TRANSFORMING GENES IN HUMAN LEUKEMIA CELLS

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High-molecular weight DNAs of fresh bone marrow cells from 32 patients with fresh leukemia were assayed for the presence of transmissible activated transforming genes by a DNA-mediated gene transfer technique using NIH/3T3 cells. DNAs of bone marrow cells from four of the 32 patients induced transformation of NIH/3T3 cells. Two of the four cases, a chronic myelogenous leukemia and an acute lymphocytic leukemia, contained activated N-ras oncogenes. Molecular cloning and nucleotide sequence analysis revealed that the lesion responsible for the transforming activity was localized to a single nucleotide transition from guanine to thymine in codon 12 of the predicted protein in each of the two cases. These observations indicate that activation of N-ras oncogenes is independent of the specific stage of cell differentiation or the leukemia phenotype. The other two transforming genes associated with an acute myelogenous leukemia and an acute lymphocytic leukemia showed homology neither with members of the ras gene family nor with the human Blym-1 gene. Thus, the NIH/3T3 transfection assay frequently detects activated N-ras oncogenes in human leukemias, while other transforming genes, distinct from the ras gene family, can be detected in some leukemias by the transfection assay.

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MATERIALS AND METHODS

Cells. Fresh primary human leukemia cells were prepared from heparinized marrow from 32 leukemia patients: eight patients with acute myelogenous leukemia (AML), two patients with acute promyelocytic leukemia (APL), two patients with acute monocytic leukemia (AMoL), eight patients with Ph1-positive chronic myelogenous leukemia (CML) in the chronic phase, two patients with CML in the blastic phase, eight patients with acute lymphocytic leukemia (ALL), and two patients with chronic lymphocytic leukemia (CLL). The normal marrow cells from eight healthy volunteers were obtained with informed consent and were examined as normal controls.

Transfection assay. High-molecular weight DNAs were extracted fromuffy coat cells of heparinized marrow from the patients and the normal subjects. The transforming activity of high-molecular weight DNAs was assayed by transfection of NIH/3T3 cells as described. 5 Briefly, NIH/3T3 cells were plated at a density of 2 x 10^3 cells per 60-mm culture dish in Dulbecco’s modified Eagle’s medium supplemented with 10% calf serum. After incubation at 37°C overnight, the cells were inoculated with DNA using a previously described modification 6 of the calcium phosphate co-precipitation method originally described by Graham and van der Eb. 7 DNAs were precipitated at a final concentration of 40 µg/mL. The recipient culture was inoculated with 0.5 mL of DNA precipitate without removing the culture medium and incubated for eight to 12 hours at 37°C. Media were changed at two-to-three-day intervals and foci of transformed cells were counted 13 to 15 days after exposure to DNA. One hundred micrograms of each DNA sample was assayed on five recipient cultures in an experiment. Three experiments were performed for each DNA sample. SV40 and v-H-ras DNAs were also included as positive controls in all assays. The clonal transformed cells (primary transformants) were grown to mass culture and their DNAs were used in a subsequent cycle of transfection assay. Tumorigenicity of transformant clones was determined by injecting subcutaneously 1 to 2 x 10^6 cells into female congenitally athymic nude mice (BALB/c-nu/nu mice, 7 weeks old, background congenitally athymic).

Analysis of DNA from transformed cells. DNAs isolated from representative clones were subjected to Southern blot analysis for detection of human repetitive sequences and human proto-oncogene sequences. Twenty micrograms of high-molecular weight DNAs was digested with appropriate restriction endonucleases, electrophoresed on agarose gels, and blotted to nitrocellulose filters. The resulting blots were hybridized with a nick-translated 32P-labeled DNA probe in a mixture containing 50% formamide, 0.2% polyvinyl-pyrrolidone, 0.2% bovine serum albumin, 0.2% Ficoll, 0.05 mol/l. Tris-HCl (pH 7.5), 1 mol/l NaCl, 0.1% sodium pyrophosphate, 0.1% sodium dodecyl sulfate (SDS), 10% dextran sulfate, and denatured salmon sperm DNA (100 µg/mL) for 24 hours at 42°C. After sequential washing with 0.2 x SSC (1 x SSC is 0.15 mol/l NaCl, 0.015 mol/l sodium citrate) in 0.1% SDS at 42°C, Hybridized DNA was revealed by autoradiography.

Molecular cloning. DNAs from NIH/3T3 transformants 29L-6-4 and 31L2-3 were digested to completion with restriction enzymes.}

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endonuclease EcoRI, and fragments of different sizes were fractionated by agarose gel electrophoresis and purified by the electrophoretic method. The DNA fractions hybridized to each N-ras probe for 9.2-kb or 7.0-kb EcoRI fragments were separately collected. DNA of the kg1WES-AB phage vector was digested with EcoRI and purified by preparative sucrose density gradient centrifugation. Purified phage arms and cellular DNA fragments were ligated at a 1:1 molar ratio by T4 DNA ligase at 14°C for 16 hours and packaged in vitro into phage particles. The phages were screened for N-ras sequence by in situ hybridization of phage plaques with each N-ras probe. Recombinant DNA experiments were conducted under EKI/P2 containment conditions as specified by the National Institutes of Health guidelines.

**Chimeric gene constructions.** The 9.2-kb and 7.0-kb EcoRI fragments were separated from normal N-ras clones, ACM7, ACM9, ACM7, AAL9, and AAL7. Chimeric gene constructions between 9.2-kb and 7.0-kb EcoRI fragments were performed using T4 DNA ligase. To assess the biological activity of the genes, the resulting concatamers were tested for their ability to transform NIH/3T3 cells.

**Nucleotide sequence analysis.** Sequences were determined by the chain terminator method after subcloning suitable restriction enzyme-generated DNA fragments into phage M13 mp10 and mp11 replicative form DNAs. Single-strand DNAs from recombinant phages were used as templates. DNA synthesis was carried out using DNA polymerase 1 (Klenow fragment) and a synthetic oligonucleotide primer.

**Probes.** Human repetitive sequences were analyzed using a probe prepared from plasmid Blur-8, kindly provided by T. Friedmann (University of California, California). Probes specific for v-H-ras, v-K-ras, and Blym-1 were generously provided by E.M. Scolnick (Merck & Dohme Research Laboratories, Pennsylvania) and G.M. Cooper (Dana-Farber Cancer Institute, Massachusetts). Each N-ras probe specific for 9.2-kb or 7.0-kb EcoRI fragments (Fig 2) was isolated from normal N-ras clones kindly provided by S.A. Aaroson (National Institute of Health, Maryland).

**RESULTS**

**Transforming activity of human leukemia cell DNAs.** High-molecular weight DNAs of bone marrow cells from 32 leukemia patients and eight normal volunteers were subjected to transfection analysis utilizing NIH/3T3 cells. A DNA sample exhibiting transforming activity of >0.01 foci per microgram of DNA in both primary and secondary transfection assays was evaluated as “positive by transfection assay.” DNAs from AML-1, CML-1, ALL-1 and ALL-2 exhibited positive transforming activity (Tables 1 and 2). The leukemia cells of the two ALL patients positive by transfection assay were of intermediate T cell phenotype. The DNAs of marrow cells from the other leukemia patients or from normal subjects did not exhibit significant transforming activity (<0.01 foci per microgram of DNA) (Table 2). Individual primary transfectants were grown to mass culture and used as donors of DNA in secondary transfection assays. These DNAs all induced transformation of NIH/3T3 cells with higher efficiencies (Table 2), indicating that these DNAs contained transmissible and dominant activated transforming genes. All of the primary and secondary transformants obtained were transplantable into nude mice.

**Identification of the oncogenes in CML-1 and ALL-1.** In order to confirm that the transforming activity was due to human DNA sequences, DNAs from primary and secondary transformants of CML-1 and ALL-1 were analyzed by Southern blotting for the presence of human repetitive sequences. In primary transformants, a large number of DNA fragments was detected that hybridized to the probe Blur-8, representative of human Alu sequences (Fig 1A and B). Secondary transformants retained relatively few Alu-related fragments. As shown in Fig 1A and B, 9.2-kb and 7.0-kb conserved EcoRI bands appeared in the DNAs from secondary transformants of CML-1 and ALL-1. These results indicated that transformation of NIH/3T3 cells was correlated with the transfer of human DNA.

In view of evidence relating transforming genes of a number of human tumors to ras genes, we analyzed the transformant DNAs of CML-1 and ALL-1 for sequences homologous to these onc genes. Neither H-ras nor K-ras probes detected any fragments other than their endogenous mouse related fragments (data not shown). The N-ras probe, a mixture of probes A and B in Fig 2, specifically detected the presence of human N-ras EcoRI fragments of 9.2-kb and 7.0-kb in transformant DNAs of CML-1 and ALL-1 (Fig 3).
Fig 1. Identification of the oncogenes in CML-1 and ALL-1 by Southern blot analysis. Twenty micrograms of high-molecular weight DNA was digested with EcoRI, electrophoresed, blotted, and hybridized (A and B) to ^32P-labeled human repetitive DNA fragments purified from Blur-8 or (C) to ^32P-labeled N-ras-specific DNA fragments (a mixture of probes A and B in Fig 2). Lanes a, g, and m, NIH/3T3 DNA; lanes b (31L2-1), c (31L2-3), and n (31L2-3), CML-1 NIH/3T3 primary transformant DNAs; lanes d (33L3-2), e (33L3-3), f (33L3-4), and o (33L3-2), CML-1 NIH/3T3 secondary transformant DNAs; lanes h (29L6-3), i (29L6-4), and p (29L6-4), ALL-1 NIH/3T3 primary transformant DNAs; lanes j (35L3-4), k (35L3-5), l (35L3-8), and q (35L3-4), ALL-1 NIH/3T3 secondary transformant DNAs; lane r, normal human cellular DNA.

Only a 7.4-kb mouse cell specific band was detected in NIH/3T3 cell DNA digested with EcoRI. The present results demonstrate that the transforming genes observed in CML-1 and ALL-1 were identified as the activated N-ras genes.

Molecular cloning of the oncogenes of CML-1 and ALL-1. For characterization of the N-ras oncogenes in CML-1 and ALL-1, we cloned these sequences from primary NIH/3T3 transformants designated 31L2-3 and 29L6-4. EcoRI-digested DNAs of these transformants showed 9.2-kb and 7.0-kb fragments with probes A and B, respectively (data not shown). The transformant DNAs digested completely with EcoRI were fractioned by agarose gel electrophoresis. Fractions that hybridized with probe A or B were ligated individually to the EcoRI arms of AgtWES-XB DNA and packaged in vitro. For each fragment from 500,000 plaques, over ten positive clones were obtained using probe A or B. For confirmation that the cloned DNAs carried the fragments of the N-ras gene, they were digested with EcoRI and subjected to Southern blot analysis using probes A and B. Clones designated XCM9 and XCM7 were cloned from 31L2-3 DNA, and clones XAL9 and XAL7 were obtained from 29L6-4 DNA. XCM9 and XAL9 hybridized with probe A, and XCM7 and XAL7 hybridized with probe B (data not shown). The physical restriction map of each N-ras gene cloned was identical with that of the normal human N-ras gene5-8 (Fig 2).

Biological activity of the cloned oncogenes. To determine the location of the transforming activity of the cloned N-ras oncogenes, chimeric genes were constructed and tested for their ability to transform NIH/3T3 cells. Cloned 9.2-kb and 7.0-kb EcoRI fragments of each transforming N-ras gene were ligated to 7.0-kb and 9.2-kb EcoRI fragments of normal N-ras gene, respectively, and the resultant

Fig 2. Cloned fragments (lower portion), restriction map (middle portion), and sequencing strategy (upper portion) for the N-ras genes of CML-1 and ALL-1 NIH/3T3 transformants. XCM9 and XAL9 were cloned using probe A (a 0.9-kb PvuII fragment), and XCM7 and XAL7 were obtained using probe B (a 1.5-kb PvuII fragment). Coding exons are indicated as solid blocks. Restriction endonuclease sites are as follows: E, EcoRI; H, Hind III; P, PvuII; Ps, PsI; S, SstI. The map is not complete for the restriction sites that are underlined. The three small DNA fragments containing exons 1 and 2 were sequenced by the chain terminator method.
Table 3. Transforming Activity of Ligated N-ras Fragments

<table>
<thead>
<tr>
<th>Source of 9.2-kb EcoRI Fragment</th>
<th>Source of 7.0-kb EcoRI Fragment</th>
<th>Transforming Activity (No. of Foci/μg DNA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal N-ras</td>
<td>Normal N-ras</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Normal N-ras</td>
<td>λAL7</td>
<td>47</td>
</tr>
<tr>
<td>Normal N-ras</td>
<td>λAL7</td>
<td>55</td>
</tr>
<tr>
<td>Normal N-ras</td>
<td>λCM7</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>λCM9</td>
<td>Normal N-ras</td>
<td>61</td>
</tr>
<tr>
<td>λCM9</td>
<td>λCM7</td>
<td>48</td>
</tr>
</tbody>
</table>

Chimeric gene constructions between 9.2-kb EcoRI fragments and 7.0-kb EcoRI fragments were made using T4 DNA ligase and gel-purified EcoRI fragments from the normal N-ras gene, λAL9, λAL7, λCM9, and λCM7. NIH/3T3 transfection assays were performed using 1 μg of each construct per 10⁶ NIH/3T3 cells.

Transforming activity was compared with the reconstituted normal or activated N-ras gene. As shown in Table 3, the chimeric genes containing the 9.2-kb fragment of the N-ras gene of CML-i or ALL-i were active, whereas those containing the normal 9.2-kb fragment did not show detectable transforming activity. Thus, the lesions responsible for activation of these N-ras genes could be localized to their 9.2-kb EcoRI fragments.

Identification of the lesions responsible for activation of the CML-i and ALL-i oncogenes. The 9.2-kb EcoRI fragment of the N-ras gene contains the first and second exons of the p21 coding sequence. To localize the lesions responsible for activation of the CML-1 and ALL-1 oncogenes, the DNA sequence and predicted amino acid sequence of the entire first and second exons of the N-ras oncogenes in CML-1 and ALL-1 were analyzed. The complete nucleotide sequence of both exons was identical between the N-ras genes in CML-1 and ALL-1. A single base change (G to T) and the consequent amino acid change of glycine to cysteine were observed. The known RNA splice sites are marked by arrows.

![Fig 3. Autoradiograms of sequencing gels containing the lesion of codon 12 in the first exon of the CML-1 N-ras gene. Plus (+) and minus (−) strands were sequenced by the chain terminator method. The single nucleotide transition in codon 12 is represented by arrows.](image)

![Fig 4. The DNA sequence and predicted amino acid sequence of the entire first and second exons of the N-ras oncogenes in CML-i and ALL-i. The complete nucleotide sequence of both exons was identical between the N-ras genes in CML-1 and ALL-1. A single base change (G to T) and the consequent amino acid change of glycine to cysteine are boxed. The known RNA splice sites are marked by arrows.](image)
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Fig 5. Southern blot analysis of human repetitive sequences in NIH/3T3 transformants derived from AML-1 and ALL-2. Twenty micrograms of high-molecular weight DNA was digested with EcoRI, electrophoresed, blotted, and hybridized to a Blur-8 probe. Lanes a, d, and g, NIH/3T3 primary transformant (29L7-5) DNA; lane c, AML-1 NIH/3T3 primary transformant (32L7-3) DNA; lanes e and f, ALL-2 NIH/3T3 secondary transformant DNAs; lanes h and i, AML-1 NIH/3T3 secondary transformant DNAs.

Fig 6. Southern blot analysis of ras genes in AML-1 and ALL-2 NIH/3T3 transformants. Twenty micrograms of high-molecular weight DNA was digested with EcoRI, electrophoresed, blotted, and hybridized (A) to an H-ras-specific probe, (B) to a K-ras-specific probe, or (C) to an N-ras-specific probe (a mixture of probes A and B in Fig 2). Lanes a, e, and i, normal human cellular DNA; lanes b, f, and j, ALL-2 NIH/3T3 primary transformant (29L7-5) DNA; lanes c, g, and k, AML-1 NIH/3T3 primary transformant (32L7-3) DNA; lanes d, h, and i, NIH/3T3 DNA.

genes, we determined the nucleotide sequence of their first and second exons (Fig 2). Comparison of their sequence with that of the normal N-ras gene revealed that both CML-1 and ALL-1 N-ras oncogenes were activated by the same single point mutation at position 34, which corresponds to the first base of codon 12 of the predicted amino acid sequence (Figs 3 and 4). The transversion of guanine to thymine results in the substitution of cysteine for glycine at codon 12 of the predicted p21 protein (Figs 3 and 4). From these observations, we conclude that the single nucleotide substitution has altered the product of an N-ras gene, thereby activating its transforming potential in either case.

Analysis of the AML-1 and ALL-2 oncogenes. Primary and secondary transformants of AML-1 and ALL-2 were transplantable into nude mice. DNAs from primary and secondary transformants of AML-1 and ALL-2 were analyzed by Southern blotting for human repetitive sequences using Blur-8. Positive results were obtained for primary transformants 32L7-3 and 29L7-5 derived from AML-1 and ALL-2 DNAs, respectively, and for secondary transformants derived from 32L7-3 and 29L7-5 DNAs (Fig 5). Secondary transformants of ALL-2 retained only a common fragment that hybridized to the Blur-8 probe (Fig 5B). In secondary transformants of AML-1, a few common Alu-related bands were conserved (Fig 5C). DNAs from 32L7-3 and 29L7-5 were further analyzed by Southern blotting and hybridization to probes for v-H-ras, v-K-ras, N-ras (Fig 6) and Blym-1 (data not shown). In each case, the hybridization pattern obtained was identical to that of normal NIH/3T3 DNA under the standard stringency conditions described, indicating that these transformants contain none of human c-H-ras, c-K-ras, N-ras, and Blym-1 sequences.
A number of human tumors and tumor cell lines have been shown to contain oncogenes capable of transforming NIH/3T3 cells. In the present study, 12.5% of fresh human leukemia cells (four of 32 cases) were found to contain transforming genes. The majority of these oncogenes thus far reported have been identified as members of the human ras gene family, which is a family of evolutionarily well-conserved genes in eukaryotes encoding immunologically related p21 proteins. Although the normal function of these proteins in physiologic conditions is unknown, the genetic alteration that the ras proto-oncogenes undergo in these tumors confers oncogenic capabilities to the ras p21 proteins in the NIH/3T3 assay. In human hematopoietic tumors, all of the oncogenes thus far detected have been identified as N-ras genes by using the NIH/3T3 assay, except that K-ras and Blymph-1 oncogenes have been detected in only a T-ALL cell line and a few cases of Burkitt’s lymphomas, respectively. Moreover, activated N-ras oncogenes can be frequently detected in lymphocytic leukemia cells that have an intermediate T cell phenotype. In our studies, N-ras oncogenes were activated in two of four leukemias that were identified as positive in NIH/3T3 transfection assay, and the transforming genes of the other two leukemias were not members of the ras gene family.

In all of the cases thus far examined, activation of a human cellular ras gene depends on a point mutation in either codon 12 or 61 of the gene, resulting in a single amino acid substitution in the ras protein it encodes. The random in vitro mutagenesis of a cloned wild-type human H-ras gene was performed by using sodium bisulphite, and the resulting mutants were tested for transforming activity in NIH/3T3 cells. DNA sequencing of the transforming mutants revealed three new targets for activation (codons 13, 59, and 63) flanking codon 12 and 61. What is not clear is why ras oncogenes with mutants other than in codons 12 and 61 have not yet been detected in tumor cells. The mutations leading to the activation of N-ras oncogenes thus far analyzed in human tumors are shown in Table 4. Of these, the mutations observed in our two cases of human leukemias were localized in codon 12. These findings demonstrate that the mechanism of activation of N-ras genes in leukemias is similar to that in neoplastic solid tumors. The reports that activated N-ras genes have been detected not only in neuroblastoma and lung carcinoma cell lines but frequently in human leukemias as well, and our observations that the mutation of the N-ras oncogene in CML-1 is the same as that in ALL-1, indicate that activated N-ras genes determine neither tissue types of tumors nor phenotypes of leukemias. Accordingly, the differences of the levels in proliferation and the stages in differentiation of leukemia cells between CML and ALL might depend on causes other than ras genes.

In experimental animals, rat mammary carcinomas induced by nitrosomethylurea and mouse skin papillomas induced by dimethylbenzanthracene and 12-O-tetradecanoylphorbol-13-acetate contained activated c-H-ras genes. Furthermore, there appears to be selectivity in the activated gene because mouse lymphomas induced by nitrosomethylurea have activated the N-ras gene, and those induced by radiation have activated the K-ras gene. The reproducible detection of specific transforming genes in animal model systems strongly suggests that oncogenes must play a significant role in the development of those tumors in which they have been detected. If these demonstrations apply to human leukemias, the N-ras oncogene may play an important role in human leukemogenesis.

Recently, human H-ras mutants were constructed which specified all 20 possible amino acids at position 12 of p21. The degree of transformation induced by each of the mutants was highly variable both in morphology of transformed cells and in transforming efficiencies. These findings indicate that we may thus far have underestimated the proportion of tumors containing activated ras genes. Furthermore, new transforming genes, distinct from the ras genes reported, may be detected by analyzing the transfected NIH/3T3 cells that do not display a striking change in morphology. The secondary transformants of AML-1 and ALL-2 retain conserved Aftu-related fragments. These observations suggest that NIH/3T3 transformation by AML-1 and ALL-2 DNAs is associated with the transmissible and activated transforming sequences. The transformants of AML-1 and ALL-2 contain no human cellular ras genes. However, whether these transforming genes are novel is a question that must await molecular cloning and sequence analysis.

Acknowledgment

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References


Table 4. Mutations Leading to the Activation of N-ras

<table>
<thead>
<tr>
<th>Origin</th>
<th>Codon Affected</th>
<th>Base Change</th>
<th>Amino Acid Change</th>
<th>Reference</th>
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<tbody>
<tr>
<td>Neuroblastoma (SK-N-SH)*</td>
<td>61</td>
<td>C → A</td>
<td>Gin → Lys</td>
<td>36</td>
</tr>
<tr>
<td>Lung carcinoma (SW-1271)*</td>
<td>61</td>
<td>A → G</td>
<td>Gin → Arg</td>
<td>25</td>
</tr>
<tr>
<td>Teratocarcinoma (PA-1)*</td>
<td>12</td>
<td>G → A</td>
<td>Gly → Asp</td>
<td>24</td>
</tr>
<tr>
<td>Fibrosarcoma (HT1080)*</td>
<td>61</td>
<td>C → A</td>
<td>Gin → Lys</td>
<td>42</td>
</tr>
<tr>
<td>CML††</td>
<td>12</td>
<td>G → T</td>
<td>Gly → Cys</td>
<td>Present study</td>
</tr>
<tr>
<td>T-ALL††</td>
<td>12</td>
<td>G → T</td>
<td>Gly → Cys</td>
<td>Present study</td>
</tr>
</tbody>
</table>

*Cell lines.
††Patients.
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Transforming genes in human leukemia cells

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