Endonuclease Digestion on Transforming Activity of Transformant DNA. The transforming DNAs of NB-1-JCK, GL-5-JCK, and FH were characterized by examining their sensitivities to inactivation with a series of restriction endonucleases: *Bam*HI, *Bgl* II, *Eco*RI, *Hin*dIII, *Kpn* I, *Pst* I, *and Xho* I (Table 2). The DNAs tested were from an NB-1-JCK-derived secondary transformant, a GL-5-JCK-derived secondary transformant, and an FH-derived primary transformant. The transforming activities of the three transformant DNAs were inactivated by digestion with all seven restriction endonucleases. Results on the transforming DNA of GL-5-JCK and FH were confirmed with DNA from an additional clone of each transformant. The inactivation patterns of the three transforming DNAs with a series of restriction endonucleases were different from that reported for a human mammary carcinoma transforming gene (8) and human B- and T-cell lymphoma transforming genes (12).

Table 2. Transforming activity of DNA digested with restriction endonucleases

Restriction endonuclease	NB-1-JCK-S1 DNA, foci per 75 μg	GL-5-JCK-S6 DNA, foci per 75 μg	FH-P2 DNA, foci per 75 μg
None	9	204	5
*Bam*HI	0	0	0
Bgl II	0	0	0
*Eco*RI	0	0	0
*Hin*dIII	0	0	0
Kpn I	0	0	0
Pst I	0	0	0
Xho I	0	4	0

High molecular weight DNA (75 μg) was digested to completion with each of the indicated restriction endonucleases. The digested DNA was purified and assayed for transforming activity on NIH 3T3 cells as described for Table 1. Transformed foci were counted 18 days after transfection. NB-1-JCK-S1, a secondary transformant derived from NB-1-JCK Ewing sarcoma DNA; GL-5-JCK-S6, a secondary transformant derived from GL-5-JCK glioblastoma DNA; FH-P2, a primary transformant derived from FH leiomyosarcoma DNA.

DISCUSSION

Our findings that only one of four transforming DNA sequences detected was a known member of the *ras* gene family and that another one was a *raf*-related transforming DNA were in contrast with many reports of detection of mainly *ras* transforming genes in human tumor cell lines (12–22), although we used a wide variety of human tumor lines as DNA donors. Experiments on inactivation of the other two transforming DNAs with a series of restriction endonucleases showed that they were distinct from reported transforming genes in a human mammary carcinoma cell line (8) and in human B- and T-cell lymphomas (12). In addition, the patterns of *Eco*RI-generated *Alu*-containing DNA fragments of the two transforming genes appeared to be different from those recently reported for a transforming gene in a chemically transformed human osteosarcoma-derived cell line (39) and a human melanoma transforming gene that showed slight homology with members of the *ras* gene family (40). The following possibilities may explain the relatively frequent detection of these unique transforming genes.

First, the sizes of two of the three transforming DNAs were estimated to be much more than those of reported transforming genes: the largest human transforming gene reported so far is the Ki-*ras* gene of ≈40 kbp (26, 35, 41), whereas the transforming DNAs of the NB-1-JCK Ewing sarcoma line and FH leiomyosarcoma line appeared to be much larger than the Ki-*ras* gene when estimated from the sum of the *Alu*-containing DNA sequences conserved in common in secondary and tertiary transformant DNAs. When the DNAs prepared from tumors or tumor cell lines were degraded or sheared before transfection experiments, only relatively small transforming genes were detectable.

Second, we detected the three transforming DNAs in a Ewing sarcoma line, a glioblastoma line, and a leiomyosarcoma line. There has been no report of detection of transforming genes in these three types of tumors. On the other hand, we detected the Ki-*ras* transforming DNA in a lung carcinoma line. Although the Ki-*ras* transforming gene was detected in a variety of human tumors (13, 15, 18, 21, 41), it was detected most often in lung carcinomas (13, 15, 41). Specific transforming genes were suggested to be present in a human mammary carcinoma cell line and human B- and T-cell lymphomas (8, 12). Therefore, there may be a relationship between a specific transforming gene and a certain type of human tumor in some cases.

In attempts to obtain possible genes responsible for induction and maintenance of the malignant phenotypes of human tumors by NIH 3T3 transformation assay, we detected a *raf*-related and two unique transforming DNAs, suggesting that other distinct transforming genes in human tumors may

be detected by this method. However, the activity of a gene to transform NIH 3T3 mouse cells does not necessarily prove its oncogenic role in human tumor cells: the transforming DNAs detected in our study may have arisen in the course of tumor progression in the host or during propagation of tumor cells in nude mice. In fact, two reports suggest that activation of the *ras* gene is not involved in either initiation or maintenance of some human tumors (22, 42). The relationship between the functions of these transforming genes and the phenotypes of the human tumors in which they were detected should be clarified, together with the precise structure and origin of the transforming genes.

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