MOLECULAR AND CELLULAR BIOLOGY, May 1987, p. 1776–1781
0270-7306/87/051776-06$02.00/0

Vol. 7, No. 5

Molecular Cloning and Characterization of an Activated Human c-*raf*-1 Gene

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Received 17 November 1986/Accepted 7 February 1987

Results of previous studies have shown that a *raf*-related transforming DNA sequence is present in NIH 3T3 transformants that are derived from GL-5-JCK human glioblastoma DNA transfection. The transforming DNA was molecularly cloned by using cosmid vector pJB8 to determine its structure and origin. Analyses of selected clones revealed that the transforming DNA consisted of three portions of human DNA sequences, with the 3′ half of the c-*raf*-1 gene as its middle portion. This *raf* region was about 20 kilobases long and contained exons 8 to 17 and the poly(A) addition site. RNA blot analysis showed that the *raf*-related transforming DNA was transcribed into 5.3-, 4.8-, and 2.5-kilobase mRNAs; the 2.5-kilobase transcript was thought to be the major transcript. Immunoprecipitation analyses revealed that a 44-kilodalton *raf*-related protein was specifically expressed in the NIH 3T3 transformants. The *raf*-related transforming DNA was considered to be activated when its amino-terminal sequence was truncated and the DNA was coupled with a foreign promoter sequence. On hybridization analysis of the original GL-5-JCK glioblastoma DNA, no rearrangement of c-*raf*-1 was detectable in the tumor DNA. The rearrangement of c-*raf*-1 may have occurred during transfection or may have been present in a small population of the original tumor cells as a result of tumor progression.

We previously detected a transforming DNA sequence in NIH 3T3 cells that were transformed by GL-5-JCK human glioblastoma DNA and showed that the transforming DNA was a human homolog of the retroviral oncogene v-*raf* (8). Among the transforming DNA sequences so far detected by NIH 3T3 transformation assay, a few were found to be related to retroviral oncogenes. Besides the transforming *raf* gene mentioned above, a transforming Ha-*ras* gene (21, 25) and a transforming Ki-*ras* gene (7) were identified as cellular homologs of the oncogenes of Harvey murine sarcoma virus and Kirsten murine sarcoma virus, respectively. A transforming N-*ras* gene (9, 29) and a human melanoma transforming gene, *mel* (20), showed homology to v-Ha-*ras* and v-Ki-*ras*. In addition, a transforming gene of rat neuroblastomas and glioblastomas, *neu*, showed homology to v-*erbB* (27). The transforming *ras* genes Ha-*ras*, Ki-*ras*, and N-*ras* have been well characterized. These three transforming *ras* genes and the two viral *ras* genes were demonstrated to have a similar coding sequence and point mutations that are responsible for their transforming activity (22, 33, 36, 39). The v-*raf* gene was originally detected in a murine sarcoma virus, 3611-MSV (22). A functional human gene homologous to v-*raf* was cloned and termed c-*raf*-1. This c-*raf*-1 gene contained 17 exons, 9 of which were homologous to v-*raf* (4). The v-*raf* gene was a truncated gene containing only the 3′ portion of the cellular homolog, and its translational products were identified as *gag*-fused proteins (22). Activation of *raf*-related transforming DNA derived from GL-5-JCK glioblastoma DNA might occur in the same way as activation of v-*raf*. In this study we performed molecular cloning of the *raf*-related transforming DNA and analyzed the transcripts and the protein that is specifically expressed in NIH 3T3 cells that are transformed by the transforming DNA.

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MATERIALS AND METHODS

Cell lines. Most of the cell lines and the media used in this study have been described previously (8). NIH 3T3 transformants obtained by transfection with HL-60 human promyelocytic leukemia DNA and with the complete Harvey murine sarcoma virus genome clone (pH-1) were also produced in this laboratory and contained N-*ras* and v-Ha-*ras*, respectively.

Preparation of DNA and RNA. DNAs were prepared from cultured cells or tumors as described previously (8). Total cellular RNA was prepared by the guanidinium thiocyanate-cesium chloride method, as described previously (5). Poly(A)$^+$ RNA was purified by oligo(dT)-cellulose chromatography, as described previously (2).

Construction and screening of cosmid libraries. High-molecular-weight DNA prepared from a secondary transformant, S1-3, that was derived from GL-5-JCK human glioblastoma DNA transfection was partially digested with *Mbo*I and size fractionated by centrifugation through a sucrose density gradient, and 35- to 45-kilobase (kb) fragments were isolated as described previously (18). Vector arms were prepared from cosmid pJB8 by the protocol described by Ish-Horowicz and Burke (11), and then the donor DNA fragments were ligated into the *Bam*HI site of the cosmid. The recombinant DNA was packaged in vitro and transduced into *Escherichia coli* 490A. The cells were plated onto nitrocellulose filters (diameter, 82 mm; ATF; Millipore Corp., Bedford, Mass.) on LB agar plates containing 50 μg of ampicillin per ml at a density of about 10,000

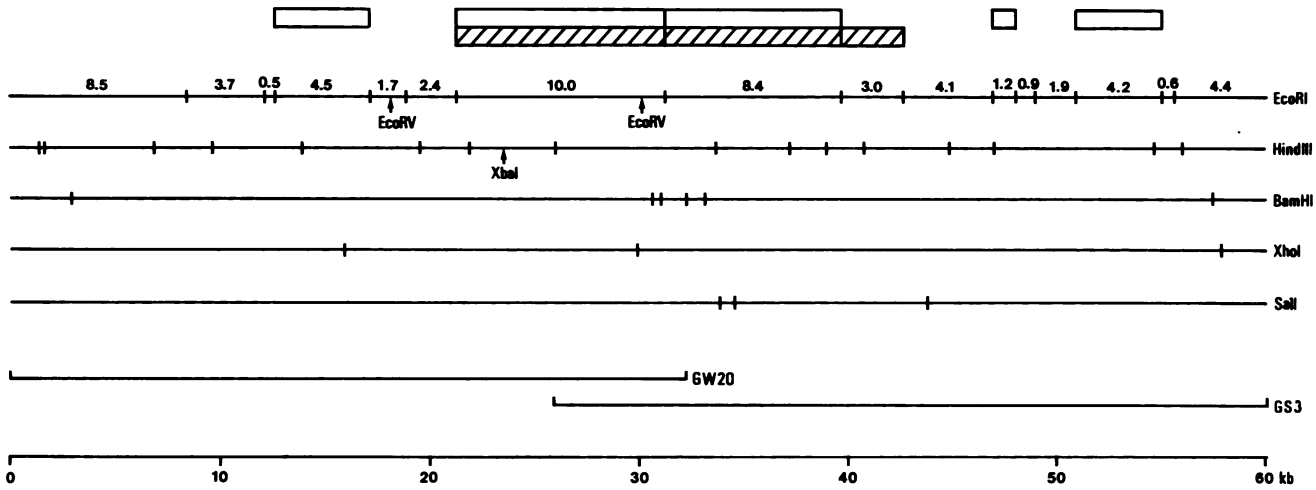

FIG. 1. Restriction endonuclease map of transforming DNA derived from GL-5-JCK glioblastoma DNA transfection. Open boxes above the map indicate *Eco*RI fragments containing human *Alu* sequences hybridized to the human *Alu* clone BLUR8. Hatched boxes indicate *Eco*RI fragments containing *raf* sequences hybridized to the cDNA clone of the human c-*raf*-1 gene, which contains all the coding exons. In addition, *Eco*RV sites in the 1.7- and 10.0-kb *Eco*RI fragments and the *Xba*I site in the 4.2-kb *Hin*dIII fragment are marked. Sizes of restriction fragments are indicated in kilobases.

colonies per filter, and duplicate sets of filters were screened as described by Hanahan and Meselson (10) with the human *Alu* probe BLUR8 (13).

Hybridization analyses of DNA and RNA. DNAs were digested with restriction endonucleases; electrophoresed on a 0.7, 0.9, or 1.2% agarose gel; and blotted onto nitrocellulose filters by the method described by Southern (31). The blots were hybridized with nick-translated (24), ^{32}P-labeled probes as described previously (8).

RNAs were denatured in 50% formamide at 55°C and electrophoresed on 1.2% agarose gel containing 2.2 M formaldehyde, as described previously (17). The RNAs were blotted onto a nitrocellulose filter by the method described by Thomas (37). The blots were hybridized with a ^{32}P-labeled probe in 50% formamide–5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate [pH 7.0])–10× Denhardt solution (1× Denhardt solution is 0.02% polyvinylpyrrolidone, 0.02% Ficoll, 0.02% bovine serum albumin)–200 μg of salmon testis DNA per ml–50 mM sodium phosphate buffer (pH 6.5) at 42°C for 18 h. The filter was washed at 50°C for 2 h with four changes of 0.1× SSC–0.1% sodium dodecyl sulfate (SDS), and the ^{32}P-labeled blots were detected by autoradiography.

Preparation of antisera. NIH 3T3 transformants were irradiated with γ-rays to eliminate the tumorigenicity of the cells and were injected subcutaneously into NFS mice (age, 4 to 6 weeks). Injections of 10^7 cells per animal were repeated every 2 weeks, and serum samples were prepared from the mice after 13 weeks.

Immunoprecipitation and SDS-polyacrylamide gel electrophoresis. Approximately 1.5×10^6 growing cells in a culture dish (diameter, 60 mm) were labeled with 100 μCi of [^{35}S]methionine (500 Ci/mmol; Amersham Corp., Arlington Heights, Ill.) for 7 h, and the cell lysates were immunoprecipitated with antisera, as described previously (14). SDS-polyacrylamide gel electrophoresis of immunoprecipitated samples was performed as described by Laemmli (15). Gels were processed for fluorography (16) and exposed to XAR-5 film (Eastman Kodak Co., Rochester, N.Y.) with an intensifying screen at −70°C.

RESULTS

Molecular cloning of a *raf*-related transforming DNA. Results of previous analyses of GL-5-JCK-derived transformant DNAs digested with *Eco*RI revealed that four human DNA fragments containing *Alu* are conserved in the transformants (8). These human DNA sequences were thought to be closely associated with the *raf*-related transforming DNA. A cosmid library was constructed from *Mbo*I partial digests of a GL-5-JCK-derived secondary transformant S1-3 DNA and was screened with human *Alu* probe BLUR8 for human sequences. We obtained 32 *Alu*-positive cosmid clones from the approximately 400,000 recombinant clones that were screened. These clones were characterized by restriction analyses, followed by hybridization analyses with BLUR8 and a cDNA clone of human c-*raf*-1, which contains all the coding exons (4) as probes. Several clones were characterized further by a series of partial digestions and double digestions with restriction endonucleases to construct a restriction enzyme map of the transforming DNA.

The restriction enzyme map of about 60-kb DNA sequences contained in clones GW20 and GS3 is shown in Fig. 1. In addition to the 10.0-, 8.4-, 4.5-, and 4.2-kb *Eco*RI fragments that hybridized to the *Alu* probe BLUR8, there was a 1.2-kb *Eco*RI fragment that hybridized weakly to BLUR8. The 10.0- and 8.4-kb fragments and a 3.0-kb *Eco*RI fragment contained sequences that hybridized to the *raf* probe. The restriction enzyme map of the normal human c-*raf*-1 gene and the positions of all the coding exons (exons 2 to 17) were determined (4). Comparison of our map with that of normal human c-*raf*-1 showed that the map of the sequence from the *Hin*dIII site located in the middle of the 10.0-kb *Eco*RI fragment to the *Sal*I site in the 4.1-kb *Eco*RI fragment was identical in the two. In this region, the 8.4-kb *Eco*RI fragment contained exons 11 to 13, and the 3.0-kb *Eco*RI fragment contained exons 14 to 17 and the site of the poly(A) signal. Furthermore, the *Xba*I site that is 2.5 kb to the 5′ side of the *Hin*dIII site in the middle of the 10.0-kb *Eco*RI fragment was also present in both of the transforming DNAs and normal c-*raf*-1. This 2.5-kb region contained

exons 8 to 10. Thus, the 5′ and 3′ ends of the conserved *raf* region were estimated to be located between the *Xba*I site mentioned above and the *Hin*dIII site that is 1.7 kb to the 5′ side of the *Xba*I site, and between the *Sal*I site and *Hin*dIII site in the 4.1-kb *Eco*RI fragment, respectively. Because DNA sequences containing human *Alu* were present on both of the 5′ and 3′ sides of the *raf* region, DNA recombination was thought to occur in at least two points within human DNA sequences.

Analyses of the c-*raf*-1 gene in the GL-5-JCK glioblastoma line. For determination of whether the truncation and rearrangement of the c-*raf*-1 gene described above was present in the original tumor, GL-5-JCK glioblastoma DNA was subjected to hybridization analysis with the cDNA clone of the c-*raf*-1 gene (Fig. 2). A 14-kb fragment was detected in *Xho*I digests of the transformant DNAs (Fig. 2A, lanes c and d), and a 12-kb fragment was detected in *Eco*RV digests of the transformant DNAs (Fig. 2B, lanes c and d). The 5′ and 3′ ends of the 12-kb *Eco*RV fragment were located in the 1.7-kb *Eco*RI fragment and the 10.0-kb *Eco*RI fragment, respectively. The 14-kb *Xho*I fragment and the 12-kb *Eco*RV fragment were located within the block of human DNA sequences and encompassed the 5′ rearranged point of the *raf* region in the transformant DNA. If the 5′ rearrangement of c-*raf*-1 was present in the original tumor, the 14-kb *Xho*I fragment and the 12-kb *Eco*RV fragment should be detected in the tumor DNA. However, these fragments were not detectable in the tumor DNA (Fig. 2A, lane b, and Fig. 2B, lane b). A 4.1-kb fragment was detected in *Hin*dIII digests of the transformant DNAs (Fig. 2C, lanes a and b). This *Hin*dIII fragment was also located within the block of human DNA sequences and contained the 3′ rearranged point of the *raf* region. This *Hin*dIII fragment was not detected in the tumor DNA (Fig. 2C, lane c). Thus, no rearrangement of the c-*raf*-1 gene was detectable in the GL-5-JCK glioblastoma line.

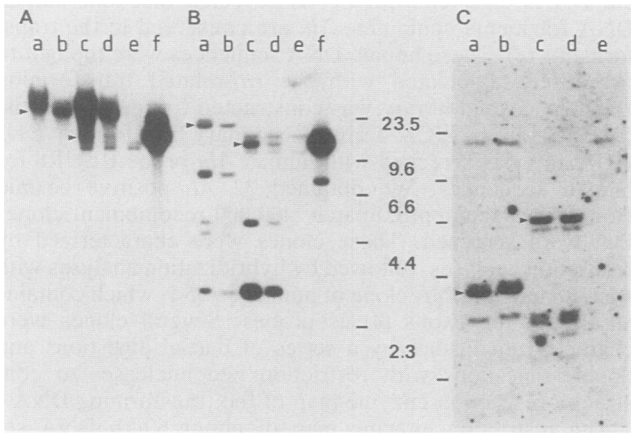

FIG. 2. Analyses of *raf*-related sequences in a GL-5-JCK human glioblastoma line and in NIH 3T3 transformants derived from GL-5-JCK glioblastoma DNA transfection. Cellular DNAs (12 or 6 μg for lanes b in panels A and B, respectively) were digested with *Xho*I (A), *Eco*RV (B), or *Hin*dIII (C); subjected to electrophoresis on a 0.9% agarose gel; blotted onto a nitrocellulose filter; and hybridized with the ^{32}P-labeled cDNA clone of human c-*raf*-1. The filter was washed at 50°C for 2 h with four changes of 0.1× SSC–0.1% SDS, and ^{32}P-labeled blots were exposed to XAR-5 film (Kodak) with an intensifying screen at −70°C. Lanes in panels A and B: a, human placenta DNA; b, GL-5-JCK glioblastoma DNA; c, GL-5-JCK-derived primary transformant DNA; d, GL-5-JCK-derived secondary transformant S1-3 DNA; e, NIH 3T3 DNA; f, NIH 3T3 DNA with 10 ng of cosmid clone GW20. Lanes in panel C: a and b, GL-5-JCK-derived independent tertiary transformant DNAs; c, GL-5-JCK glioblastoma DNA; d, human placenta DNA; e, NIH 3T3 DNA. Arrowheads indicate restriction enzyme fragments encompassing the rearranged point or fragments containing corresponding *raf* region in unrearranged human c-*raf*-1. The sizes of marker fragments of *Hin*dIII-digested λ DNA are indicated in kilobases.

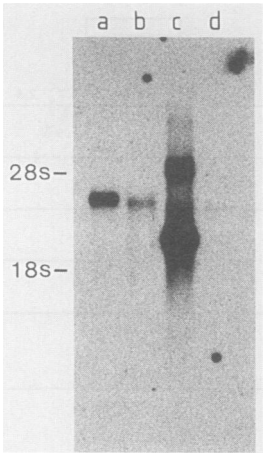

FIG. 3. Detection of altered *raf* transcripts in the GL-5-JCK-derived secondary transformant S1-3. Poly(A)$^+$ RNAs (2 μg in lanes a, c, and d) or total cellular RNA (20 μg in lane b) were denatured, subjected to electrophoresis on a 1.2% agarose gel containing 2.2 M formaldehyde, blotted onto a nitrocellulose filter, and hybridized with the ^{32}P-labeled cDNA clone of human c-*raf*-1. Lanes: a, human embryo fibroblast RNA; b, GL-5-JCK glioblastoma RNA; c, GL-5-JCK-derived secondary transformant S1-3 RNA; d, NIH 3T3 RNA. The positions of 28S and 18S rRNAs are indicated.

Analysis of *raf*-related transcripts in the GL-5-JCK-derived transformant. Northern blotting was used to examine the transcription of the *raf*-related transforming DNA. RNAs prepared from the secondary transformant S1-3, human embryo fibroblasts, the GL-5-JCK glioblastoma line, and NIH 3T3 cells were analyzed by using the cDNA clone of human c-*raf*-1 as a probe (Fig. 3). In human embryo fibroblasts and the GL-5-JCK glioblastoma line, a single transcript of 3.5 kb was detected (Fig. 3, lanes a and b). In the transformant, however, 5.3-, 4.8-, and 2.5-kb transcripts were detected, in addition to the 3.3-kb transcript of mouse c-*raf* (Fig. 3, lane c). These 5.3-, 4.8-, and 2.5-kb transcripts with homology to human c-*raf*-1 were thought to be transcribed from the *raf*-related transforming DNA. The 2.5-kb transcript was overexpressed much more than the 5.3- and 4.8-kb transcripts in the transformant.

Detection of a protein specifically expressed in GL-5-JCK-derived transformants. The *raf*-related transforming DNA should produce a specific protein that is responsible for the transformation of NIH 3T3 cells. We irradiated the secondary transformant S1-3 cells with γ-rays to reduce their viability and cause loss of their tumorigenicity. We then repeatedly injected these irradiated cells into NFS mice and prepared serum samples from the mice, which had no tumors. Serum samples from 10 animals were pooled and used for the next set of experiments. A series of GL-5-JCK-derived transformants containing the *raf*-related transforming DNA and a variety of NIH 3T3 transformants containing other transforming DNAs were tested by immunoprecipitation for expression of a specific protein (Fig. 4). The 44-kilodalton (kDa) protein was detected in all transformants derived from GL-5-JCK glioblastoma DNA (Fig. 4, lanes a

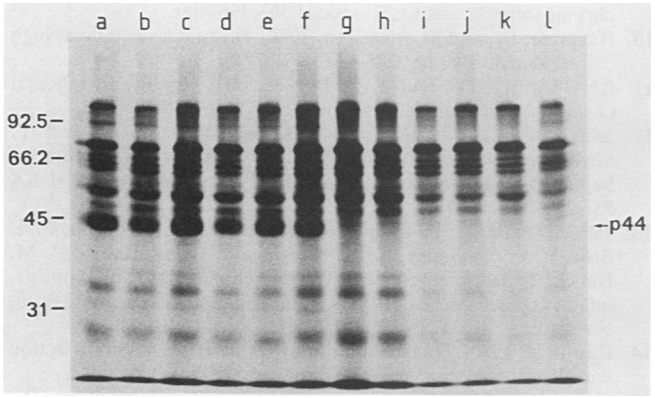

FIG. 4. Detection of a 44-kDa protein in NIH 3T3 transformants derived from GL-5-JCK glioblastoma DNA transfection. [^{35}S]methionine-labeled cell extracts were immunoprecipitated with sera raised against the GL-5-JCK-derived secondary transformant S1-3 and analyzed by electrophoresis on 10% SDS-polyacrylamide gel. Lanes: a, a primary transformant derived from GL-5-JCK DNA; b, a secondary transformant S1-3 derived from GL-5-JCK DNA; c to f, independent tertiary transformants derived from GL-5-JCK DNA; g, NIH 3T3 cells; h, a primary transformant derived from FH human glioblastoma DNA; i, a primary transformant derived from NB-1-JCK human Ewing sarcoma DNA; j, a primary transformant derived from LC-12-JCK human lung carcinoma DNA; k, a primary transformant derived from HL-60 human promyelocytic leukemia DNA; 1, a primary transformant derived from transfection with the complete Harvey murine sarcoma virus clone pH-1. Coelectrophoresed molecular weight markers were as follows (10^3): phosphorylase B, 92.5; bovine serum albumin, 66.2; ovalbumin, 45; carbonic anhydrase, 31.

to f), but not in normal NIH 3T3 cells (Fig. 4, lane g) or other NIH 3T3 transformants (Fig. 4, lanes h to l). The 44-kDa protein was specifically expressed in the transformants containing the *raf*-related transforming DNA, and so it seemed to be induced by the transforming DNA. To examine whether the 44-kDa protein was *raf* related, a *raf*-specific antibody was obtained from U. R. Rapp, and similar immunoprecipitation analyses of the transformants were carried out by using the antibody. The antibody used was raised against a synthetic peptide that is specific for the carboxy terminus of c-*raf*-1 (28). The 44-kDa protein was again specifically detected in the transformants containing the *raf*-related transforming DNA (data not shown) and was confirmed to be encoded by the transforming DNA.

DISCUSSION

Molecular cloning and structural analyses of the *raf*-related transforming DNA derived from GL-5-JCK glioblastoma DNA transfection revealed that the transforming DNA consists of three portions of human DNA sequences, with the 3′ half of the c-*raf*-1 gene as its middle portion. The *raf* region was estimated to be about 20 kb long and to contain exons 8 to 17 and the site of the poly(A) signal. The *raf*-related transforming DNA was transcribed into three kinds of mRNA (5.3, 4.8, and 2.5 kb). The 2.5-kb transcript was much more abundant than the other two transcripts. The 5.3- and 4.8-kb mRNA might be immature transcripts containing introns. Immunoprecipitation analysis revealed that a 44-kDa *raf*-related protein was specifically expressed in NIH 3T3 transformants containing the *raf*-related transforming DNA. The human DNA sequence located upstream of the *raf* region was expected to provide the transforming DNA with a transcriptional promoter and coding sequences, if any. In fact, one of the cDNA clones of the transforming DNA contained a sequence encoding 16 amino acids on its 5′ end, in addition to the sequence corresponding to exons 8 to 17 of the c-*raf*-1 gene (unpublished data). A human DNA sequence was also present downstream of the *raf* region of the transforming DNA. The 4.2- and 1.2-kb *Alu*-containing *Eco*RI fragments were detected in all six tertiary transformants that were derived from the secondary transformant S1-3 DNA (data not shown). The presence of the human DNA sequence downstream of the *raf* gene region did not seem to be a coincidence. This human DNA sequence may play some essential role in the activation of the transforming DNA, such as in transcriptional enhancement.

Six examples of activated *raf* genes have been reported. In murine retrovirus 3611-MSV, mouse c-*raf* is activated by truncation of its amino-terminal sequence and its fusion with a viral sequence containing a potent transcriptional promoter (3). An avian homolog of the *raf* gene in the MH2 virus (v-*mil*) was thought to be activated in a similar manner (32). Activation of mouse c-*raf* by retroviral promoter insertion has also been reported (long terminal repeat-activated c-*raf*) (19). In addition, two activated forms of human c-*raf*-1 and one activated form of rat c-*raf* were detected by NIH 3T3 transformation assays with DNAs from a human glioblastoma line (8), a human stomach tumor (30), and a rat hepatocellular carcinoma (12). As suggested by Bonner et al. (4), all six activated *raf* genes appeared to be truncated at their amino termini. The amino termini of the truncated *raf* genes varied, but were estimated to be confined to the region in or around exons 6 to 9. Nucleotide differences were present between v-*raf* sequences and human c-*raf*-1 sequences. However, the nucleotide changes of v-*raf* were demonstrated not to be essential for its transforming ability (3), in contrast to the cases with *ras* genes. The translational product of our *raf*-related transforming DNA was expected to be a hybrid protein, like those of the v-*raf* and v-*mil* genes. On the other hand, the translational product of long terminal repeat-activated c-*raf* was not a hybrid protein but a truncated *raf* protein (19). c-*raf* may be activated by truncation of only limited amino-terminal sequences and combination with a potent transcriptional promoter.

Our *raf*-related transforming DNA was derived from GL-5-JCK human glioblastoma DNA. However, the rearrangement of c-*raf*-1 that was present in the transforming DNA was not detectable in the original GL-5-JCK tumor DNA. Thus, rearrangement of c-*raf*-1 might occur during the process of transfection of the tumor DNA. Alternatively, the genetic alteration might be present in a small population of the original tumor cells as a result of tumor progression. The observation that only one of the three primary transformants derived from GL-5-JCK glioblastoma DNA contained the *raf*-related transforming DNA supports both possibilities. Recently, two transforming genes, *ret* (35) and rat c-*raf* (12), have been reported to be activated by DNA rearrangement. As in the case of the *raf*-related transforming DNA described here, the genetic alterations of the two transforming genes were not detected in the original tumor cells.

Cooper et al. (6) reported that sonicated DNA fragments (0.5 to 4.5 kb) of normal mouse and chicken cells induce transformation of NIH 3T3 cells at a low efficiency and that high-molecular-weight DNAs from the transformants can transform NIH 3T3 cells at high efficiency in the second-cycle transfection. Schäfer et al. (26) obtained similar results in studies on transfection by using sonicated DNA fragments of normal human lymphocytes. They confirmed the presence

of human DNA sequences containing *Alu* in the transformants. These results suggest that potential transforming genes can be activated by DNA rearrangement during transfection. It should be remembered, however, that there have been no reports on the detection of transforming genes by transfection with normal high-molecular-weight DNAs, although these DNAs are usually used as controls in transfection experiments.

In the case of *ras* genes, there are some examples of activation during tumor progression. Of five human melanoma cell lines that were derived from separate metastatic deposits of a single patient, only one contained an activated N-*ras* gene (1). An activated Ki-*ras* gene was demonstrated in a metastatic variant of a mouse T-lymphoma cell line, but not in the parental cell line (38). An activated N-*ras* gene was detected in a human teratocarcinoma cell line at late but not early passages (34). Tumor cells are so-called hypermutable cells and are thought to have various genetic rearrangements. Potential transforming genes may be involved in such rearrangements and may be activated in some cases. If the tumor cells that acquire a transforming gene proliferate predominantly, their total population in the tumor will gradually increase. Thus, we cannot exclude the possibility that the *raf*-related transforming DNA is activated during tumor progression.

ACKNOWLEDGMENTS

We thank U. R. Rapp for the cDNA clone of human c-*raf*-1 and the antibody specific for the carboxy terminus of c-*raf*-1.

This study was supported by Grants-in-Aid for Special Project Research, Cancer-Bioscience, from the Ministry of Education, Science and Culture of Japan.

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