Acquisition of Interleukin-3 Independence in FDC-P2 Cells After Transfection With the Activated c-H-ras Gene Using a Bovine Papillomavirus-Based Plasmid Vector

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Since the ras family of proto-oncogenes is supposed to be involved in leukemogenesis by point-mutational activation, we studied the effect of the activated ras gene on the growth of a murine interleukin-3 (IL-3)-dependent cell line, FDC-P2. The human activated c-H-ras gene was transfected into FDC-P2 cells by electroporation using a high-level expression vector, BMGhph, which contains a partial DNA sequence of a murine interleukin-3 (IL-3) dependent cell line, FDC-P2. The activated c-H-ras gene was introduced into a factor-dependent hematopoietic cell line, FDC-P2, using the high-expression vector, BMGhph, which contains a sequence from bovine papillomavirus (BPV) and a hygromycin B (hmB)-resistant gene as a selectable marker. The transformed FDC-P2 cells showed a high incidence of IL-3-independent growth and tumorigenicity in nude mice. These clones did not express or secrete IL-3, suggesting the acquisition of IL-3 independence by a nonautocrine mechanism. The high incidence of autonomous growth may be due to the use of the BMG vector, because (1) the activated ras gene in pBR322 vector (pHs-49) was not so efficient in the induction of IL-3 independence, (2) the c-H-ras genome copies per cell increased in number up to about 50 copies by using the BMG vector, and (3) cotransfection with the activated ras gene and the BPV gene in separate plasmids partly enhanced the incidence of autonomous growth without increasing the copy number of the ras gene compared with transfection with the activated ras gene alone. The present study supports the idea that the activation of ras gene is an important step in malignant transformation of hematopoietic cells and suggests that the BPV gene products may cooperate with ras gene activation probably by affecting the cellular genes that may be involved in multistep leukemogenesis. The BMG vector may be useful to test the transforming ability of oncogenes whose oncogenic potential is relatively low.

LEUKEMOGENESIS is supposed to be the result of a multistep process that may include activation of proto-oncogene(s) and/or inactivation of tumor suppressor gene(s). The transforming ability of various activated oncogenes has been studied by means of DNA transfection into NIH3T3 cells. However, it is difficult to transform normal hematopoietic cells by introducing activated oncogenes, because leukemic transformation is the consequence of accumulation of more than one genetic alteration. Thus, factor-dependent hematopoietic cell lines have been used as target cells of DNA transfection and the effects on factor dependence and tumorigenicity have been examined. The introduction of several activated oncogenes such as v-abl, v-fms, v-src, and v-myc efficiently induces autonomous growth of factor-dependent cell lines. On the other hand, when the activated ras gene was transfected into factor-dependent cell lines, the efficiency of induction of factor-independent growth was relatively low, despite the facts that point mutations of the ras family of proto-oncogenes often occur in leukemic cells, and that the transforming activity is high when NIH3T3 cells are used as target cells. Recently, it was demonstrated that overexpression of activated ras gene in factor-dependent cells induced a high incidence of autonomous growth. In the present study, therefore, we introduced the activated c-H-ras gene into a murine bone marrow–derived interleukin-3 (IL-3)–dependent cell line, FDC-P2, using the high-expression vector, BMGhph, which contains a sequence from bovine papillomavirus (BPV). As a result, IL-3–independent clones were generated at a high incidence in the activated ras–introduced FDC-P2 cells without evidence of an autocrine mechanism, and these clones were apparently tumorigenic in nude mice. Subsequent cotransfection experiments suggested that the BMGhph vector contributed to the efficient induction of factor independence by the activated ras gene.

MATERIALS AND METHODS

Cell lines and culture. FDC-P2" and 32DCl are IL-3-dependent myeloid cell lines established from mouse bone marrow. WEHI-3B is a mouse myelomonocytic leukemia cell line that secretes IL-3. The IL-3–dependent cell lines were maintained in Iscove’s modified Dulbecco’s medium (IMDM; GIBCO Laboratories, Grand Island, NY) containing 10% heat-inactivated fetal calf serum (FCS; GIBCO), 100 U of penicillin per milliliter, 100 μg of streptomycin per milliliter and 10% (optional mitogenic concentration) WEHI-3B cell-conditioned medium (WEHI-CM) as a source of IL-3 at 37°C in a humidified atmosphere with 5% CO2.

Plasmid construction. All enzymes and linkers used were purchased from Takara Shuzo Co (Kyoto, Japan). BMGneo is a complementary DNA (cDNA) expression vector containing 69% of the sequence of BPV DNA, including replication origin, an enhancer element, and open reading frames (ORFs) for E1 through E817 and a neomycin-resistance gene (Neo8) as a selectable marker, which has previously been described in detail. BMGhph was generated from BMGneo by replacing Neo8 with the hygromycin B (hmB) phosphotransferase (hph) gene derived from py3. In brief, the hph gene with a Moloney sarcoma virus long-terminal repeat (LTR) as a promoter was excised as an HindIII/KpnI fragment from py3, linked to 0.85 kb of simian vacuolating virus-40 (SV40) processing signal sequence, and then ligated at HindIII and XbaI sites of BMGneo after the removal of Neo8, thymidine kinase (TK) promoter, and TK poly(A) signal sequences. To construct BMGhpIL-3 (BMGL-3), a SpI 1/NcoI
fragment of mouse IL-3 cDNA (a gift from Dr Hiroshi Matsui, Ajinomoto Co, Kawasaki, Japan) with the Rous sarcoma virus LTR promoter was inserted between the Xba I and Sal I sites of BMGhph after the removal of the metallothionein promoter. BMGhphH-ras (BMGH-ras) was also constructed from BMGhph and pHs-49 (Japanese Cancer Research Resources Bank [JCRB]-Gene), which carries the human activated c-H-ras gene with a point mutation in the sixty-first codon (see Fig 2B). Briefly, 4.7 kb of the EcoRI/Sph I fragment of c-H-ras gene was excised from pHs-49, the 5’ end was blunted, and a Sal I linker was attached to the 3’ end. The fragment was inserted between the Xba I site (blunted) and the Sal I site of the BMGhph vector. The expression of c-H-ras gene in BMGH-ras is driven by its own promoter.

Transfection, selection, and cloning in methycellulose medium. The plasmids were introduced into FDC-P2 cells using electroporation. Twenty micrograms of plasmid DNA and 5 x 10^6 viable cells were combined in 0.4 mL of phosphate-buffered saline (PBS) in a disposable cuvette. The cell mixture was pulsed with a field of 300 V and a capacitance of 500 μF using a Gene Pulser apparatus (BioRad Laboratories, Richmond, CA), held at room temperature for 5 minutes, and then transferred to 20 mL of culture medium. After 48 hours, the cells were selected in medium containing 1.5 mg/mL of hmB (GIBCO) and IL-3 using multiwell culture plates (Falcon; Becton Dickinson Labware, Lincoln Park, NJ; approximately 2 x 10^5 cells/well). After an appropriate period, the number of wells in which hmB-resistant cells appeared was scored. The hmB-resistant cells in each well were divided into two wells, in one of which the cells were cultured in the absence of exogenous IL-3. The number of wells in which IL-3-independent cells appeared was scored. BMGH-ras–transformed IL-3–independent cells in some wells were cloned in methylcellulose medium using 35 mm tissue culture dishes (Falcon). Cells (4 x 10^6) were plated in 1 mL of IMDM containing 0.8% methylcellulose (Dow Chemical Co, Midland, MI), 20% FCS, and 10% WEHI-CM. Colonies were harvested with a micropipet (Gilson Medical Electronics, Villiers-Le-Bel, France) under an inverted microscope after 7 days of culture and expanded in suspension cultures in the absence of IL-3.

Growth kinetics study. Parent and transfected cells were washed three times in IMDM containing 10% FCS, adjusted to 1 x 10^5 cells/mL in complete IMDM with or without IL-3, and seeded in 24-well culture plates. Viable cells in triplicate cultures were periodically counted by trypan blue dye exclusion.

Assay for IL-3 in conditioned media. The IL-3 activity in the conditioned media of transformed cells (1 x 10^9/mL) was assayed using IL-3–dependent 32D cells. The 32D cells were washed, adjusted to 1 x 10^6 cells/200 μL/well in 96-well microtiter plates (Falcon), and cultured in the presence of serially diluted test samples for 18 hours. Subsequently, 1 μCi of [3H]thymidine (Amersham, Buckinghamshire, UK) was added to each well and cells were cultured for an additional 6 hours. Samples were harvested with an automated cell harvester onto filter paper and [3H]thymidine incorporation was estimated by a liquid scintillation counter.

Extraction and analysis of cellular DNA and RNA. DNA and RNA were extracted using guanidinium HCl from parent and transformed FDC-P2 cells. For Southern analysis, 10 μg of DNA was completely digested with BamHI or Xba I (Takara Shuzo Co, Kyoto, Japan), size fractionated by 0.8% agarose gel electrophoresis, and transferred to nylon membranes (GeneScreen-plus; New England Nuclear, Boston, MA) according to the manufacturer’s protocol. Prehybridization and hybridization were done in a solution containing 6x standard saline citrate (SSC), 0.5% sodium dodecyl sulfate (SDS), 5 x Denhardt’s solution, and 100 μg/mL denatured salmon sperm DNA at 65°C overnight. The c-H-ras probe was labeled with the [32P]dCTP (Amersham) using the Multiprime DNA-labeling system (Amersham). The filters were washed and autoradiographed with Fuji RX film (Fuji Photo Film Co, Kanagawa, Japan) at -70°C. To estimate the copy number of the activated c-H-ras gene per cell, a series of appropriate amounts of BMGH-ras plasmid was digested with BamHI and subjected to Southern blotting simultaneously. The amount of plasmid DNA per lane was calculated based on the size of BMGH-ras plasmid DNA (about 18.9 x 10^6 bp) and chromosomal DNA per cell (about 6 x 10^9 bp). For Northern blot analysis, 40 μg of total RNA was size fractionated on 1.5% agarose gels containing 2.2 mol/L formaldehyde. The RNA was transferred to a nylon membrane (GeneScreen-plus), then hybridized with a 32P-labeled IL-3 cDNA probe at 60°C. The RNase protection assay was performed according to the protocol of the Riboprobe system (Promega Co, Madison, WI). In brief, 50 μg of total RNA was hybridized with 32P-labeled antisense RNA generated by in vitro transcription using T7Z19R, which carries an 860-bp Sac I-Xba I fragment containing the first exon from the c-H-ras DNA. Hybridization was performed in a solution containing 80% deionized formamide, 40 mmol/L piperazine-N,N’-bis-2-ethane-sulfonic acid (PIPES), 0.4 mol/L NaCl, and 1 mmol/L EDTA, at 45°C overnight. The hybridized samples were then digested with 40 μg/mL of RNase A and 800 U/mL of RNase T1 for 60 minutes at 30°C, and the reaction was stopped by adding 20 μL of 10% SDS and 50 μg of proteinase K. After phenol/chloroform extraction and ethanol precipitation, protected RNA fragments were dissolved, denatured, and fractionated on a 5% polyacrylamide/urea sequencing gel, followed by autoradiography with Fuji RX film.

Tumorigenicity assay. Young adult BALB/c athymic nude mice were injected subcutaneously with 5 x 10^9 each of parent and transformed FDC-P2 cells and monitored for up to 12 weeks thereafter. Animals that formed tumors at the injection sites were killed. Cells from tumor tissue and bone marrow of some mice were cultured in vitro to examine IL-3 independence and its production.

Cotransfection. (1) Each plasmid combination, ie, 10 μg of BMGhph plus 10 μg of pUC19 as a mock plasmid, 10 μg of pHs-49 plus 10 μg of BMGhph, as well as 10 μg of BMGH-ras plus 10 μg of pUC19, were transfected into FDC-P2 cells with 1 μg of pRSVneo as a selectable marker gene. Transfection was performed using electroporation as described above. After selection in medium containing 1 mg/mL of G418 (GIBCO) using multiwell culture plates, the G418-resistant cells in each well were further tested for IL-3 independence. The number of wells in which G418-resistant cells appeared and the number of wells in which IL-3-independent cells appeared were then scored. (2) Each plasmid combination, that is, 40 μg of pUC19, 20 μg of BMGneo plus 20 μg of pUC19, 20 μg of pHs-49 plus 20 μg of pUC19, 20 μg of BMGneo, were transfected into FDC-P2 cells with 2 μg of pY3 as a selectable marker gene. After selection with hmB using multiwell culture plates, the hmB-resistant cells in each well were further tested for IL-3 independence. The number of wells in which hmB-resistant cells appeared and the number of wells in which IL-3-independent cells appeared were then scored.

RESULTS

Transfection of FDC-P2 cells with the activated c-H-ras gene using the BMG vector. BMGH-ras, BMGIL-3 (a positive control), or BMGhph vector plasmid (a negative control) was transfected into FDC-P2 cells by electroporation. HmB-resistant cells emerged at a high incidence after selection of the transfected cells using hmB (Table 1). Among them, IL-3–independent growth was observed in 56 of 69 wells for BMGH-ras, in 69 of 69 wells for BMGIL-3, and in 1 of 66 wells for the BMG vector. A similar result was
obtained in a separate experiment (data not shown). BMGhph-transfected IL-3–dependent cells (FD-BMG), BMGIL-3-transfected IL-3–independent cells (FI-IL-3), and BMGhRas-transfected IL-3–independent cells (FI-Ras) were harvested, cloned in methylcellulose culture, and studied as follows. The growth kinetics of parent and transformed FDC-P2 cells are shown in Fig 1. In the presence of IL-3, parent and all transformed cells grew at similar rates (doubling time, 10 to 11 hours). However, in its absence, their growth rates differed. Almost all parent and FD-BMG cells died within 2 days. On the other hand, FI-IL-3 cells grew in an identical fashion with that in the presence of exogenous IL-3, and FI-Ras (clone 1) grew more slowly (doubling time, 15 hours) (Fig 1).

**Southern analysis of FI-ras clones.** The presence of the human activated c-H-ras gene in FI-ras clones was demonstrated by Southern blotting (Fig 2A). DNA extracted from FI-ras cells was digested with BamHI or Xba I. The BMGhRas plasmid, which contains two BamHI sites and two Xba I sites (Fig 2B), was similarly digested to function as the standard. A c-H-ras DNA probe hybridized with the 5.9-kb fragment of the BamHI-digested BMGhRas plasmid and with the 2.2-kb and 16.8-kb fragments of the Xba I-digested plasmid. Southern analysis of BamHI- or Xba I-digested DNA from three FI-ras clones had similar profiles to the standard. Two distinct bands of about 17.0 and 2.2 kb were observed after Xba I digestion, which cut the c-H-ras gene internally. This finding is compatible with the concept that the BMGhRas plasmid replicates as an episome in transfected cells, although Hirt extraction will be required to confirm episomal replication of the plasmid. Moreover, comparison of the intensity of bands with that of the standard after BamHI digestion indicated that FI-ras clones 1 and 2 contained about 50 copies of the c-H-ras gene per cell, and that FI-ras clone 3 had a smaller number of copies of the ras gene.

**RNA studies of FI-ras clones.** The expression of c-H-ras gene in FI-ras clones was studied by the RNase protection assay (Fig 3). A 32P-labeled antisense RNA complementary to Sac I-Xba I fragment (860 bp) containing exon 1 of c-H-ras genomic DNA was used as a probe to protect the 150-base fragment (exon 1) of c-H-ras messenger RNA (mRNA). As shown in Fig 3A, a significant amount of c-H-ras mRNA was detected in FI-ras clones. The expression levels of c-H-ras mRNA in clones 1 and 2 were higher than that of clone 3, which correlates with the copy number of c-H-ras gene in each. As expected, the growth rate of clone 3 was lower than that of clone 1 (data not shown).

**Ability of IL-3 production of FI-ras clones.** To determine whether abrogation of IL-3 dependence after transfection with the activated c-H-ras gene is due to an autocrine mechanism or not, IL-3 production by the FI-ras clones was examined. A biologic assay of the conditioned media using an IL-3–dependent cell line (32D) showed that FI-IL-3 secreted a large amount of IL-3, but the activity was undetectable in the conditioned media of clones 1 and 2 of FI-ras (Fig 4). Moreover, Northern blotting showed that IL-3 mRNA was expressed in the FI-IL-3 but not in the FI-ras cells (clones 1, 2, and 3) (Fig 5). These findings suggest a nonautocrine mechanism of autonomous growth of the FI-ras cells.

**Tumorigenicity of transformed FDC-P2 cells.** FI-ras and parent FDC-P2 cells were morphologically similar when examined with Wright-Giemsa stain (data not shown). To test their tumorigenicity, 5 × 10⁶ cells were injected subcutaneously into nude mice. FI-IL-3 and FI-ras cells formed tumors at all injected sites, but the parent and FD-BMG cells were nontumorigenic (Table 2). The latent periods to tumor formation were different between FI-IL-3 and FI-ras. The growth rate of FI-ras tumors in vivo was slower than that of FI-IL-3 tumor, which was consistent with the finding in liquid cultures. Tumor-bearing mice were killed to check for organ involvement. Superficial lymph node swelling and splenomegaly were observed macroscopically. Infiltration of abnormal cells in the liver, spleen, bone marrow, and superficial lymph nodes were documented histologically (data not shown). When cells from bone marrow and subcutaneous tumors of FI-ras–injected mice were cultured without IL-3 in the presence of hmB in vitro, IL-3–independent and hmB-resistant cells died within 2 days, as expected, the growth rate of clone 3 was lower than that of clone 1 (data not shown).
proliferated. The conditioned media of these cells did not contain IL-3 (data not shown).

Involvement of BMG vector in the high incidence of autonomous growth induced by BMGH-ras. To investigate whether the BMG vector contributed to increase the incidence of autonomous growth, cotransfection experiments were performed. First, the activated c-H-ras gene in pBR322 vector (pHs-49) and BMGHph plasmid were cotransfected into FDC-P2 cells with a selectable marker gene, and the incidence of IL-3-independent growth was compared with that of BMGH-ras. Cotransfection with pHs-49 plus BMG vector induced IL-3 independence in 9 of 68 wells containing G418-resistant cells, but the incidence was significantly lower than that of BMGH-ras, which induced IL-3 independence in 26 of 87 wells (Table 3). Second, pHs-49 was transfected into FDC-P2 cells with or without the BPV gene (BMG vector) in the presence of a selectable marker gene, and the incidence of IL-3-independent growth was compared. The activated ras gene (pHs-49) alone induced IL-3 independence in 3 of 57 wells containing hmB-resistant cells, whereas the incidence increased up to 11 of 59 after cotransfection with pHs-49 plus BMG vector (Table 4). This finding was confirmed by two other independent experiments (data not shown). In addition, the expression of a significant amount of BPV mRNA in the cells transfected with BMG vector was demonstrated by Northern analysis (data not shown).

The copy number of activated c-H-ras gene per cell was compared among the representative IL-3-independent clones by Southern analysis. The result showed that a clone transfected with pHs-49 and a clone transfected with pHs-49 plus BMG vector contained only a few copies of the ras gene, which were apparently fewer than that in a FI-ras clone transfected with BMGH-ras (Fig 6). Because pHs-49 has only one BamHI site, the size of BamHI-digested c-H-ras fragments (about 10.0 and 10.5 kb) in the clones transfected with pHs-49 (with or without BMG vector) was variable and dependent on their integration sites.

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Fig 2. Southern analysis of FI-ras clones using a c-H-ras probe. (A) Autoradiograph of Southern blot. The probe was a Sac I fragment from pHs-49. Genomic DNA (10 µg) from each FI-ras clone and BMGH-ras plasmid DNA as a standard were digested with BamHI or Xba I. The copy number of the ras gene per cell was calculated by comparison with the standard. (B) Structure of BMGH-ras plasmid.

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Fig 3. RNase protection analysis of c-H-ras mRNA expression in transformed FDC-P2 cells. (A) Autoradiograph of RNase protection analysis. The molecular weight markers were 5' end labeled pBR322-HaelIII fragments. Arrow indicates the protected band of about 150 bases. (B) Predicted size of protected fragment (about 150 bases) is illustrated.
Fig 4. Biologic assay for IL-3 in media conditioned by transformed FDC-P2 cells. The method is described under Materials and Methods. The vertical line indicates \(^{3}H\)-TdR incorporation of indicator cells (320 cells), and the horizontal line indicates diluting magnification (log) of samples. (●) Conditioned media of two separate cell lines of Fl-IL-3; (△) conditioned media of clones 1 and 2 of Fl-ras.

**DISCUSSION**

Activation of the ras family of proto-oncogenes by point mutation has been frequently detected in leukemic patients, suggesting their involvement in multistep leukemogenesis. Transformation of factor-dependent myeloid cells by DNA transfection is a valuable approach for studying the role of individual genes. Several investigators reported that activated ras genes failed to induce factor independence. However, it was recently suggested that the activated c-H-ras gene may induce factor independence under some conditions. Andrejauskas et al reported that high-level expression of activated c-H-ras gene in a PB-3c mast cell line led to the complete abrogation of the exogenous IL-3 requirement for their growth, and that most of the transformed cells produced IL-3 and GM-CSF, suggesting that their autonomous growth may have been due to an autocrine mechanism. On the other hand, Boswell et al reported that incorporation of multiple copies of the activated c-H-ras gene into FDC-P1 cells induced high-level ras expression and IL-3-independent growth without evidence for autostimulatory growth factor production. In the present study, we attempted to introduce multiple copies of the activated c-H-ras gene into FDC-P2 cells using a high-level expression BMG vector. As a result, IL-3-independent and tumorigenic clones emerged at a high incidence, and these clones did not express or secrete IL-3, suggesting the acquisition of IL-3 independence by an autocrine mechanism. Whether the activated ras-transformed cells produce autocrine growth factors or not may be related to the inherent nature of parent cell lines.

The high incidence of autonomous clones induced by the activated c-H-ras gene in our experiment may be explained by the use of the BMG vector. The BMG vector is known to replicate as a multicopy episome in transfected cells. Southern analysis demonstrated that the representative Fl-ras clone transfected with BMGH-ras contained a large number of copies of the activated c-H-ras gene. In contrast, only a few copies of the ras gene were detected in clones transfected with pHs-49 or pHs-49 plus BMG. This difference in the copy number may be one of the factors that influenced the efficiency of induction of factor independence by the activated ras gene, as previously suggested by other investigators. Actually IL-3 independence was much more efficiently induced by BMGH-ras compared with cotransfection with the activated ras gene and the BPV.
TRANSFORMATION OF FDC-P2 BY ACTIVATED \textit{RAS}

**Table 4. Incidence of HmB-Resistant and IL-3-independent FDC-P2 Cells After Cotransfection**

<table>
<thead>
<tr>
<th>Test Plasmid</th>
<th>Selectable Marker Gene</th>
<th>HmB Resistant*</th>
<th>IL-3 Independent†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mock</td>
<td>None</td>
<td>0/24</td>
<td>—</td>
</tr>
<tr>
<td>Mock</td>
<td>pY3</td>
<td>120/120</td>
<td>0/115</td>
</tr>
<tr>
<td>BMG</td>
<td>pY3</td>
<td>54/120</td>
<td>0/53</td>
</tr>
<tr>
<td>pHs-49</td>
<td>pY3</td>
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<td>3/57</td>
</tr>
<tr>
<td>BMG + pHs-49</td>
<td>pY3</td>
<td>59/120</td>
<td>11/59</td>
</tr>
</tbody>
</table>

*Number of wells in which hmB-resistant cells appeared/number of wells tested.
†Number of wells in which IL-3-independent cells appeared/number of wells tested.

gene in separate plasmids. Moreover, cotransfection with the activated \textit{ras} gene and BPV gene in separate plasmids induced a higher incidence of IL-3 independence in FDC-P2 cells without increasing the copy number of the \textit{ras} gene compared with transfection with the activated \textit{ras} gene alone (pHs-49 plus BMG v pHs-49). This finding suggests that the BPV gene in BMG vector is involved in the efficient induction of factor independence, because the BPV gene itself is known to have oncogenic potential. Although the BPV gene reportedly cooperates with the \textit{ras} gene to transform fibroblasts, to our knowledge, this is the first demonstration of a similar phenomenon in hematopoietic cells. The mechanisms of this cooperation remain to be determined, but there are several studies on the functions of BPV gene products. First, it has been reported that BPV E2 protein acts as a transactivator of genes. Therefore, BPV E2 protein may have transactivated some critical genes in transformed FDC-P2 cells. Second, BPV E5 protein has been reported to cooperate with receptors for epidermal growth factor and colony-stimulating factor-1 to transform NIH3T3 cells in a ligand-independent manner. The other group also reported that receptor for platelet-derived growth factor was activated by BPV E5 protein in BPV E5 gene–transfected murine fibroblast cell lines. In our study, it is possible that BPV E5 protein activated some growth factor receptors and thereby cooperated with the \textit{ras} gene to transform FDC-P2 cells. Third, BPV oncoproteins may have cooperated with the \textit{ras} gene by inactivating the products of tumor suppressor genes, because it is supposed that oncoproteins of several DNA tumor viruses function in transformation by inactivating the products of tumor suppressor genes such as the retinoblastoma susceptibility (RB) and p53 genes. Such a mechanism may participate in transformation of FDC-P2 cells, because some BPV oncoprotein possesses the p105 binding domains. It is noteworthy that abnormalities of tumor suppressor genes have been detected in some hematopoietic malignancies. We are currently investigating possible functional changes in some tumor suppressor genes in FDC-P2 cells transformed with the BPV gene. In any case, our present data suggest that the activated \textit{ras} gene cooperates with functional changes in some cellular genes caused by BPV gene products, and thus support the concept of multistep leukemogenesis.

The \textit{ras} genes encode a 21-Kd membrane-associated protein (p21) that is a homologue of the GTP-binding proteins involved in receptor-mediated signal transduction. Several investigators have reported that p21 activates protein kinase C through increasing the level of diacylglycerol. The mutational activation of the \textit{ras} genes is thought to modify these signal transduction pathways and induce uncontrolled cellular proliferation. Moreover, recent studies suggested that activation of the \textit{ras} p21 protein is involved in IL-3 signal transduction. Therefore, activation of the \textit{ras} gene may replace some part of IL-3 signal transduction. However, the exact role of the \textit{ras} genes in the signaling pathway and in leukemogenesis remains poorly understood. Because most FI-\textit{ras} clones had nonautocrine properties in vitro and in vivo in this study, they may provide a valuable opportunity for studying the effect of the activated \textit{ras} gene on the post–IL-3–receptor signal transduction pathway.

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REFERENCES


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**Figure 6.** Southern analysis of activated c-H-ras gene in the representative IL-3–independent clones. The probe was a Sac I fragment from pHs-49. Genomic DNA (10 μg) from each clone and BMGH-ras plasmid DNA as a standard were digested with BamHI. Lane 1, a clone transfected with pHs-49; lane 2, a clone transfected with pHs-49 plus BMG plasmid; lane 3, a clone transfected with BMGH-ras (FI-ras C1).


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