A v-H-ras-Dependent Hemopoietic Tumor Model Involving Progression from a Clonal Stage of Transformation Competence to Autocrine Interleukin 3 Production

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Autocrine interleukin 3 (IL-3)-secreting tumors were generated from an IL-3-dependent mouse mast cell line (PB-3c) after introduction of the v-H-ras oncogene. Tumor progression was characterized by four distinct phenotypes. The first corresponded to immortalized mast cells unresponsive to the oncogenic effect of v-H-ras. The second was expressed in a clonable subpopulation of PB-3c cells and was marked by the competence to form v-H-ras-dependent tumors (immortalized transformation competence). The third was a direct effect of v-H-ras expression on all PB-3c cells and was characterized in vitro by a reduced IL-3 requirement. Upon injection of v-H-ras-expressing, transformation-competent cells into mice, the final, fully malignant phenotype developed with a long latency period and was marked in vitro by independence of exogenous IL-3 and by autocrine IL-3 stimulation. Northern (RNA) blot analysis and an RNase A-T₁ protection assay showed that IL-3 production was strictly associated with the tumor phenotype. Two of six tumors showed an alteration at the 5' region of the IL-3 gene. We conclude that v-H-ras requires complementation by IL-3 gene rearrangement or an alternate event to generate autocrine mastocytomas.

Clinical, epidemiological, and experimental studies on cancer have shown the emergence of malignant cells to be the result of a multistep process, frequently involving the proto-oncogenes (7, 32, 33). Several mechanisms such as point mutations, translocations, or gene amplification can activate these genes, rendering them oncogenic in a genetically dominant fashion. In contrast, mutations in a second set of genes important for the carcinogenic process are recessive; both alleles appear to be nonfunctional in cancer cells (11). Thus, a stepwise accumulation of complementing dominant and recessive mutations appears to underlie the process of carcinogenesis.

One event occurring frequently during spontaneous tumor formation is a point mutation in codon 12, 13, or 61 of one of the three *ras* genes (H-, K-, or N-*ras*) (4, 5). Such mutations have been found in a large variety of tumors of different origin, reflecting the ubiquitous expression of *ras* genes in animal cells. *ras* p21 proteins are structural homologs of G proteins and are thought to be involved in receptor-mediated signal transduction. Their role in this process and in tumorigenesis is poorly understood.

Earlier studies from our laboratory (23, 47, 53) and others (8, 19, 39) showed that mutated *ras* genes are frequently detected in human hemopoietic malignancies. These mutations may appear either early (28) or late (20, 47a) in the development of the disease. Although N-*ras* alterations predominate, mutations in the H-*ras* gene have also been reported (50). In vitro, activated *ras* genes can enhance the proliferative potential of hemopoietic progenitor cells belonging to the lymphoid (37), myeloid (40), erythroid (25, 29), and mast (44) cell lineages. In addition, v-H-*ras* in combination with v-*myc* has been shown to induce neoplastic trans-

formation in lymphoid (46) and myeloid (51) cells, mirroring earlier results obtained with fibroblasts (34).

Although clinical and experimental studies show that *ras* activation may be a tumorigenic step in hemopoietic cells, neither its role nor the number and nature of complementing steps are known. To elucidate the process further, we have studied the effect of v-H-*ras* expression in an interleukin 3 (IL-3)-dependent, pseudodiploid, nontumorigenic mast cell line, PB-3c (3, 13). We have now shown that tumorigenesis in these cells proceeds through at least four steps, and we have identified a cellular state of transformation competence and an autocrine IL-3 loop as elements complementing the v-H-*ras* oncogene.

MATERIALS AND METHODS

Cell lines and tissue culture. PB-3c is a cloned, IL-3-dependent mastocyte line isolated from mouse bone marrow (3, 13). FDCP-1 cells express the macrophage-specific marker F4/80 and are dependent either on granulocyte-macrophage colony-stimulating factor or on IL-3 for growth (18, 27). WEHI-3B (D⁻ subline) is a mouse myelomonocytic leukemia cell line that secretes IL-3 (36). All of these cell lines and the tumor cells derived from infected PB-3c cells were maintained in Iscove modified Dulbecco medium (IMDM) supplemented with 10% fetal calf serum, 100 U of penicillin per ml, 100 µg of streptomycin per ml, and 50 µM β-mercaptoethanol. IL-3-dependent cells further required addition of medium conditioned by WEHI-3B cells (WEHI-3CM). To determine the optimal mitogenic concentration, each batch of WEHI-3CM was titrated on PB-3c cells. Cell lines secreting retroviral vectors were grown in Dulbecco modified Eagle medium and 10% fetal calf serum.

Infection and selection procedures. Rash-1 cells (46) secrete a retroviral Zip vector carrying the v-H-*ras* gene in addition to the *neo* gene which confers resistance to the drug G418.

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Viral titers on NIH 3T3 cells were around 5×10^3 CFU/ml, and the virus is referred to as Zip-*ras-neo* in this paper. For controls we used a *neo*-carrying virus without insertions, namely, the N2 virus (31). Culture supernatants, derived from confluent cultures and kept frozen at -70°C , were used as viral stocks. Infection of 10^6 PB-3c cells was carried out in 5 ml of complete medium in the presence of polybrene (16 $\mu\text{g/ml}$) and WEHI-3CM. The medium was changed 2 h later. After 48 h the cells were subjected to G418 selection (1 mg/ml).

Methylcellulose cloning. Cloning in methylcellulose was performed in 35-mm tissue culture dishes by the method of Davis et al. (17). The cloning mixture was made up in IMDM and contained methylcellulose (1.2%, wt/vol), bovine serum albumin (1%, wt/vol), fetal calf serum (20%, vol/vol), iron-saturated human transferrin (300 $\mu\text{g/ml}$), G418 (1 mg/ml), and, when indicated, saturating amounts of WEHI-3CM. At 48 h after infection, 10^6 cells were plated per ml of cloning mixture. Macroscopic colonies were picked about 10 days later and expanded in suspension cultures containing G418 and, when required, WEHI-3CM. In cloning experiments involving tumor cells, the numbers of visible colonies were determined after 15 to 20 days. Anti-IL-3 antibody (5.7 $\mu\text{g/ml}$) or normal rabbit serum (5.7 $\mu\text{g/ml}$) was included wherever indicated.

Mitogenicity assay. PB-3c or FDCP-1 cells were washed three times in IMDM–10% fetal calf serum and adjusted to 2×10^5 cells per ml; 100- μl samples were dispensed into microdilution plates. After the addition of 10 μl of the test samples, incubation was carried out at 37°C for 18 to 24 h. [^3H]thymidine (87 Ci/mol; Amersham Corp.) was then added (0.5 μCi per well), and the incubation was continued for an additional 6 h. The cells were finally processed with a semiautomated harvester, and the incorporation of [^3H]thymidine was determined in a standard toluene-based scintillation cocktail.

Tumorigenicity assay. Young adult syngeneic DBA/2 mice were injected subcutaneously with 2×10^6 or 5×10^6 cells washed in phosphate-buffered saline. Mice were inspected weekly for the appearance of palpable tumors at the site of injection. When tumors reached a diameter of 0.5 to 1.0 cm, they were excised and minced with scissors, and the cells were plated into culture medium.

Immunoprecipitation of p21 protein. Cells (10^6) were incubated with 400 μCi of [^{32}P]phosphate per ml in 2 ml of phosphate-free IMDM containing 10% dialyzed fetal calf serum for 13 h. Cells were washed once with normal IMDM and twice with phosphate-buffered saline and then lysed at 4°C by incubating them for 30 min in 1 ml of lysis buffer (120 mM NaCl, 50 mM Tris hydrochloride [pH 8.0], 1% Triton X-100, and 3 mM phenylmethylsulfonyl fluoride as a protease inhibitor). To each lysate, 50 μl of pansorbin (Calbiochem) was added. After 10 min on ice, the samples were centrifuged at 13,000 rpm for 10 min in a Eppendorf centrifuge, and the supernatants were directly processed or stored at -70°C . Samples from the above supernatants containing 5×10^6 cpm of trichloroacetic acid-precipitable material were precipitated for 40 min on ice with the monoclonal anti-p21 antibody Y13-259 (21) or, as a control, with normal rat serum. Protein A-Sepharose 4B coated with rabbit anti-rat immunoglobulin G (Nordic) was added for another 2 h at 4°C . Precipitates were subjected to five washes with the following: (i) 1% Triton X-100–0.5% sodium deoxycholate–0.1% sodium dodecyl sulfate–50 mM Tris hydrochloride (pH 7.7)–120 mM NaCl; (ii) 50 mM Tris hydrochloride (pH 7.4)–150 mM NaCl–5 mM EDTA–1% Triton X-100; (iii) 50

mM Tris hydrochloride (pH 7.4)–150 mM NaCl–5 mM EDTA; (iv) LiCl buffer (0.5 M LiCl, 0.1 M Tris hydrochloride [pH 7.6]); and (v) H_2O . They were finally dissolved in sample buffer (62.5 mM Tris hydrochloride [pH 8.0], 2% sodium dodecyl sulfate, 5% β -mercaptoethanol, 10% glycerol) and separated by gel electrophoresis in 19% polyacrylamide–6 M urea. The gels were dried, and the protein bands were identified by fluorography.

Anti-IL-3 antibody inhibition. A 10- μl sample of culture supernatant was preincubated with 10 μl of rabbit anti-IL-3 antibody preparation (30 ng/ml) (9) or control rabbit antimouse immunoglobulin in a final volume of 70 μl of medium. After 2 h at 37°C , 2×10^4 PB-3c cells were added in 50 μl and incubated for 24 h. [^3H]thymidine was then added, and incorporation was determined as described above.

Northern (RNA) blot analysis. RNA was isolated by the acid guanidinium thiocyanate-phenol-chloroform method as described by Chomczynski and Sacchi (12). Samples of 100 μg of total RNA were fractionated on 1.2% agarose gels containing 2.2 M formaldehyde. Gels were treated with 50 mM NaOH for 30 min, equilibrated in $10\times$ SSC ($1\times$ SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and transferred to GeneScreen-plus membranes (Dupont, NEN Research Products) according to the protocol of the manufacturer. Prehybridization and hybridization were done at 42°C in the presence of 50% formamide, 10% polyethylene glycol 6000, and 7% sodium dodecyl sulfate (2). Washing was done once in $2\times$ SSC for 5 min at room temperature, twice in 0.25 M sodium phosphate buffer (pH 7.2) containing 2% sodium dodecyl sulfate and 1 mM EDTA for 20 min at 65°C , and twice in 0.05 M sodium phosphate buffer containing 1% sodium dodecyl sulfate and 1 mM EDTA for 20 min at 65°C . RNA probes were transcribed from linearized pSP6-IL-3, containing 368 base pairs of mouse IL-3 cDNA, in the presence of [^{32}P]GTP (New England Nuclear Corp.) by the SP6 transcription procedure in the Promega Biotech (Madison, Wis.) instruction manual. Specific activity was 2×10^8 cpm/ μg of DNA. Loading 25, 50, or 100 μg of total RNA showed a linear increase in IL-3 band intensity.

RNase A- T_1 protection assay. Poly(A) $^+$ RNA was hybridized to 2×10^4 to 3×10^4 cpm of acrylamide-purified, ^{32}P -labeled antisense RNA (45) generated from pSP6-multi CSF which carries a 368-base-pair fragment from the cDNA for IL-3. The probe that is generated by SP6 polymerase is 395 base pairs long. The protected fragment observed in the two mastocytomas and WEHI-3B preparations corresponds to the expected size of 368 base pairs.

Preparation of anti-IL-3 antibody column and IL-3 depletion. An antiserum against recombinant IL-3 was obtained through repeated immunizations of rabbits and was affinity purified over a column of recombinant IL-3 immobilized on Affi-Gel 10 (Bio-Rad Laboratories) (16). A 5-ml sample of this fraction (4 mg of pure immunoglobulin) was coupled to 2 ml of CNBr-activated Sepharose 4B (Pharmacia, Uppsala, Sweden) as recommended by the manufacturer.

For IL-3 depletion, the conditioned media ($20\times$) were loaded onto the column, which was previously equilibrated with 0.1 M Tris hydrochloride (pH 8.0). The column was subsequently washed with 0.1 M Tris hydrochloride (pH 8.0)–0.5 M NaCl to remove unbound material. The adsorbed IL-3 was eluted with 0.58% acetic acid–150 mM NaCl; the flowthrough fractions were subjected to five cycles of binding and dialyzed extensively against phosphate-buffered saline. These were now devoid of IL-3 and were tested for colony-stimulating activity.

Southern blot analysis. DNA was isolated by Nucleic Acid

FIG. 1. IL-3 responsiveness of V lines (○) and N lines (●). (A) [³H]thymidine incorporation in response to decreasing concentration of IL-3. (B) Growth curve at saturating (0.2% WEHI-CM) IL-3 concentration. (C) Growth curve at limiting (0.02% WEHI-CM) IL-3 concentration. The same six cultures were analyzed in all three panels.

Extractor 340A from Applied Biosystems. DNA samples (20 µg) were digested with enzymes from Boehringer Mannheim Biochemicals or Biofenix as recommended by the suppliers. Digested DNA was separated on 0.8% agarose gels, transferred to Genescreen Plus (Dupont, NEN) according to the protocol of the manufacturer. Prehybridization and hybridization were done as in the case of Northern blots. The probe used was the IL-3 cDNA derived from pUC/Mu 2A.1. The 1.1-kilobase (kb) *Eco*RI fragment was random primed to an efficiency of 1×10^9 to 2×10^9 cpm/µg of DNA with the kit supplied by Boehringer.

RESULTS

Reduction of the IL-3 requirement follows *v-H-ras* expression. To test whether *v-H-ras* expression has an effect on IL-3 dependency in PB-3c cells, cultures were infected with either a recombinant retrovirus carrying the *v-H-ras* oncogene together with a *neo* gene (*Zip-ras-neo* virus) (46) or with the N2 virus carrying the *neo* gene alone (31). Both cultures were then selected for resistance to the neomycin antibiotic G418 in the presence of saturating amounts of IL-3. Outgrowing resistant cell populations derived from *Zip-ras-neo* and N2 infections were (designated as V lines and N lines, respectively) tested for survival in the absence of IL-3. Like the parental cells, these cells did not survive in IL-3-free medium, indicating that *v-H-ras* did not abrogate factor dependency (data not shown). However, V lines showed higher incorporation of [³H]thymidine at limiting IL-3 concentrations compared with N lines (Fig. 1A). Uninfected PB-3c cells behaved like N lines (data not shown). When cell proliferation was measured, V and N lines grew identically at saturating IL-3 concentration (Fig. 1B), but at a limiting IL-3 concentration the V lines displayed shorter doubling times and higher saturation densities than the N lines (Fig. 1C). This reduction in IL-3 requirement correlated with the presence of the viral p21 protein as shown by immunoprecipitation of ³²P-labeled cell lysates of three representative V lines with a monoclonal anti-p21 antibody (21) (Fig. 2).

To estimate the clonal frequency of the IL-3 effect, PB-3c cells were plated in methylcellulose in the presence of G418 48 h after *Zip-ras-neo* infection, and the resultant clones (designated as V clones) were analyzed as above. All the 33 V clones isolated showed a reduced IL-3 requirement (data not shown), indicating this to be a direct effect of *v-H-ras* expression.

***v-H-ras* expression leads to tumorigenicity.** PB-3c cells do not form tumors in syngeneic mice (3, 13). To test whether this phenotype was altered in V and N lines, cells were inoculated subcutaneously into syngeneic mice. N lines, like PB-3c cells, were nontumorigenic, but nine of nine V lines generated tumors at the site of inoculation in 89% of injected animals with a latency of 5 to 12 weeks (Table 1, experiment 1). Histologically, these tumors were classified as mastocytomas (F. Gudat, unpublished data).

In contrast to the V lines, only 42% of the 33 V clones with reduced IL-3 requirement formed tumors (Table 1, experiment 2), although the tumor incidence induced by the positive clones remained high (76%) and the latency time remained long.

Mastocytoma formation requires complementation by a clonally expressed state of competence. The fact that about half of the V clones were unable to form tumors suggested

FIG. 2. Immunoprecipitation analysis of p21 protein. ³²P-labeled lysates of PB-3c cells and three different V lines immunoprecipitated with monoclonal anti-p21 antibody Y13-259 (Y) or control rat immunoglobulin (C).

TABLE 1. Tumorigenicity of Zip-*ras-neo*-infected PB-3c lines

Exp ^a	Cells injected ^b	No. of independent cell lines tested	Incidence (%) of tumorigenic cell lines ^c	Penetrance (%) of tumorigenic cell lines ^d	Latency in wks (range) ^e
1	PB-3c	1	0		
	N lines	5	0		
	V lines	9	9/9 (100)	40/45 (89)	5–12
2	V clones	33	14/33 (42)	32/42 (76)	8–24
3	Preinfection V clones	20	3/20 (15)	7/9 (78)	4–12

^a In experiment 1, 2×10^6 cells were injected per mouse (five mice per group); in experiments 2 and 3, 5×10^6 cells were injected per mouse (three mice per group).

^b N and V lines represent G418-selected, PB-3c mass cultures after infection with N2 and Zip-*ras-neo* virus, respectively; V clones represent cell lines derived from methylcellulose cloning of PB-3c mass culture freshly infected with Zip-*ras-neo* virus; preinfection V clones represent cell lines derived from PB-3c cells subcloned before infection with Zip-*ras-neo* virus.

^c Number of cell lines that formed a tumor in at least one mouse/total number of cell lines tested.

^d Number of tumor-bearing mice/total number of mice injected with tumorigenic cell lines.

^e The animals were observed for 40 weeks in experiments 1 and 2 and for 24 weeks in experiment 3.

the presence of two subpopulations, one progressing toward tumorigenicity in response to the *ras* oncogene and the other not. To test this possibility, PB-3c cells were subcloned in methylcellulose before Zip-*ras-neo* virus infection. Twenty such different clones were infected, selected in the presence of G418 (designated as preinfection V clones), and tested for tumor formation (Table 1, experiment 3). Only 3 (clones 6, 8, and 15) out of 20 clones (15%) were able to generate tumors after infection, with latencies ranging from 4 to 12 weeks, whereas animals inoculated with the remaining 17 clones remained tumor free even after 24 weeks. However, tumorigenic clones again displayed a high penetrance (78%). Two positive (no. 8 and 15) and two negative (no. 20 and 22) clones were retested in a larger number of animals, and the same results were obtained (data not shown), revealing the coexistence of two PB-3c subpopulations, one competent and the other refractory to v-*H-ras*-induced tumorigenesis.

In vivo progression leads to an IL-3-secreting tumor phenotype. A hallmark of the observed tumorigenicity (Table 1) was the long latency period. To test whether this is due to an intrinsically slow proliferation capacity of PB-3c-derived tumor cells, five primary tumors were transplanted into secondary hosts. Inoculation of 10^6 cells induced tumors within 2 weeks (data not shown). These results were a first suggestion that competent cells underwent a phenotypic alteration after v-*H-ras* expression during passage through the animals.

To further analyze the tumor cells, a series of mastocytomas was excised, and the cells were dispersed in culture medium. All tumors were found to grow in the absence of IL-3, and a series of IL-3 independent cell lines (designated as VxDy) was established. The donor origin of the tumor cell lines was confirmed by the observed growth in the presence of G418 as well as by detecting the v-*H-ras* gene by Southern blot analysis (data not shown). As expected, they expressed immunoglobulin E receptor (C. Heusser, unpublished results).

To determine whether these IL-3-independent tumor cells produced their own growth factor, media conditioned by several such lines were tested for mitogenic activity on

PB-3c cells and on cells of another IL-3-dependent line, FDC-P1 (18) (Fig. 3A). A strong mitogenic effect was observed on both lines with medium from more than 50 different tumor cell lines (Fig. 3A; data not shown), although the mitogenic potency varied between cell lines.

To test whether the mitogenic agent was IL-3, three culture supernatants were incubated with FDCP-1 cells in the presence of anti-IL-3 antibody (9). In all cases (V4D6, V3D7, and V2D1) mitogenic activity was inhibited, as was IL-3 derived from WEHI-3B cells (36) included as positive control (Fig. 3B).

IL-3 gene expression is a step after v-*H-ras* expression. It was conceivable that the competent cells within the PB-3c stock culture already produced IL-3 and, after v-*H-ras* expression, could generate mastocytomas. Alternatively, IL-3 production could occur either as a direct consequence of v-*H-ras* expression or as the result of an event taking place subsequent to v-*H-ras* expression. To distinguish between these possibilities, PB-3c cells and one of the competent subclones, PB-3c-8, as well as their corresponding V- (V2, V4, 8V) and tumor-derived (V3D7, V4D6, 8VD3) cells were analyzed for the presence of IL-3 mRNA by Northern blot analysis. The mastocytomas, derived from PB-3c cells (Fig. 4A) or subclone PB-3c-8 (Fig. 4B), expressed an IL-3 transcript of a size similar to that found in WEHI-3B cells. By contrast, neither V cells nor PB-3c cells produced IL-3 mRNA. The same conclusion was reached when the IL-3 probe was hybridized in solution with poly(A)⁺ RNA and analyzed by gel electrophoresis after an RNase A-T₁ protection assay (Fig. 4C). A protected fragment of the expected size was detected in the mastocytomas (V4D6 and 8VD3) but not in the V cells. Thus, the tumor cells had acquired a new phenotype, most probably the result of an event occurring in vivo after injection of V cells into mice.

Evidence for an autocrine mechanism. It seemed possible that IL-3 secretion formed part of an autocrine loop capable of providing the required growth stimulus. Because cloning of autocrine cells below a critical cell concentration often requires the addition of the appropriate growth factor, decreasing numbers of cells derived from two of the tumor lines (V4D6 and V2D1) were plated in methylcellulose either in the presence or the absence of their own conditioned medium (auto-CM). Colony numbers in the presence of auto-CM increased linearly with the number of cells plated, yielding a constant cloning efficiency of 6–12% at all cell densities, a value typical of uninfected PB-3c cells in the presence of IL-3 (Fig. 5). However, in the absence of auto-CM, colonies formed only when more than 10^3 cells were plated. Similarly, when 10^3 cells from four tumor lines were plated with auto-CM, WEHI-3CM, or no additions, three lines (V2D1, V3D8, and V4D6) formed colonies only in the presence of auto-CM (Fig. 6A), and one tumor line (8VD2) grew without added factor, but the colony formation was increased by the addition of auto-CM. That WEHI-3CM stimulated colony formation to a similar extent was consistent with the idea that the autostimulating activity was IL-3. To further test this point, medium conditioned by two tumor cell lines (V2D1 and V4D6) was passed through an affinity column containing anti-IL-3 antibody and examined for residual activity. In earlier experiments, this column was shown to adsorb IL-3 specifically and bound IL-3 could be eluted in a biologically active form (data not shown). The results showed that the auto-CM and WEHI-CM were unable to induce colony formation after depletion of IL-3 (Fig. 6B). Direct proof for an autocrine role of IL-3 was

FIG. 3. Evidence for IL-3 secretion. (A) Media conditioned by seven tumor cell lines were tested for mitogenic activity on FDCP-1 cells (□) or PB-3c cells (■). Background values were below 1,000 cpm. (B) Conditioned media were tested for mitogenic activity on FDCP-1 cells after preincubation with control rabbit immunoglobulin (□) or anti-IL-3-antibody (■).

obtained from the following experiment. V4D6 mastocytoma cells were plated at high cell number (4×10^3 cells per ml) (where no auto-CM is required) in the presence and absence of the affinity-purified anti-IL-3 antibody preparation. No colonies formed in the presence of the antibody, whereas normal rabbit serum had no effect (3.5×10^2 colonies per plate, compared with 3.2×10^2 colonies per plate in controls with no addition to the medium).

Southern blot analysis. To test whether the production of IL-3 was accompanied by a rearrangement of the gene, genomic DNA was digested with various restriction enzymes and hybridized to IL-3 cDNA (full-length cDNA of 1.1 kb). Both of the IL-3 alleles were unaltered in all the tumors derived from V clones (Fig. 7A and B, lanes 4 through 6).

Two tumors obtained from infected mass cultures (V3D7 and V4D6) gave evidence for gene rearrangement. An *Eco*RI digest shows an additional 3.5-kb band (Fig. 7A, lanes 8 and 9) of a size similar to that of the band seen in DNA from WEHI-3B cells (lane 10). This DNA is known to carry a rearranged IL-3 gene, with an alteration 125 base pairs upstream to the transcription start (54). DNA from V3D7 and V4D6 tumors, like DNA from WEHI-3B cells, when digested with *Hind*III (Fig. 7B), showed a 5.5-kb band spanning entire exons II, III, and IV, suggesting no alteration affecting the 3' end of the gene. *Bgl*II (Fig. 7C) and *Pvu*II (Fig. 7D) digests of V3D7 and V4D6 DNAs confirmed our conclusion that these two tumors carry a rearranged IL-3 gene.

DISCUSSION

In this work we describe a novel *v-H-ras*-dependent hemopoietic tumor model in which a terminal autocrine stage with IL-3 secretion is preceded by at least three preneoplastic steps.

Immortalization. Immortalization of PB-3c cells appears to be the initial step in the tumor transformation pathway in this system. The original PB-3 cell line from which the PB-3c clone was later derived was grown out of a primary mast cell culture undergoing terminal crisis. In another report (44) infection of nonimmortalized mast cells with *v-H-ras* led to an extended lifespan of the cells rather than to the sequence of events described here. Whereas in primary fibroblasts transfection of the *v-myc* gene led to immortalization (34, 38), the *c-myc* gene is not constitutively expressed in PB-3c cells but is under the control of IL-3 (14).

Acquisition of transformation competence. Although the immediate effect of *v-H-ras* (reduction in IL-3 requirement) is exerted in all infected cells, the tumorigenic potential was restricted to a subpopulation that could be cloned out before or after infection. This argues that the formation of mastocytomas depends on a property that preexists in a subpopulation of PB-3c cells. On the basis of these results, a step essential for tumorigenesis in a hemopoietic cell line can be defined as the acquisition of competence to progress to the tumor phenotype after *v-H-ras* expression. It is possible that the competent cells arise in culture from noncompetent parental PB-3c cells as a result of minute chromosomal alterations.

FIG. 4. Expression of IL-3 mRNA. (A) Northern analysis of subclone PB-3c-8, its V line (8V), and tumor 8VD3 derived from 8V. V3D7 served as a positive control. (B) Northern analysis of two tumor cell lines (V3D7, V4D6), two V lines, and the PB-3c mass culture. WEHI-3B cells served as a positive control. (C) RNase A-T₁ protection assay of poly(A)⁺ RNA. The band in the mastocytoma V4D6 and 8VD3 lanes corresponds to the expected size of IL-3 cDNA (see Materials and Methods).

v-H-ras reduction of IL-3 requirement. The third step in this tumor system is expression of the v-H-ras gene, leading to an immediate characteristic change in phenotype: the oncogene-expression cells were able to proliferate optimally at only 10% of the IL-3 concentration needed for uninfected cells. Since neither the generation time nor the saturation density was affected by v-H-ras at the optimal concentration of IL-3 (Fig. 1B), it is unlikely that a subpopulation of

FIG. 5. Cloning efficiency to tumor cells in methylcellulose in the presence (▲) and absence (△) of auto-CM. (A) Mastocytoma V4D6. (B) Mastocytoma V2D1.

FIG. 6. IL-3 acts as an autocrine regulator. (A) Cells (10^3) of the indicated tumor cell lines were plated with no addition (■), auto-CM (▨), or WEHI-CM (□). (B) Cells (10^3) of the two indicated tumor cell lines were plated with auto-CM (▨) or WEHI-CM (□) or the same samples after passage over an anti-IL-3 antibody column (■).

IL-3-hypersensitive cells was selected. In contrast to this *ras* oncogene effect, infection of other IL-3-dependent lines with v-*abl* (15, 41), v-*myc* (43), and v-*src* (52) genes led to factor independence in vitro.

What is the mechanism of the v-H-ras effect observed in vitro? As in fibroblasts, activated *ras* genes have been

FIG. 7. Southern blot analysis of IL-3 gene. (A and B) Lanes: 1, DBA mouse liver; 2, C18; 3, 8V; 4, 8VD3; 5, 8VD10; 6, 8VD7; 7, V2D1; 8, V3D7; 9, V4D6; 10, WEHI. (C) Lanes: 1, V4D6; 2, V3D7. (D) Lanes: 1, V4D6; 2, V3D7. (A) *EcoRI* digest. An 8.5-kb band is present in all DNAs; a 3.5-kb band is present in V3D7, V4D6, and WEHI DNAs. The uppermost bands are due to a partial digestion. (B) *HindIII* digest. A 5.5-kb band results from the hybridization of exons II, III, and IV. The upper bands result from a partial digestion. (C) *BglII* digest. The 4.5-kb band is from the 5' end, and the 2.5-kb band is from the 3' end of the unaltered allele. The 6.5-kb band in V4D6 and the 5.1-kb band in V3D7 are due to rearrangements. (D) *PvuII* digest. The 4.4-, 3.2-, and 1.2-kb bands and the lowest (0.7-kb) bands arise from the normal IL-3 allele, whereas the 0.86-kb band in V4D6 and the 0.9-kb band in V3D7 result from the rearranged allele.

reported to induce tumor growth factors (48). It could be that expression of v-H-ras also induces directly or indirectly a growth factor in PB-3c cells. The only factor known to stimulate PB-3c cells is IL-3, but we obtained no evidence for IL-3 gene expression in V cells. Recently, Pierce et al. (42) have shown that transfection of the epidermal growth factor receptor gene into an IL-3-dependent cell line conferred the ability to utilize epidermal growth factor for transduction of mitogenic signals. It is an interesting possibility that v-H-ras could similarly activate an alternate mitogenic pathway, physiologically nonoperative in mast cells, which then synergizes with IL-3.

Autocrine IL-3 secretion. Since production of IL-3 was a property of all tumors tested (over 50), it appears that this trait was selected for in the tumorigenic process. The illegitimate activation of a growth factor gene in a normally nonproducing target cell is considered to be an important feature of many natural and experimental tumors (1, 6, 24, 30). Expression of cDNA for granulocyte-macrophage colony-stimulating factor (35) or IL-3 (26) in factor-dependent, nontumorigenic cells was sufficient for tumorigenicity. In Abelson virus-transformed mast cells, IL-4 production has been reported, and the possibility that this factor may provide an autocrine stimulus has been suggested (10).

Insertional mutation has been shown to be a cause for the activation of IL-3 as well as granulocyte-macrophage colony-stimulating factor (49, 54) genes. Two tumor cell lines (V3D7 and V4D6) from infected PB-3c mass cultures showed insertions near one of the IL-3 genes. However, the IL-3-secreting tumors from V clones showed no rearrangement of the IL-3 gene, suggesting the possibility of alternate steps.

The role of the v-H-ras gene in the establishment of the autocrine state in the described system is not known. Overexpression of p21 has been ruled out as the cause of IL-3 secretion, because the tumor cell lines showed no increase in the amount of p21 (data not shown). Two hypotheses merit consideration. (i) IL-3 expression is induced spontaneously and independently of v-H-ras, but the oncogene product is required for signal amplification. In this case, both viral p21 and IL-3 autoproduction represent tumor maintenance function. (ii) The oncogene, with the obligatory help of factors present *in vivo*, could lead to the induction of the growth factor, a trait which would then be selected. In this model, v-H-ras may or may not additionally provide a tumor maintenance function as in model one. Nevertheless, it appears that the induction of IL-3 gene expression with concomitant autostimulation is the key feature in the multistep process described in this work. The mechanisms by which the preceding steps complement IL-3 gene expression remain to be elucidated.

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